Application News

High Performance Liquid Chromatography

Improving Data Reliability and Laboratory Efficiency Testing of Fluoroquinolones in Fatty and Non-Fatty Fish Samples with One Method using Automated Solid Phase Extraction and New Consumable Technologies

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Toni Hofhine, Horizon Technology, Inc., Salem, NH USA; Curtis Hedman and David Rogers, Wisconsin State Laboratory of Hygiene, Madison, WI, USA; Robert Buco, Richard Koeritz, and Zachary Lilla, Shimadzu Corporation, Marlborough, MA, USA; Michael McGinley, Phenomenex, Torrance, CA, USA; Rob Freeman, Ty Kahler, and Rick Lake, Restek, Bellefonte, PA, USA

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Introduction

The global demand for fish as a natural source of fresh animal protein, essential fats, minerals, and vitamins continues to rise with the human population. It is estimated that natural fish resources will not be sustainable, creating the need to increase the available supply of fish through aquaculture. According to the Food and Agricultural Organization of the United Nations (FAO), growth in the global fish supply was at an average growth rate of 3.2% per year during 1961–2009, outpacing the global population growth of 1.7% per year within the same time frame1. To keep up with the global demand, fish farming is projected to increase to an annual 6.4% growth rate through 2018, with a projected market value of $164 billion2.

Fish farming is a growing commodity; however, the regulation of small and large fish farms will be critical for food safety on a global scale. According to the FAO, 37% of the fish produced is internationally traded1. China leads the world in exports of aquaculture at 61%3, while the USA leads the world in imports of fish, with over 50% sourced from aquaculture4. Grown in close proximity, fish within an aquaculture environment can develop disease and impact aquaculture economic trade. The mechanisms for keeping fish healthy and free of disease is regulated within each country, often prompting the use of antibiotics in the water; however, the misuse of large antibiotic doses has increased attention on the impact to human health and thus the testing of aquaculture according to country specific food safety regulations.

Fluoroquinolones are a commonly used fluorine subclass group of quinolones that are effective against gram negative bacteria5. Popular for their use in aquaculture, fluoroquinolones consumed in high levels have contributed to the development of antibiotic resistance in the human population5,6. Concerns prompted the FDA to ban the use of fluoroquinolones in food producing animals in 19977. To regulate the use and level of fluoroquinolone antibiotics in fish from imports or aquaculture within the USA, the FDA routinely inspects domestic and imported aquaculture operations and recommends a standard method for the routine testing of four frequently used fluoroquinolone antibiotics in fish8.

Since the October 2003 implementation of the FDA regulatory method, many new consumable technologies and improved automated systems are widely available to increase laboratory efficiency and generate more confidence in obtained results. Through the use of more effective sample preparation consumables, automated solid phase extraction of fatty fish and non-fatty fish samples was performed using the SmartPrep® Extractor to compare overall recovery and matrix cleanup effectiveness from two solid phase extraction cartridges of differing sorbent properties. Sample extracts were analyzed on an uHPLC system using enhanced column sorbent properties. The results demonstrate improved recovery and superior chromatography peak shape for more reliable identification of fluoroquinolones in less run time than the standard FDA method.
Experimental
Fish samples were selected based on their fat content to provide both a low fat and high fat matrix for method development testing across a large range of fat content in fish. Only the fillets of each fish matrix were used for testing of fluoroquinolones. Fat content was tested separately following the grinding process for each matrix using gravimetric analysis. White bass fillets represented the low-fat fish matrix, with tested levels of fat content at 0.66%. Trout fillets represented the high-fat fish matrix, with tested levels of fat content at 18.2%.

Every effort was made to follow the existing steps and procedures outlined in the October 2003 fluoroquinolone FDA method, substituting consumables and thus respective solvents. Below is an overview of the multiple steps performed. Details for each step (pre-preparation, liquid-liquid extraction, solid phase extraction, evaporation, and analysis) are outlined below.

Instrumentation

- Solvents
  - Purified water—Milli-Q™
  - Solvents—laboratory grade
  - Acetic Acid—Sigma Aldrich, ≥99%
  - Ammonium Hydroxide—JT Baker, 30%
- Standards—stock solutions prepared in methanol; working solutions prepared in mobile phase
  - Ciprofloxacin HC1—Fluka, ≥98.0%
    - Note: 5 drops of 2% acetic acid solution was added prior to QS (i.e., a sufficient quantity) with methanol to dissolve standard
  - Enrofloxacin HC1—Fluka, ≥ 98.0%
  - Sarafloxacin HC1—Riedel-deHaen, 99.7%
  - Difloxacin HC1—Fluka, 99.0%
- Solutions
  - Extracting Solution—absolute ethanol:water:acetic acid (98 mL:1 mL:1 mL)
  - SPE Equilibration Solution—extracting solution + 1% acetic acid (35 mL +20 mL)
  - Eluting Solution—ammonium hydroxide + methanol (1 mL + 3 mL)
- Samples—acquired from Wisconsin lakes
  - White bass fillets
  - Trout fillets
- SPE
  - Horizon Technology SmartPrep Extractor—automated SPE cartridge system
  - Phenomenex SPE Cartridge—polymeric-based strong cation mixed mode sorbent
    - Strata™-X-C, 3 mL, 500 mg, 33 µm particle size, 85 Å pore size
    - Strata™-X-C, 3 mL, 500 mg, 100 µm particle size, 300 Å pore size
- uHPLC
  - Whatman nylon syringe filter, 13 mm, 0.45 micron
  - Shimadzu Nexera XR uHPLC system with Fluorescence detection
  - Restek Raptor™—Biphenyl, 2.7 µm, 2.1 mm x 50 mm
Sample Pre-Preparation and Storage

Whole white bass and trout fish were first filleted and the skin removed. The belly tissue portion was not included in the fillet. A meat grinder was used to further homogenize the fillet with a ±60 g sample taken and frozen. The second step further homogenized the sample by slightly thawing the sample prior to blending with an industrial blender using dry ice. The resulting tissue was fractured into 10-60 mesh-size frozen particles, allowing for thorough extraction efficiency of fats, proteins, and targeted contaminants with an extraction solvent. Before extracting, the dry ice was allowed to sublime overnight to eliminate a weight bias during extracting.

Sample Preparation—Liquid-Liquid Extraction Procedure

Each prepared fish matrix tested included a control sample (non-spiked sample) to test for the presence of any fluoroquinolones and to have a representative control chromatogram for each matrix. Fish samples were prepared according to the FDA method—weighed (2 grams) and then spiked for a fortification level of 20 ng/g of each fluoroquinolone.

Liquid-liquid extraction (LLE) was performed twice with extracting solution prior to an additional two LLE steps with 1% acetic acid. Procedure 1 indicates the steps followed according to the FDA method (blue) and the modified steps (green). Following each LLE step, the supernatant was decanted and combined. The resulting final LLE supernatant was ~80 mL. Photographs at each step show the difficulties in transferring the final supernatant (Figures 1-4).
Sample Preparation – Automated SmartPrep Extractor SPE Procedure

The SmartPrep Extractor automated the solid phase extraction process. For each SPE cartridge tested, a separate 2-gram fish sample was extracted. Each ~80-mL combined supernatant was automatically loaded onto separate Strata-X-C and Strata-XL-C cartridges using the SmartPrep Extractor (Procedure 2).

Where possible, the original October 2003 FDA method for solid phase extraction was followed; however, the use of new cartridge technology with differing pore sizes and particle sizes required a few modifications to enable proper extraction. The use of automation with the SmartPrep Extractor allowed the SPE process to be unattended.

The Strata-XL-C and Strata-X-C cartridges contain 500 mg of polymeric-based strong cation exchange sorbent to help clean up the zwitterionic properties of the four fluoroquinolones present in the fish samples. To properly wet the sorbent in each cartridge, two separate conditioning steps were used for methanol and the...

Procedure 2: SmartPrep Extractor Automated Solid Phase Extraction Procedure

![Figure 5: Phenomenex Strata-XL-C (top) and Strata-X-C (bottom)](image)

Figure 5: Phenomenex Strata-XL-C (top) and Strata-X-C (bottom)

![Figure 6: Linear regression of four fluoroquinolones](image)

Figure 6: Linear regression of four fluoroquinolones
Fish samples were spiked at a fortification level of 20 ppb for both high-fat and low-fat fish matrices. Spiked samples were injected between bracketed external standards representing a 5-point standard curve from 10–160 ppb (20–320 ng/mL). Linear regression of all four fluoroquinolones met the FDA criteria of >0.995 (Figure 6).

Prepared control fish samples were injected to obtain any levels of fluoroquinolone and to obtain a chromatogram for each matrix. Control samples did not contain any fluoroquinolones. Reagent spikes were performed on each cartridge to gauge recovery of the fluoroquinolones without fish matrix, allowing the sorbent retention to be evaluated using the original FDA SPE method steps (Figures 7 and 8). Recoveries from the reagent spikes showed that recoveries were consistent for both Strata-X-C and Strata-XL-C SPE cartridges (Table 2).

Recoveries from the reagent spikes were used to optimize the original FDA method for the low-fat and high-fat fish sample spikes. Reagent spike recoveries for Enrofloxacin, Sarafloxacin, and Difloxacin on both cartridges were within the FDA specified range of >60%. Ciprofloxacin showed a lower recovery value on both cartridges, despite meeting the FDA criteria with the Strata-XL-C cartridge. No changes were made to test recoveries of the four fluoroquinolones with the fish matrices. Resulting recoveries for both fish matrices dropped significantly using the original FDA method.

Sarafloxacin was the most difficult fluoroquinolone to extract, and as a result, was the compound that was used to mark progress over several method modifications for the LLE and SPE steps. The recoveries obtained from the Strata-X-C SPE cartridges did not improve with method modifications like the recoveries from the Strata-XL-C SPE cartridges. This is likely the result of the smaller pore and particle sorbent size not allowing the high fat and protein compounds to fully elute from the cartridge during the load and wash steps, causing the fluoroquinolones to be bound on the cartridge with the fat and protein compounds.

Further modifications to the LLE and SPE steps were performed using only the Strata-XL-C SPE cartridges. Under the same optimized LLE and SPE conditions, acceptable recoveries for both low fat and high fat fish spiked samples were obtained. Control samples and spiked samples are represented, along with the equivalent standard at 20 ppb. Extracted control matrices of low-fat and high-fat fish matrices extracted from the Strata-XL-C SPE cartridges did show some matrix effects. Low fat fish extracts contain an additional unknown peak at 3.4-3.5 minutes that was visible as a result of the improved sensitivity of the optimized HPLC method. While present, this unknown peak did not impact peak identification and quantitation (Figures 9-13).
Recoveries and %RSD values were within the required October 2003 FDA method specification levels for all fluoroquinolone peaks for both low-fat and high-fat spiked samples (Table 3).

Table 3: Recoveries of Four Fluoroquinolones at 20 ppb using Strata-XL-C SPE Cartridges

<table>
<thead>
<tr>
<th>n=3</th>
<th>Ciprofloxacin</th>
<th>Enrofloxacin</th>
<th>Sarafloxacin</th>
<th>Difloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish Matrix</td>
<td>Low Fat</td>
<td>High Fat</td>
<td>Low Fat</td>
<td>High Fat</td>
</tr>
<tr>
<td>% Recovery</td>
<td>92.5</td>
<td>87.5</td>
<td>121.3</td>
<td>129.9</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.88</td>
<td>1.90</td>
<td>0.42</td>
<td>3.94</td>
</tr>
</tbody>
</table>
Conclusion
The optimized method parameters showed both high fat and low fat fish samples were effectively recovered following LLE and SPE extraction steps. The larger pore and particle size of the Strata-XL-C SPE cartridges in combination with the SmartPrep Extractor System efficiently automated the combined LLE sample extracts to achieve consistent %RSD values across both high and low-fat fish matrices. Using new column technology with the uHPLC system improved peak resolution for increased sensitivity at the specified 20-ppb FDA monitored level. Elution of all four fluoroquinolone peaks occurred in 7 minutes compared to the 20 minutes using the original column technology (Table 4).

Table 4: Comparison of Original FDA Method Parameters to Optimized FDA Method Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Original FDA Method</th>
<th>Optimized FDA Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>&gt;0.995</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>Recovery – Low Limit</td>
<td>&gt;60%</td>
<td>&gt;87%</td>
</tr>
<tr>
<td>Recovery – High Limit</td>
<td>NA</td>
<td>&lt;130%</td>
</tr>
<tr>
<td>%RSD</td>
<td>&lt;20%</td>
<td>&lt;4%</td>
</tr>
<tr>
<td>Run Time (min)</td>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

The optimized method passed all FDA criteria for % recovery, % RSD and linearity within less chromatographic time and using current automation and consumable technology. Data presented concludes that while the four fluoroquinolones are structurally similar and exhibit zwitterionic properties, using current consumable chemistries improved overall method efficiency and effectiveness. Improving on work flow by implementing automation for the SPE process with the SmartPrep Extractor enabled objective sample handling across all samples by reducing scientist bias. This improved the reliability of results and the ability to transfer the method to multiple sites for similar results.

References