Determination of Veterinary Drug Residues in Fish by an Automated SPE-HPLC System

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Introduction

As aquaculture has developed, a range of fish and shellfish diseases have been encountered that have led to major economic losses leading to failure of the viable industry in some parts of the world. This has lead to the increased use of veterinary drugs and vaccines in intensive production systems to combat disease in farmed fish. Antibiotics are used in the aquaculture worldwide to treat infections caused by a variety of bacterial pathogens of fish including *Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda, Pastuerella piscidida, Vibrio anguillarum, Vibrio salomonicida and Yersinia ruckeri*. They are commonly used as in-feed medications or surface coated onto feed pellets and dispersed in the water.

The use of antibiotics in fish farming is associated with a new hazard in fish production not encountered in wild captured species. The main hazards are antibiotic residues and development of antimicrobial resistance in bacteria that may be transferred to consumers. Malachite green, the anti-fungal, is another matter. The chemical dye battles fish parasites and fungal infections. Lab tests have showed increased cancer rates in rats and mice fed malachite green and leucomalachite green, which is formed from malachite green, at doses ranging from 100-600 ppm for two years.

- **The FDA began testing for the residues in 2001, triggered by concerns of chloramphenicol in shrimp. China banned malachite green in 2002, yet current violations indicate that it is still widely used. Last year the FDA restricted imports of eel from china after FDA testes found 91% of those sampled contained leucomalachite green- some levels up to 3,239 ppb.**
- **This application presents a simple and automated procedure employing SPE to extract and concentrate the residues from fish tissue prior to direct HPLC analysis.**

List of Antibiotics used in Aquaculture

Group	Compound	Comments
Sulphonamides	Sulphamerazine Sulphaimidine Sulfadimethoxine[1]	Bacteriostatic agents with broad-spectrum activity against furunculosis in salmonids (trout and salmon).
Potentiated Sulphonamide	Co-trimazine/Sulfatrim[1,2,3] (combination of trimetho-prim and sulfadiazine)	Used for treating diseases in salmon and trout (furunculosis, vibriosis and enteric red mouth).
Tetracyclines	Chlortetracycline Oxytetracycline[1,2,3,4]	Wide use in aquaculture. Effective against several fish pathogens and is relatively cheap. Used in salmon, trout, turbot and shrimp farming. Approved for prevention of "red tail" in lobsters in Canada.
Penicillins (Beta-lactams)	Ampicillin[4] Amoxycillin[2,4]	Used to treat furunculosis in salmon and rainbow trout fry syndrome (RTFS) in Europe.
	Benzyl penicillin[3]	Used for yellowtail and sea bream in Japan
Quinolones	Ciprofloxacin	Used in shrimp farms in Asia
	Enrofloxacin	Used in shrimp farms in Asia
	Norfloxacin Oxolinic acid[2,3,4] Perfloxacin Flumequine[3,4]	Used in shrimp farms in Asia
	Sarafloxacin[2]	EU MRL 150ug/kg fish muscle
Nitrofurans	Furazolidone	Broad-spectrum antimicrobial agent. Used in shrimp farms in Asia. Use discouraged as it is a potential carcinogen.
Macrolides	Erthromycin[4] Spiramycin	
Aminoglycosides	Gentamycin	
Other antibiotics	Chloramphenicol	Residues in foods may cause aplastic anaemia in man[5]. Use banned in the European Union.
	Florfenicol[1,3,4] Thiamephenicol[4] Tiamulin Nalidixic acid Milozacin	Used to treat RTFS and furunculosis in salmon.

1. *Use permitted in Canada (http://www.syndel.com/msds/canada_approved.htm)*
2. *Licensed for use in the UK (Alderman and Hastings 1998)*
3. *Use permitted in Norway (Alderman and Hastings 1998)*
4. *Use permitted in Japan (Okamoto 1992)*
5. *Tan (1999).*

www.fao.org/DOCREP/006/Y4743E/y4743e0e.htm

Fish Residue Instrumentation

- Gilson GX-271 ASPEC, Direct Inject with 50 uL loop, and 5 port preparative solvent delivery station flow rates up to 50 mL/min, septum piercing probe
- Gilson Orbital Shaker up to 700 RPM
- 3 mL/500 gm Chromabond SA, SPE cartridges, 60 Å, 45 um, 0.75 meq/g; strong acid cation exchange resin based on silica, Macherey-Nagel GmbH & Co. KG
- Gilson 322 H2, HPLC pump, 0.030- 30 mL/min
- Atlantis dC18, 5 um, 4.6 x 150 mm, Waters, Inc.
- Gilson UV/Vis variable wavelength detector, 210/254, analytical flow cell (5 mm path length); Sensitivity: 0.005/0.005
- Trilution LC® v1.4 data acquisition and liquid handler software

Overview of the Entire Extraction and Analysis Process

Manual:

- Mix 10 gm ground tissue (salmon) with 25 mL Ethyl acetate, sonicate 10 min below 40 ^{o}C, repeat x 2 with 25 mL ethyl acetate, rinse tissue with 25 mL ethyl acetate
- Mix the filtrates and filter through filter paper, smooth fluted, 313-folded, add 0.5 ml of acetic acid and fill to 100 mL with ethyl acetate

SPE Method

- Condition SPE cartridge with n-Hexane 2 x 3 mL, dry column
- Condition cartridge with Ethyl acetate (0.5% acetic acid), do not dry column
- Load sample through column, 5-8 mL/min, dry column 10-12 min
- Wash column with 10 mL Methanol
- Elute column with 5 mL 10% Triethanolamine in Methanol
- Sample can be analyzed directly by HPLC

GX-271 ASPEC

Positive Pressure Extraction

The probe introduces the sample or solvent to the SPE cartridge, once delivery is complete a gas valve is turned on by the software and Nitrogen is introduced to the cartridge which pushes the liquid through the cartridge. The time that the cartridge is exposed to the gas pressure is settable, hence DRYING or NOT DRYING the cartridge is not an issue and since every cartridge is treated exactly the same, consistency is optimized from the 1st cartridge to the last cartridge.

Mobile SPE Racks

- **The GX-ASPEC automated instruments have movable SPE racks that do not require additional electronics or gripper arms to move back and forth from the drain to the collection tubes. The probe itself moves the rack holding the SPE cartridges to its correct location. Individual steps are also available via the probe allowing a set of SPE cartridges to be placed over each row of collection tubes, in order to collect each step in the extraction process (optimization) or unique fractions based on classes of compounds.**

HPLC Method and Conditions:

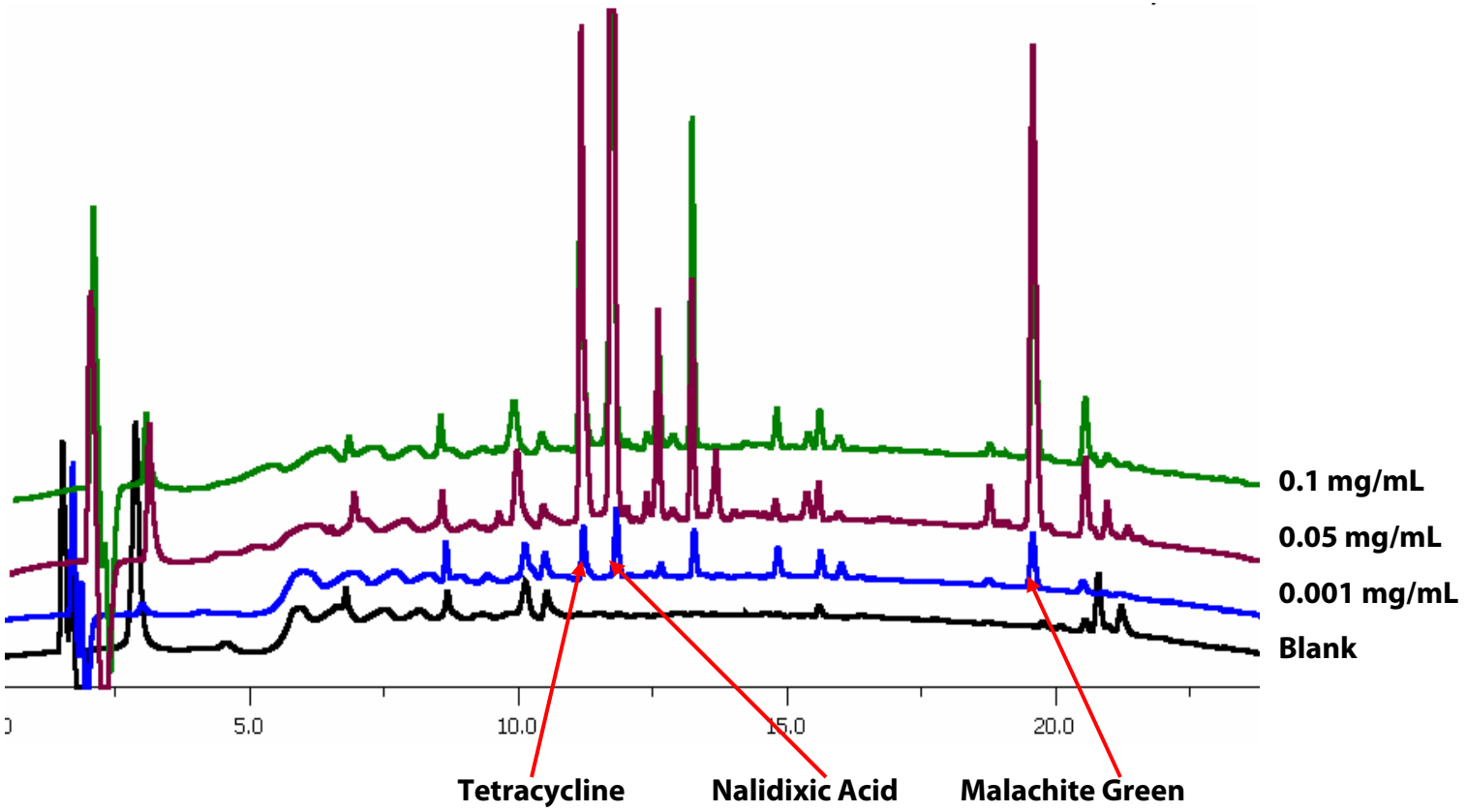
Time (min)	% A (Aqueous)	% B (Organic)
0	95	5
1.3	95	5
26.5	5	95
30.0	5	95
31.5	95	5
34.0	95	5

RP HPLC: Atlantis dC18
4.6 x 150 mm;

mobile phase:
Water(0.1% TFA) (A)
Acetonitrile(0.1%TFA) (B)

flow rate :
1.5mL/min, 50 uL injection

Chromatographic Analysis of Fortified Salmon



Data Analysis: Recoveries

- Tetracycline 104%
- Nalidixic Acid 85%
- Malachite Green 102%
  - Data was based on area counts relative to standard solutions made in acidic ethyl acetate and extracted by SPE
  - Recoveries are very consistent within and between days

Conclusion

- The automated SPE/HPLC presented was capable of extracting and analyzing the fish (salmon) for veterinary drug residues
- Recoveries for the three residues tested was consistent and the extraction yielded high recoveries (85-100%)
- The extraction process is time consuming, with required dry times and sample volumes of 100 mL, requiring a load rate 5-8 mL/min automating the process alleviated the tedious manual process and offered good results

- The capabilities of the system are not limited to SPE and HPLC analysis, but also include various liquid handling capabilities e.g. addition of acetic acid to the extracted fish sample
- Addition of peripherals, e.g. orbital shaker adds additional versatility to the system