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Abstract

AQUI-S is a fish anesthetic/sedative that is approved for use in a number of countries throughout the world and has the potential for use in the United States. The active ingredient in AQUI-S is isoeugenol. A method for determining isoeugenol concentrations in edible fillet tissue is needed for regulatory purposes, including surveillance and potential use in studies fulfilling human food safety data requirements if U.S. Food and Drug Administration approval is pursued. A method was developed and evaluated for determining isoeugenol concentrations in fillet tissue using relatively common procedures and equipment. The method produced accurate and precise results with fillet tissue from 10 freshwater fish species. The percentage of isoeugenol recovered from samples fortified with isoeugenol at nominal concentrations of 1, 50, and 100 g/g for all species was always >80 and <97. Within-day precision for samples fortified at those same concentrations was 10, and day-to-day precision was 4.0. Method precision with fillet tissue containing biologically incurred isoeugenol was 8.1. There were no or minimal chromatographic interferences in control fillet tissue extracts from 9 of the 10 species. The method detection limits for all but one species ranged from 0.004 to 0.014 g/g, and the quantitation limits ranged from 0.012 to 0.048 g/g.

Disciplines

Agriculture | Aquaculture and Fisheries | Veterinary Toxicology and Pharmacology

Comments

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VETERINARY DRUG RESIDUES

Evaluation of a Method for Determining Concentrations of Isoeugenol, an AQUI-S™ Residue, in Fillet Tissue from Freshwater Fish Species

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AQUI-S™ is a fish anesthetic/sedative that is approved for use in a number of countries throughout the world and has the potential for use in the United States. The active ingredient in AQUI-S is isoeugenol. A method for determining isoeugenol concentrations in edible fillet tissue is needed for regulatory purposes, including surveillance and potential use in studies fulfilling human food safety data requirements if U.S. Food and Drug Administration approval is pursued. A method was developed and evaluated for determining isoeugenol concentrations in fillet tissue using relatively common procedures and equipment. The method produced accurate and precise results with fillet tissue from 10 freshwater fish species. The percentage of isoeugenol recovered from samples fortified with isoeugenol at nominal concentrations of 1, 50, and 100 μg/g for all species was always >80 and <97%. Within-day precision for samples fortified at those same concentrations was ≤10%, and day-to-day precision was ≤4.0%. Method precision with fillet tissue containing biologically incurred isoeugenol was ≤8.1%. There were no or minimal chromatographic interferences in control fillet tissue extracts from 9 of the 10 species. The method detection limits for all but one species ranged from 0.004 to 0.014 μg/g, and the quantitation limits ranged from 0.012 to 0.048 μg/g.

Presently, Finquel (tricane methanesulfonate or MS-222), the only fish anesthetic approved by the U.S. Food and Drug Administration (FDA), is constrained by a 21 day withdrawal period. AQUI-S™ (AQUI-S New Zealand Ltd, Lower Hutt, NZ) is a fish anesthetic/sedative approved in a number of countries as an anesthetic with a zero withdrawal period.

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The primary use of AQUI-S is for reducing the physical response of fish during harvest operations when they are taken from commercial aquaculture net pens. Use of AQUI-S during these operations minimizes harvest-induced damage, ultimately providing a quality product for the consumer. Pending approval in the United States, additional AQUI-S uses would include any aquaculture or fishery management activity in which fish are handled or transported.

Because AQUI-S is used by other countries, there is a need for an analytical method to determine AQUI-S residue concentrations in imported fish fillet tissue. In addition, because AQUI-S could be considered for FDA approval, an analytical method for a primary AQUI-S residue will be needed to conduct studies fulfilling human food safety data requirements. As part of the approval process concerning human food safety data requirements, the FDA selects a residue of a drug to serve as the marker (surrogate) to represent all of the drug's residues in fish fillet tissue after exposure. After selection, a method for the marker compound must be developed and validated according to FDA guidelines (1).

Isoeugenol is the active ingredient in AQUI-S (Figure 1) and is also the primary residue in fillet tissue extracts from rainbow trout (*Oncorhynchus mykiss*) exposed to AQUI-S [Upper Midwest Environmental Sciences Center (UMESC), LaCrosse, WI, unpublished data]. Analytical methods for determining isoeugenol concentrations in various biological matrixes have been reported. A method for determining phenolic compounds, including isoeugenol, in smoked herring used solid-phase microextraction techniques with analysis by gas chromatography (GC; 2). Although precision was adequate, method accuracy was very poor (<1%). A method for isoeugenol in rat blood used a simple ethyl acetate extraction with analysis by liquid chromatography (LC; 3). The method was used in an isoeugenol disposition and metabolism study where method precision and accuracy data were not reported. An internal standard method for isoeugenol in silver perch (*Bidyanus bidyanus*) skinless fillet tissue used a dichloromethane Soxhlet extraction with analysis by GC (4). The time-consuming method was used in an isoeugenol accumulation and clearance study where, again, method precision and accuracy data were not reported. Although adequate for their specific applications, the methods did not

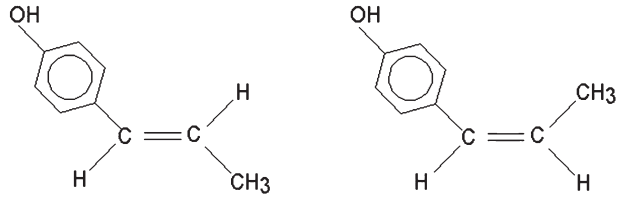

Figure 1. Chemical structures of the isoeugenol isomers.

appear moderately robust or provide adequate precision and accuracy data.

The objectives of this study were to evaluate method performance and robustness in the following areas: interferences from natural endogenous compounds extracted from control fillet tissue; accuracy (percent recovery) with fillet tissue fortified with isoeugenol at 1, 50, and 100 μg/g; repeatability (within-day and day-to-day precision) with fillet tissue fortified with isoeugenol at 1, 50, and 100 μg/g; repeatability (precision) with biologically incurred isoeugenol residues; detection and quantitation limits; isoeugenol stability in methanol–water (90 + 10, v/v) fillet tissue extracts and fillet tissue stored at <–60°C; interferences from other aquaculture drugs; and method ruggedness.

Experimental

Chemicals and Reagents

(**a**) *AQUI-S*.—Active ingredient, isoeugenol, 50% by weight (AQUI-S New Zealand Ltd).

(**b**) *Isoeugenol*.—2-Methoxy-4-[1-propenyl] phenol; molecular weight, 164.2; purity, ≥99% (PT Indesso Aroma, Bogor, Indonesia).

(**c**) *Acetonitrile and methanol*.—LC grade (Fisher Scientific, Pittsburgh, PA).

(**d**) *Water*.—Deionized to a specific resistance of >17.8 mΩ/cm with a water purification system (Barnstead E-pure; Dubuque, IA).

(**e**) *Eluting solvent*.—Methanol–water (9 + 1, v/v).

Apparatus

(**a**) *Commercial blender*.—Waring bar blender (Dynamics Corp. of America, New Hartford, CT).

(**b**) *Freezer storage bags*.—Ziploc® freezer bags (Dow Chemical Co., Indianapolis, IN).

(**c**) *Analytical balances*.—Sartorius Model 1712 MP8 (for preparing chemical solutions) and Model LC3201D (for preparing tissue samples; Sartorius AG, Goettingen, Germany).

(**d**) *Extraction tubes*.—Glass, 25 mL, screw-cap tubes.

(**e**) *Rotary evaporation flasks*.—Glass, 100 mL, pear-shaped.

(**f**) *Wrist action shaker*.—Lab Line Model 3589 (Lab Line Instruments, Inc., Melrose Park, IL).

(**g**) *Centrifuge*.—Beckman Model Avanti 30 (Beckman Instruments, Inc., Palo Alto, CA).

(**h**) *Rotary evaporator system*.—Buchi Model RE121 rotovapor, Model 461 water bath, and Model B-171 vacobox (Buchi Corp., New Castle, DE).

(**i**) *Glass wool*.

(**j**) *Solid-phase extraction (SPE) accessories*.—25 mL reservoir, stopcocks, adaptors, and stainless steel needles (J.T. Baker, Phillipsburg, NJ).

(**k**) *SPE column*.—Phenomenex Strata phenyl 55 μm, 70 Å, 500 mg, 3 mL column (Phenomenex, Torrance, CA).

(**l**) *SPE manifold*.—Baker SPE-24G column processor and vacuum chamber (J.T. Baker).

(**m**) *Vacuum pump*.—Gast Model DOA-102-AA (Gast Manufacturing Corp., Benton Harbor, MI).

(**n**) *Volumetric flask*.—Clear glass, class A, 5 mL.

(**o**) *Transfer pipet*.—Glass, 5.75 in.

(**p**) *Syringe filter*.—13 mm, 0.45 μm Acrodisc® CR PTFE (Gelman Sciences, Ann Arbor, MI).

(**q**) *Syringes*.—Plastic, 1 cc tuberculin (Becton Dickinson and Co., Franklin, NJ).

(**r**) *LC sample vials*.—Amber glass, 12 × 32 mm, 1.5 mL, open-top cap with 8 mm Teflon/silicon septum (Sun Brokers, Inc., Wilmington, NC).

LC System

The LC system was an Agilent Model HP1090 system with Chem Station chromatography software, Version A.10.01 (Agilent Technologies, Wilmington, DE). The following LC parameters were used to determine isoeugenol concentrations: isocratic mobile phase, 49% water and 51% acetonitrile (both solvents helium sparged); flow rate, 1.5 mL/min; injection volume, 45 μL; column temperature, 50°C; guard column, YMC, ODS-A, 5 μm, 4.0 × 23 mm (YMC, Inc., Wilmington, NC); analytical column (Phenomenex) Synergi Max-RP, 4 μm, 4.6 × 250 mm; and detector wavelength setting, 261 nm. The concentration of isoeugenol in each extract was determined from the isoeugenol peak area and the linear regression equation was developed from a calibration graph created with 5 isoeugenol working solutions.

Preparation of Test Chemical Working Solutions

Stock solutions of isoeugenol were prepared by weighing 100 or 500 mg (±30 mg) of the chemical in a 100 mL volumetric flask and dissolving the chemical in acetonitrile. Working solutions (i.e., solutions with isoeugenol concentrations suitable for developing a calibration graph) were prepared by diluting the stock solution to appropriate concentrations with eluting solvent.

Experimental Animals

Cold water fish species included brown trout (*Salmo trutta*), Chinook salmon (*O. tshawytscha*), lake trout (*Salvelinus namaycush*), and rainbow trout. Cool water fish species included northern pike (*Esox lucius*), walleye (*Sander vitreus*), and yellow perch (*Perca flavescens*). Warm water fish species included channel catfish (*Ictalurus punctatus*),

hybrid striped bass (*Morone saxatilis* x *M. chrysops*), and largemouth bass (*Micropterus salmoides*).

All live fish designated for exposure to AQUI-S were reared at the UMESC fish culture facility. Fish reared at the UMESC were fed Sterling Silver Cup Trout Food (Nelson and Sons, Inc., Murray, UT) at a rate that maintained fish growth (5).

Fillets of Chinook salmon were acquired by Garrison Dam National Fish Hatchery (Riverdale, ND) personnel during the Lake Sakakawea fall spawning run. All fish of all species were used without regard to gender.

Acquisition of Control Fillet Tissue

Skin-on fillets were taken from each fish (exception, catfish fillets were skinless). If a fish was relatively large (>100 g), only the right side fillet was taken; otherwise, fillets from each side were removed. Fillets were hardened in a freezer before being homogenized in a blender with dry ice (6). The fillet tissue/dry ice matrixes were poured into plastic freezer bags and stored at about –15°C overnight in unsealed bags to allow the dry ice to sublime. Thereafter, the bags were sealed and the homogenized tissue was stored at <–60°C. Fillet homogenates from individual fish were stored separately from fillet homogenates of other fish.

Method to Determine Isoeugenol Concentrations in Fillet Tissue

Frozen homogenized tissue was thawed on the laboratory bench at ambient room temperature (about 21°C). Homogenized fillet tissue was weighed into an extraction tube (accuracy, 5 ± 0.2 g; precision, 0.001 g) and subjected to 4 sequential extractions with acetonitrile. *Extraction 1*.—10 mL acetonitrile was added to the sample and the sample shaken for 5 min; the sample was centrifuged at a relative centrifugal field (rcf) of 950 × *g* for 5 min at ambient temperature, and the supernatant was poured into a 100 mL pear-shaped rotary evaporation (roto-vap) flask. *Extraction 2*.—5 mL acetonitrile was added to the sample, and the sample shaken for 5 min, centrifuged at rcf 2630 × *g* for 5 min, and the supernatant poured into the 100 mL pear-shaped roto-vap flask. *Extractions 3 and 4*.—A 5 mL volume of acetonitrile was added to the sample and the sample was shaken for 5 min, centrifuged at rcf 2630 × *g* for 5 min, and the supernatant was poured into the roto-vap flask. The neck of the 100 mL roto-vap flask was rinsed with 3 mL acetonitrile.

Acetonitrile was evaporated from the 100 mL roto-vap flask on a rotary evaporator system with a water bath temperature of 45–50°C and a vacuum of about 220 mbar. The sample was evaporated to a volume of 5 ± 2 mL. Water (45 mL) was mixed into the 100 mL roto-vap flask and the samples poured into an SPE reservoir mounted over an SPE column with a stopcock and adapter. The SPE column was previously preconditioned with eluting solvent followed by water. The bottom of the SPE reservoir was lined with glass wool to collect tissue debris. The 100 mL roto-vap flask was quantitatively rinsed 3 times with 1 mL water and the water added to the SPE reservoir. The sample was processed through an SPE column. The extract was pulled through the column at <5 mL/min with a vacuum pump. After the extract vacated the reservoir, the reservoir was rinsed 4 times with 1 mL water. After the solution passed through the SPE column, the column was dried by pulling air through the column with the vacuum pump for about 10 min. The column was eluted with five 1 mL portions of eluting solvent into a volumetric flask. The flask volume was adjusted to 5 mL with eluting solvent. The sample was mixed, transferred to a syringe with a glass pipet, and filtered into an LC vial through a syringe filter. The extract was analyzed for isoeugenol on the LC system.

Evaluation of Chromatographic Interferences Extracted from Control Fillet Tissue

Three control tissue samples from each of 6 fish of each species (exception, 10 rainbow trout) were analyzed for isoeugenol. Control extracts were evaluated for the presence of compounds that could interfere with the chromatography of isoeugenol. Isoeugenol equivalent concentrations of chromatographically interfering peaks, with peak areas greater than the peak area of the lowest calibration standard, were determined from the linear regression equation developed from a calibration graph created with 5 working solutions of isoeugenol. In cases where the peak area of the interfering peak was less than the peak area of the lowest calibration standard, the isoeugenol equivalent concentration of the interfering peak was determined by equating the peak area to the lowest calibration standard's ratio of peak area:isoeugenol concentration.

Evaluation of Method Accuracy and Within-Day Precision

Control fillet tissue from each species was fortified with isoeugenol, resulting in nominal tissue concentrations of 1, 50, and 100 μg/g. Five tissue samples were prepared for each concentration level. All samples from one species were analyzed for isoeugenol on the same day.

Evaluation of Day-to-Day Precision

Day-to-day precision was assessed by fortifying channel catfish fillet tissue (species arbitrarily chosen) with isoeugenol, resulting in nominal tissue concentrations of 1, 50, and 100 μg/g. Five samples were prepared for each concentration level. All 5 samples from one concentration level were analyzed for isoeugenol on the same day. The process was repeated 4 more times on 4 different days.

Evaluation of Method Precision with Fillet Tissue Containing Biologically Incurred Isoeugenol

For each fish species except the fall Chinook salmon, an AQUI-S bath was prepared by weighing 1700 ± 100 mg AQUI-S in a 250 mL screw-cap flask. About 5 mL well water was added to the flask, and the contents shaken for about 15 min. The contents were shaken throughout the next 45 min with 2 periodic additions of well water (each addition about 5 mL). A stainless steel exposure tank (width at one-half the

height, 58.4 cm; length at one-half the height, 58.4 cm; height, 35.6 cm) was filled with about 100 L 17°C well water. The entire AQUI-S mixture was vigorously mixed into the exposure tank with a glass rod for about 1 min. Three water samples (5 mL) were taken from the bath to determine the initial isoeugenol concentration. Within 15 min of preparing the bath, 3 (brown trout, channel catfish, hybrid striped bass, lake trout, and northern pike) or 6 (largemouth bass, walleye, and yellow perch) fish were released into the exposure tank. The number of fish was dependent on relative size. If the fish were relatively large (>440 g), only 3 fish were needed to provide an adequate amount of tissue for assays requiring tissue containing biologically incurred isoeugenol. If the fish were relatively small (<250 g), 6 fish were needed. Fish remained in the exposure bath for 60 min. At 60 min, fish were removed and euthanized by a blow to the head. Three water samples (5 mL) were taken from the exposure tank to determine the final isoeugenol concentration.

Isoeugenol concentrations in water were determined by applying samples directly to SPE columns. Water was pulled through the columns at <5 mL/min. The columns were rinsed with 3 mL water and eluted with 4 × 1 mL portions of eluting solution into a 5 mL volumetric flask, and the flask volume was adjusted with eluting solvent. A portion of the extract was filtered through a syringe filter into an LC vial and analyzed for isoeugenol.

Euthanized fish were rinsed with flowing well water and weighed. The skin-on fillets (channel catfish were skinned before acquiring fillets) were removed from each fish and hardened in a freezer before homogenizing with dry ice as described previously. The following day, 5 fillet tissue samples from each of 3 fish were analyzed for isoeugenol.

Evaluation of Method Detection and Quantitation Limits

Method sensitivity was determined by fortifying at least 7 samples (5 g) of control fillet tissue from each species with isoeugenol and analyzing the samples for isoeugenol. Fillet tissue from all species except fall Chinook salmon was fortified with isoeugenol at concentrations ranging from 0.016 to 0.035 μg/g. Fillet tissue extracts from fall Chinook salmon contained a compound that resulted in excessive chromatographic interference at the retention time of isoeugenol; therefore, the theoretical isoeugenol concentration in fortified samples was increased to 10.13 μg/g. The method detection limit was calculated as 3*s* and the method quantitation limit calculated as 10*s* (7), where *s* was the sample standard deviation.

Evaluation of Isoeugenol Stability in Eluting Solvent

Three isoeugenol stock solutions were prepared with acetonitrile, and each stock solution was diluted with eluting solvent to nominal concentrations of 0.1 and 10 μg/mL in clear glass volumetric flasks. The isoeugenol concentrations in each of the resulting 6 working solutions were determined on the day the solutions were prepared (Day 0). Solutions were stored on the laboratory bench and exposed to ambient temperature (about 21°C) and fluorescent lighting. Isoeugenol concentrations in each solution were determined 1, 7, 14, and 21 days after preparation.

Evaluation of Isoeugenol Stability in Fillet Tissue Extract

Control fillet tissue from each species was fortified with isoeugenol, resulting in nominal tissue concentrations of 1, 50, and 100 μg/g. Three tissue samples were prepared for each concentration level and processed; the extracts were then analyzed for isoeugenol. The isoeugenol concentrations in the extracts were determined on the same day extracts were generated (Day 0). Extracts were stored in LC vials on the laboratory bench and exposed to ambient temperature (about 21°C) and fluorescent lighting. The isoeugenol concentrations in the extracts were again determined 1, 7 or 8, and 14 days after preparation.

Evaluation of Isoeugenol Stability in Fillet Tissue Stored at <–60°C

One or 2 days after exposing fish to AQUI-S (previously described), fillet tissue samples were analyzed for isoeugenol (Day 0). The remaining tissue was sealed in plastic bags and stored at <–60°C. Fillet tissue was removed from storage at about 1 month intervals through a 6 month period and analyzed for isoeugenol.

Evaluation of Chromatographic Interference from Aquaculture Chemicals

The following chemicals were evaluated for their potential to interfere chromatographically with the determination of isoeugenol:

(**a**) 3-Aminobenzoic acid ethyl ester (MS-222, Finquel™) CAS No. 886-86-2

(**b**) Formalin solution (Formalin-F™, Paracide-F™, Parasite-S™) CAS No. 50-00-0

(**c**) Ormetoprim (component of Romet 30™) CAS No. 6981-18-6

(**d**) Oxytetracycline hydrochloride (Terramycin for Fish™) CAS No. 2058-46-0

(**e**) Chloramine-T CAS No. 127-65-1

(**f**) Sulfadimethoxine sodium salt (component of Romet 30™) CAS No. 1037-50-9

The chemicals are FDA-approved and unapproved compounds known to have been used in aquaculture. Working solutions of the chemicals were prepared in eluting solvent and analyzed with the LC parameters previously described.

Evaluation of Method Ruggedness

Method ruggedness was evaluated by altering method procedures while analyzing for isoeugenol in rainbow trout fillet tissue (n = 3) fortified with isoeugenol at a nominal concentration of 50 μg/g. The following procedures were altered to evaluate method ruggedness: extraction of homogenized tissue with sample masses >5 g; extraction using volumes of acetonitrile less than and greater than the standard total volume of 25 mL; rotary evaporation of extracts

to a volume <2 and >5 mL; hydration of extracts after rotary evaporation with volumes of water less than and greater than the standard 45 mL; using various brands of frits for the SPE reservoir rather than glass wool; using various brands of SPE phenyl columns; using various sizes of prescribed SPE columns; increasing and decreasing the percentage of methanol in the eluting solvent by 5%; and filtering the extract with other brands of filters.

Data Reporting and Statistical Analyses

Accuracy was reported for each concentration level as the percent of isoeugenol recovered in the tissue extract vs the amount applied. Within-day precision was reported for each concentration level as the percent relative standard deviation (% RSD). Day-to-day precision was reported for each concentration level as the %RSD of the mean isoeugenol concentrations from the 5 analysis days.

The stability of isoeugenol in methanol–water (90 + 10, v/v), fillet tissue extract, and fillet tissue was determined by documenting the isoeugenol concentration change through time and presenting the data as a percent concentration change.

Results and Discussion

The method for determining isoeugenol concentrations in fish fillet tissue is relatively simple to perform and uses relatively common equipment and procedures. Briefly, the method procedures included extracting isoeugenol from tissue with acetonitrile, evaporating the acetonitrile from the extract with rotary evaporation techniques, changing the polarity of the extract by adding water, concentrating the isoeugenol with SPE procedures, and determining concentrations with an LC system.

Evaluation of Chromatographic Interferences Extracted from Control Fillet Tissue

The method was reasonably specific. There were no chromatographic interferences in the extracts from the fillet tissue of brown trout, channel catfish, hybrid striped bass, walleye, and yellow perch. Chromatographic interferences were found in fillet extracts from all 6 fall Chinook salmon and ranged in isoeugenol equivalent concentration from 0.0989 to 0.5153 μg/g. Interferences were also found in fillet extracts from 3 of the 6 lake trout, 3 of the 6 largemouth bass, and one of the 6 northern pike. Isoeugenol equivalent concentrations of the interfering compounds ranged from 0.0034 to 0.1096 μg/g.

Evaluation of Method Accuracy, Within-Day Precision, and Day-to-Day Precision

The method was accurate and precise. Method accuracy ranged from 80.3% (yellow perch fillet tissue with a nominal isoeugenol concentration of 1 μg/g; Table 1) to 96.5% (northern pike fillet tissue with a nominal isoeugenol concentration of 100 μg/g; Table 1). The within-day precision ranged from 0.44% RSD (walleye fillet tissue with a nominal isoeugenol concentration of 50 μg/g; Table 1) to 8.5% RSD (yellow perch fillet tissue with a nominal isoeugenol concentration of 50 μg/g; Table 1). The day-to-day precision with channel catfish fillet tissue fortified at 3 concentrations, 1, 50, and 100 μg/g, were 3.0, 1.4, and 2.4% RSD, respectively (Table 2).

Evaluation of Method Precision with Fillet Tissue Containing Biologically Incurred Isoeugenol

The isoeugenol concentration in the exposure water before exposures were initiated ranged from 8.22 to 8.73 mg/L. Isoeugenol concentrations in the water after the exposures ranged from 7.15 to 8.00 mg/L.

Table 1. Method accuracy and within-day precision with fillet tissue fortified with isoeugenol at nominal concentrations of 1, 50, and 100 μg/g

Fish species	Nominal concn, μg/g	*n*	Accuracy, %	Precision, % RSD
Brown trout	1	4	93.8	4.2
	50	5	92.2	2.8
	100	5	90.9	3.3
Channel catfish	1	5	95.6	3.2
	50	5	91.9	3.7
	100	3	90.3	3.3
Fall Chinook salmon	1	5	92.8	5.7
	50	5	93.9	1.0
	100	4	93.8	1.6
Hybrid striped bass	1	5	92.4	7.5
	50	5	94.1	0.85
	100	5	92.8	1.4
Lake trout	1	5	88.4	2.0
	50	5	90.3	1.9
	100	5	92.6	2.1
Largemouth bass	1	5	94.9	2.6
	50	4	95.0	0.83
	100	5	95.0	1.5
Northern pike	1	5	94.1	1.1
	50	5	95.4	1.2
	100	5	96.5	1.1
Rainbow trout	1	5	92.7	1.5
	50	5	85.5	5.8
	100	5	83.8	3.2
Walleye	1	4	92.8	1.4
	50	5	95.5	0.44
	100	5	89.9	7.8
Yellow perch	1	5	80.3	3.0
	50	4	81.2	8.5
	100	5	84.9	1.2

Table 2. Method day-to-day precision with channel catfish fillet tissue fortified with isoeugenol at nominal concentrations of 1, 50, and 100 μg/g

Fortification concn, μg/g	Analysis date	*n*	Daily mean accuracy, %	Overall mean, %	Overall precision, % RSD
1	October 5, 2006	5	95.6	94.2	3.0
	December 8, 2006	5	90.7		
	December 27, 2006	5	92.2		
	February 2, 2007	5	97.6		
	February 5, 2007	5	95.1		
50	October 5, 2006	5	91.9	93.2	1.4
	December 8, 2006	5	94.5		
	December 27, 2006	5	92.0		
	February 2, 2007	5	94.4		
	February 5, 2007	5	93.2		
100	October 5, 2006	5	90.3	93.7	2.4
	December 8, 2006	3	94.9		
	December 27, 2006	4	92.6		
	February 2, 2007	5	94.7		
	February 5, 2007	5	95.8		

The highest isoeugenol concentrations were found in channel catfish fillet tissue (62.2 μg/g; Table 3) and the lowest isoeugenol concentrations were found in walleye fillet tissue (18.5 μg/g; Table 3). The method precision with fillet tissue from individual fish of 9 species containing biologically incurred isoeugenol was ≤8.1% RSD with one exception (Table 3). The exception was associated with the fillet tissue from one walleye where the precision was 16% RSD. The precision values with fillet tissue from each of the other 2 walleye were 3.6 and 7.7% RSD. There was no plausible explanation for this data anomaly.

Evaluation of Method Detection and Quantitation Limits

The method was sensitive. For all species except fall Chinook salmon, the method detection limits ranged from 0.004 to 0.014 μg/g and the method quantitation limits ranged from 0.012 to 0.048 μg/g. The method accuracy with fillet tissue fortified with isoeugenol at concentrations <0.04 μg/g ranged from 91.8 to 116% (fall Chinook salmon excluded). Method precision from those same samples ranged from 3.1 to 19% RSD.

Isoeugenol interferences were found in all fall Chinook salmon fillet tissue extracts. The interferences covered a relatively wide range of isoeugenol equivalent concentrations. The fillet extract from one particular fish had an isoeugenol-equivalent interference concentration notably higher (0.52 μg/g) than was found in other extracts. The tissue from that particular fish was considered to represent a worst-case scenario for chromatographic interference, and therefore was chosen for fortification to determine method detection and quantitation limits. In order to alleviate the effect of the notable interference on method accuracy, tissue was fortified at a relatively high concentration (nominal concentration, 10 μg/g), resulting in relatively high method detection (0.99 μg/g) and quantitation limits (3.3 μg/g) for fall Chinook salmon.

Evaluation of Isoeugenol Stability in Eluting Solvent, Fillet Tissue Extract, and Fillet Tissue Stored at <–60°C

In the 0.1 and 10 μg/mL isoeugenol solutions prepared in eluting solvent, concentration decreases were ≤2.5% by Day 7, ≤6.1% by Day 14, and ≤10% by Day 21. In the extracts from fillet tissue fortified with isoeugenol at nominal concentrations of 1, 50, and 100 μg/g, isoeugenol concentration decreases were ≤2.6% by Day 1 (exception, channel catfish 1 μg/g, 4.8% decrease), ≤8.5% by Day 7 (exception, channel catfish and walleye 1 μg/g, 18 and 17% decreases, respectively), and ≤9.7% by Day 14 (exception, channel catfish, hybrid striped bass, and walleye 1 μg/g, 28, 16, and 24% decreases, respectively).

After 6 months of storage at <–60°C, isoeugenol degradation was greatest in channel catfish (7.1%) and rainbow trout fillet tissue (12%). Isoeugenol degradation in fillet tissue from all other species was negligible through the 6 month storage period.

Evaluation of Chromatographic Interference from Aquaculture Chemicals

Using the LC parameters designed for isoeugenol, only 2 of the 6 aquaculture chemicals, MS-222 and sulfadimethoxine, resulted in discernible peaks on

Table 3. Isoeugenol concentration in fillet tissue from each fish exposed to AQUI-S™ at a nominal concentration of 17 mg/L (8.5 mg/L isoeugenol) for 60 min[a]

Fish species	*n*	Isoeugenol concn, μg/g	Precision, % RSD
Brown trout	5	41.1	4.6
	5	41.9	7.4
	4	46.3	5.4
Channel catfish	5	60.8	8.1
	4	62.2	5.6
	4	51.8	4.6
Hybrid striped bass	5	52.4	3.6
	5	54.3	3.3
	5	49.0	3.7
Lake trout	4	50.1	3.0
	5	38.2	3.2
	5	37.6	0.67
Largemouth bass	5	34.5	6.4
	5	43.0	2.3
	5	37.0	5.7
Northern pike	5	30.1	1.2
	5	28.4	2.4
	5	24.3	3.0
Walleye	5	18.5	16
	5	45.0	3.6
	5	35.3	7.7
Rainbow trout	4	37.0	1.1
	5	57.5	2.4
	4	38.7	1.9
Yellow perch	5	40.9	1.9
	5	35.1	2.0
	5	36.6	4.1

[a] The data are the results from *n* subsamples from each of 3 fish.

chromatograms. Peaks from these compounds eluted more than 1 min from the isoeugenol retention time.

Evaluation of Method Ruggedness

The method was considered rugged, or robust, even though some minor parameter changes did impact its performance. In the event that an analyst would want to increase the sample size, method performance was evaluated with increases in sample size. When sample mass was increased by 1 g (prescribed mass, 5 ± 0.2 g), the method's performance (accuracy, 85.0%; precision, 8.6% RSD) was not affected. However, when the sample mass was increased by 3 g, method accuracy (77.7%; precision, 4.6% RSD) fell below a predetermined minimum acceptable limit of 80%.

Intuitively, in many instances, an analyst would assume that increasing the extracting solvent volume would increase method accuracy. However, that assumption was not true with this method. When the volume of acetonitrile used to extract the sample was decreased by 5 mL (prescribed volume, 25 mL), the method's performance (accuracy, 87.8%; precision, 6.8% RSD) was not affected. However, when the volume of solvent was increased by 5 mL, the method accuracy (76.4%; precision, 5.2% RSD) fell below the predetermined minimum acceptable limit of 80%.

The sample volume at the end of rotary evaporation was critical to method performance. When the sample was evaporated to a volume of about 8 mL (prescribed volume, 5 mL), the method performance (accuracy, 89.7%; precision, 6.1% RSD) was not affected. However, when the sample was evaporated to a volume ≤2 mL, the method accuracy (77.0%; precision, 6.4% RSD) fell below 80%. When the volume of water used to hydrate the sample after rotary evaporation was increased (accuracy, 95.2%; precision, 2.5% RSD) and decreased (accuracy, 92.0%; precision, 5.5% RSD) by 5 mL, the method performance was not affected.

An unavoidable phenomenon of this method was the decanting of tissue debris from centrifuge tubes into roto-vap flasks. Tissue debris remained in the flasks after rotary evaporation and was suspended in the sample when the sample was poured into the SPE reservoir. To alleviate the tissue debris from plugging the SPE column and virtually stopping the flow of extract through the column, glass wool was positioned on the bottom of the SPE reservoir. Polyethylene frits designed for this purpose are commercially available. Therefore, method performance was assessed with 3 different brands of polyethylene frits (Alltech, Deerfield, IL; Chrom Tech Inc., Apple Valley, MN; and Varian, Lake Forest, CA). Although method accuracy was adequate using all 3 brands, method precision was greater than the predetermined maximum acceptable limit of 10% for the Alltech (accuracy, 98.0%; precision, 12% RSD) and Varian (accuracy, 94.1%; precision, 19% RSD) brand frits. Method performance with the Chrom Tech brand frit was adequate (accuracy, 89.5%; precision, 4.2% RSD).

The prescribed SPE column to remove isoeugenol from the tissue extract was the Phenomenex Strata 55 μm, 70 Å phenyl, 500 mg, 3 mL SPE column. Although the packing material in an SPE column may be of the same type, different brands and masses can produce various results. In this instance, however, the method's performance was not affected by the brand of phenyl packing (Agilent Technologies AccuBond^{II} phenyl, 500 mg, 3 mL columns, accuracy, 82.1%; precision, 7.2%; and Varian Bond Elut-PH phenyl, 500 mg, 3 mL columns, accuracy 86.8%; precision, 8.8%). The mass of packing material in the prescribed brand of column also did not affect method performance (200 mg, accuracy, 84.9%; precision, 6.0% RSD; and 1000 mg, accuracy, 89.3%; precision, 5.8% RSD).

Because graduated cylinders are used to prepare the eluting solvent, the composition of the solvent may be slightly different from batch to batch [prescribed composition,

methanol–water (90 + 10, v/v)]. Therefore, the effects of small composition changes in the eluting solvent on methanol performance were evaluated by increasing and decreasing the percentage of methanol. Method performance was not affected when the composition of methanol was increased (accuracy, 94.1%; precision, 0.61% RSD) or decreased (accuracy, 88.6%; precision, 7.9% RSD) by 5%.

Several brands of filters are available for filtering solutions prepared for LC analyses. In addition to the prescribed brand of syringe filter (Gelman 13 mm, 0.45 μm Acrodisc® CR PTFE), Agilent 13 mm, 0.45 μm and Alltech 13 mm, 0.45 μm PTFE syringe filters did not affect method performance. Gelman, Agilent, and Alltech syringe filters are readily available in most supply catalogs.

Conclusions

A method using relatively common procedures and equipment was developed and evaluated for determining isoeugenol concentrations in fish fillet tissue. As many as 16 samples could be easily processed by one analyst in a normal work day. The method produced accurate (accuracy >80%) and precise (precision <10% RSD) results with fillet tissue from 10 freshwater fish species. The method was relatively sensitive and specific for isoeugenol in fillet tissue from most fish species. The method was rugged, i.e., relatively minor changes in the method's procedures did not grossly affect the method's performance. This validated method has good potential to serve as an analytical method to determine AQUI-S residue concentrations in imported fish fillet tissue and should be considered as an analytical method for a primary AQUI-S residue pending FDA approval for AQUI-S use in the United States.

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