Applications of High Resolution MS to Veterinary Drug Residue Analysis in Aquaculture and Animal Feed

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Aquaculture

Growing industry
By 2030 over 50% of fish for human consumption will be supplied by aquaculture

Global industry
38% fish produced globally was exported in 2010
China and Southeast Asia major producers

Varied types of species
tilapia, shrimp, salmon, catfish, frog legs, eel

**FIGURE 3.2:** Volume and Share of Capture and Aquaculture Production in Global Harvest

<table>
<thead>
<tr>
<th></th>
<th>2011 (Data)</th>
<th>2030 (Projection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Capture</td>
<td>Aquaculture</td>
</tr>
<tr>
<td>Total harvest</td>
<td>63.6%</td>
<td>90.4%</td>
</tr>
<tr>
<td>154.0 million</td>
<td>tons</td>
<td></td>
</tr>
</tbody>
</table>

Sources: FishStat and IMPACT model projections.
Veterinary drug residues in aquaculture

Use:
To prevent spread of infection in dense populations

Approval:
• Very few drugs approved for aquaculture use in the US
• More approved in the EU and Japan
• Many more drugs potentially used in other countries

Potential human health effects:
• Acute and Chronic Effects
  Chloramphenicol – aplastic anemia
  Triphenylmethane dyes - carcinogenic
• Antimicrobial Resistance
High Resolution MS:
potential advantages for residue analysis

• Full scan data collection with accurate mass allows screening for virtually unlimited number of compounds.

• Don’t preselect analytes to monitor, so target and nontarget analytes are detected.

• Data can be evaluated retrospectively.

• Fragment ions can be obtained for further characterization of analyte.
Objectives for method to screen for drugs in aquaculture

- Develop analytical screening method for veterinary drug residues in fish using HRMS.

- Initially optimize and validate method for 70 test compounds most likely to be used in aquaculture.

- Use HRMS capability with vet drug database to screen samples for hundreds of additional compounds.
Extraction procedure

**Acidic acetonitrile (ACN) extraction**

- 2 g tissue
- Add 8 mL ACN with 0.2% p-toluene sulfonic acid and 2% glacial acetic acid
- Centrifuge

**OASIS HLB PRiME SPE (200 mg)**

- Pass 3 mL of extract through SPE
- Evaporate to near dryness
  - *(Save portion of eluent to analyze directly for nonpolar compounds)*

- Reconstitute in 400 μL 10% ACN in water
- Centrifuge
- Aliquot portion to LC vial
Data acquisition

**LC:** Thermo Ultimate 3000 LC system with C18 fused-core reversed-phase column. Mobile phase gradient 0.1 % formic acid and acetonitrile (ACN)

**MS:** Thermo Q-Exactive Orbitrap High Resolution MS with a heated electrospray source (using both classic QE and QE-HF)

Two types of acquisition programs were evaluated:

**Nontargeted:** collect product ion data for all precursor ions simultaneously **All Ion Fragmentation (AIF)** or sequentially by isolating segments of precursor ions **Data Independent Analysis (DIA)**

**Targeted:** isolate and collect product ion data only if targeted precursor ion on a list has abundance above threshold **Data Dependent MS² (DDMS²)** or always when analyte is eluting **Parallel Reaction Monitoring (PRM)** using inclusion lists
Data analysis

1) Initial NonTargeted Data Acquisition with AIF or DIA

a) Targeted Data Analysis: Limit testing and identification of test compounds
   ▪ Use “TraceFinder Quant” to analyze 70-100 test compounds
   ▪ Match 5 ppm window (MS\(^1\)), 0.5 min retention time, one fragment ion (10 ppm)
   ▪ Compare to matrix-extracted standard fortified with test compounds at TTL

b) Semi-targeted Data Analysis: Expand screening for more drug residues
   ▪ Use “TraceFinder Screening” to search against larger analyte database (N > 450)
   ▪ Use 3 ppm window with higher signal criteria to limit detections
   ▪ Compare RT and fragment ions if known

2) Additional Targeted Data Acquisition

Data Analysis of Product Ion Spectra
   ▪ Examine product ion spectra for analytes on inclusion list found in sample
   ▪ Use “TraceFinder “Quant” and “Screening” to compare residues to database
   ▪ Follow up with manual evaluation of spectral data and compare to known spectra

www.fda.gov
### Example MS\(^1\) data spiked sample

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quan Peak</th>
<th>Peak Area</th>
<th>RT</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>366.11182 m/z</td>
<td>235655</td>
<td>1.80 min</td>
<td>95 ng/g</td>
</tr>
<tr>
<td>Sulfathiazole</td>
<td>256.02089 m/z</td>
<td>4698824</td>
<td>3.63 min</td>
<td>9.5 ng/g</td>
</tr>
<tr>
<td>Sarafloxacin</td>
<td>386.13107 m/z</td>
<td>9974652</td>
<td>5.15 min</td>
<td>4.6 ng/g</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>435.29030 m/z</td>
<td>83163280</td>
<td>6.00 min</td>
<td>44 ng/g</td>
</tr>
<tr>
<td>Oxolinic acid</td>
<td>262.07100 m/z</td>
<td>23549248</td>
<td>6.49 min</td>
<td>9.5 ng/g</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>461.15546 m/z</td>
<td>19533822</td>
<td>4.46 min</td>
<td>93 ng/g</td>
</tr>
<tr>
<td>Leucomalachite green</td>
<td>331.21688 m/z</td>
<td>433792</td>
<td>8.20 min</td>
<td>0.78 ng/g</td>
</tr>
<tr>
<td>Methyl testosterone</td>
<td>303.23186 m/z</td>
<td>1883594</td>
<td>9.38 min</td>
<td>0.37 ng/g</td>
</tr>
<tr>
<td>Ivermectin B1a</td>
<td>897.49708 m/z</td>
<td>9208651</td>
<td>11.87 min</td>
<td>130 ng/g</td>
</tr>
</tbody>
</table>

**Tilapia spiked with 70 compounds at target testing level.**

MS\(^1\) data shown. Also collected MS\(^2\) data and evaluated time and isotopic match.
Spiked Tilapia 1X
Oxytetracycline (100 ng/g) Product Ion Spectrum

Known fragment ions for oxytetracycline

m/z 201.0546

m/z 201.05478

m/z 426.1183

m/z 426.11853

m/z 461.0555

m/z 461.15643

m/z 443.14499

m/z 443.14499

m/z 381.06061

m/z 337.07031

m/z 337.07031

m/z 307.06003

m/z 322.04709

m/z 350.04279

m/z 381.06061

m/z 283.06024

m/z 268.03708

m/z 239.07092

m/z 226.07172

m/z 214.07092

m/z 154.04984

m/z 100

m/z 50
Validation of method

Fortified samples:

• 70 validation compounds (60 positive ion; 10 negative ion) in 5 species, 2-3 sources for each species of fish

• Fortified at target testing level (1X) to determine threshold for limits test (Semi-quantitative screen with MS identification)

• Also fortified at 2X, 0.5X, and 0.1X to determine minimum detection levels and lowest confirmation levels

• Determined false positive and false negative rates; approximate recoveries compared to solvent standards

Based on

• FDA OFVM Guidelines for Validation of Chemical Methods v2
• Acceptance Criteria for Confirmation of Identity of Chemical Residues using Exact Mass Data for the FDA FVM Program

www.fda.gov

Comparison of data acquisition

Residues confirmed at 1X target testing level
Confirmed = MH⁺ (5 ppm), one fragment (10 ppm), RT match

Nontargeted
• > 90% validation compounds confirmed at 1X with AIF
• Most confirmed with AIF at much lower levels (0.1-0.5X of target testing level)
• Recently compared different DIA methods to AIF with similar results

Targeted
• ~ 70% of validation compounds depending on matrix with DDMS²
• Compounds with low target testing levels (dyes) or low method recovery (β-lactams) don’t meet threshold to trigger DDMS²
• Some confirmed at higher levels
• Recently compared PRM (limited # of compounds) to DDMS² w/better results

Continue to improve method by exploring different data acquisition methods
Comparison of scan types

Sulfadoxine 10 ng/g in spiked eel

EIC of MS1 (m/z 311.0809)

MS2 Spectra

AIF

vDIA

mDIA

PRM

DDMS

Submitted to Rapid Commun. MS (2019)
Application of HRMS screen

• Analyze incurred aquaculture samples obtained from CVM.
  - Analyzed dosed salmon, trout, catfish
  - Detected and characterized metabolites in addition to parent compounds
• Applied method to violative regulatory samples
• Include additional analytes in method (beyond veterinary drugs)
## Application of method: incurred fish

<table>
<thead>
<tr>
<th>Fish</th>
<th>Dosed with</th>
<th>Test Compounds found by HRMS Screen (ng/g)*</th>
<th>Other compounds found by HRMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tilapia</td>
<td>Sulfadiazine</td>
<td>Sulfadiazine (220)</td>
<td>N⁴ acetyl sulfadiazine, Ethoxyquin Dimer</td>
</tr>
<tr>
<td>Catfish</td>
<td>Enrofloxacin</td>
<td>Enrofloxacin (600)</td>
<td>Desethylene enrofloxacin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciprofloxacin (30)</td>
<td></td>
</tr>
<tr>
<td>Salmon</td>
<td>Difloxacin</td>
<td>Difloxacin (102)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Sarafloxacin (1)</em></td>
<td></td>
</tr>
<tr>
<td>Salmon</td>
<td>Doramectin</td>
<td><em>Doramectin (23)</em></td>
<td></td>
</tr>
<tr>
<td>Salmon</td>
<td>Malachite green, Brilliant green, Crystal violet</td>
<td>Malachite green (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leucomalachite green (0.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brilliant Green (4)</td>
<td></td>
</tr>
<tr>
<td>Trout</td>
<td>Ampicillin</td>
<td>Ampicillin (125)</td>
<td></td>
</tr>
<tr>
<td>Trout</td>
<td>Amoxicillin</td>
<td>Amoxicillin (90)</td>
<td>Amoxicillin diketone</td>
</tr>
</tbody>
</table>

*The concentration of test compounds found by HRMS screen compared well to values obtained by QqQ methods (when available)*

Amoxicillin incurred fish

AMOX MSMS (RT = 1.9)

AMOX DIKETONE MSMS (RT = 4.7)

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Application: Imported eel sample

- Farm raised eels are susceptible to the use of chemotherapeutics because they are raised in confined spaces (tanks or barrels).

- Multiple veterinary drug residues have been found in imported eel samples using targeted LC-MS/MS method (triple quadrupole).

- Can we use HRMS screening method to determine what other residues or chemical contaminants might we be missing?

**Application: Imported eel sample**

Presumptive positive for test compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quan Peak</th>
<th>RT</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfamethazine</td>
<td>279.09102 m/z</td>
<td>4.66 min</td>
<td>85 ng/g</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>360.17180 m/z</td>
<td>4.89 min</td>
<td>58 ng/g</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>332.14050 m/z</td>
<td>4.63 min</td>
<td>44 ng/g</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>291.14517 m/z</td>
<td>4.16 min</td>
<td>22 ng/g</td>
</tr>
<tr>
<td>Ethoxyquin</td>
<td>218.15394 m/z</td>
<td>7.58 min</td>
<td>87 ng/g</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>461.15546 m/z</td>
<td>4.50 min</td>
<td>1.8 ng/g</td>
</tr>
</tbody>
</table>

Other test compounds found (< 50% TTL)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quan Peak</th>
<th>RT</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lincomycin</td>
<td>407.22103 m/z</td>
<td>3.66 min</td>
<td>11 ng/g</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>461.15546 m/z</td>
<td>4.50 min</td>
<td>1.8 ng/g</td>
</tr>
</tbody>
</table>
Data from eel sample

From screening larger database compounds (N ~450):

**Ethoxyquin Dimer**
- AA: 3341515
- RT: 11.72 min
- m/z: 433.285 (433.285)
- D m/z (ppm): 0.05

**N4-acetyl-sulfamethazine**
- AA: 4372013
- RT: 5 min
- m/z: 321.1013 (321.1016)
- D m/z (ppm): -0.85

**Desethylene Enrofloxacin**
- AA: 1424542
- RT: 4.57 min
- m/z: 334.157 (334.1562)
- D m/z (ppm): 2.4
Targeted MS2 data from eel

Product Ions of Lincomycin

- \( \text{C}_{17}\text{H}_{31}\text{O}_{6}\text{N}_{2}^+ \) at \( 359.21686 \)
- \( \text{MH}^+ \) at \( 407.22086 \)
- \( \text{C}_{18}\text{H}_{35}\text{O}_{6}\text{S}^+ \)

Product Ions of Ethoxyquin dimer

- \( \text{C}_{12}\text{H}_{14}\text{ON}^+ \) at \( 188.10765 \)
- \( \text{C}_{14}\text{H}_{18}\text{ON}^+ \) at \( 216.13934 \)
- \( \text{C}_{14}\text{H}_{19}\text{ON}_2^+ \) at \( 231.14949 \)
- \( \text{C}_{24}\text{H}_{27}\text{O}_2\text{N}_2^+ \) at \( 375.20670 \)
- \( \text{C}_{24}\text{H}_{27}\text{O}_2\text{N}_2^+ \) at \( 333.16031 \)
- \( \text{MH}^+ \) at \( 433.28546 \)
- \( \text{C}_{28}\text{H}_{37}\text{O}_2\text{N}_2^+ \)
Retrospective data analysis of sample

- Other potential hits included **2-amino mebendazole (+)** but we did not initially have retention time or known fragment ions for this compound.

- After obtaining and analyzing standards of 2-amino mebendazole we reevaluated the data from eel samples. **2-amino mebendazole was confirmed** (time and fragment ions match)

![Chemical structure of 2-amino mebendazole]

2-amino mebendazole

![Graphs of 100 ng/mL std and Eel #1](https://example.com/graphs)
2-amino mebendazole in eel

Previous Work: Residue study of mebendazole and its metabolites in eel after bath treatment, *Drug Metab Disp*. 1997

2-amino mebendazole has since been added to routine FDA QqQ regulatory method

www.fda.gov
Data from imported fish sample

Enrofloxacin, > 3000 ng/g

Ciprofloxacin, ~500 ng/g

Ofloxacin, ? ng/g
Similar area counts to ciprofloxacin
Data from imported fish sample

Ofloxacin Std
100 ng/mL
1 x e^6 counts

Croaker Sample
2 x e^7 counts

Ofloxacin

Not typically used in aquaculture, although formulations are available on-line.

www.fda.gov
Residues in the environment

- Ofloxacin has also been found in sewage water and surface water in China and many other parts of the world.

- Environmental contamination could be another potential source of residues in fish.

Table 1. Concentrations of the Four Typical Fluoroquinolone Antibiotics Detected in the Sewage Water and Surface Water Samples

<table>
<thead>
<tr>
<th>compounds</th>
<th>Sibao STP (ng/L)</th>
<th>surface water (ng/L)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>influent</td>
<td>effluent</td>
<td>site 1</td>
</tr>
<tr>
<td>ofloxacin</td>
<td>1405</td>
<td>429</td>
<td>51.6</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>248</td>
<td>96</td>
<td>7.0</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>268</td>
<td>199</td>
<td>9.3</td>
</tr>
<tr>
<td>enrofloxacin</td>
<td>108</td>
<td>54</td>
<td>10.5</td>
</tr>
<tr>
<td>total FQs</td>
<td>2029</td>
<td>778</td>
<td>78.4</td>
</tr>
</tbody>
</table>

Tong et al. J Ag Food Chem (2011) 59, 7303
Expanding method
Validating for addition chemical contaminants

- **Disinfectants/Antimicrobial Soaps**
  - Benzalkonium chlorides, triclocarban, triclosan

- **Pesticides**
  - Few dozen likely to be found in aquaculture from agricultural run-off
  - LC-MS compounds

- **Human Pharmaceuticals/Emerging Contaminants**
  - Those commonly found in surface water
  - Includes drugs for depression, hypertension, pain

- **Additional Veterinary Drug Compounds**
  - More antibiotics, anti-wormers, etc.
Example: Atrazine in shrimp

1 ng/g Solvent Standard

Atrazine MS1
(m/z 216.10105)

RT: 8.19

NL: 1.50E6

NL: 5.00E5

1 ng/g Shrimp Spike

Atrazine MS1
(m/z 216.10105)

RT: 8.19

RT: 7.33

RT: 7.79

RT: 8.18

NL: 1.50E6

NL: 5.00E5

NL: 5.00E5

Shrimp Blank

Atrazine MS2
(m/z 174.05410, 96.05562)

RT: 8.18

RT: 6.99

RT: 7.31

RT: 8.13

NL: 1.50E6

NL: 5.00E5

NL: 5.00E5

(5 ppm window)
Example: Human drugs in tilapia

Caffeine
EIC m/z 195.08765

Fluoxetine
EIC m/z 310.14133

Simvastatin
EIC m/z 419.27920

10 ng/g Tilapia Spike

10 ng/g Tilapia Spike

10 ng/g Tilapia Spike

Tilapia Matrix Blank

Tilapia Matrix Blank

Tilapia Matrix Blank

MS$^1$ (5 ppm window) AIF

www.fda.gov
Expanding method
Validating for additional chemical contaminants

- 1,3-Dibromo-5,5-dimethylhydantoin
- 1,3-Dichloro-5,5-dimethylhydantoin
- Benzalkonium chlorides
- Triclocarban
- Triclosan
- Amitraz (degradant)
- Atrazine
- Azadirachtin
- Azamethiphos
- Benzocaine
- Carbaryl
- Carbofuran
- Cypermethrin
- Dichlorvos
- Etofenprox
- Fipronil/Fipronil sulfone
- Malathion
- Phoxim
- Praziquantel
- Propazine
- Quinalphos
- Simazine
- Trichlorfon
- Trichloroisocyanuric acid
- Trifluralin
- Quinocamine
- Atenolol
- Caffeine
- Carbamazepine
- Clarithromycin
- Clofibrate acid
- Diclofenac
- Diltiazem
- Diphenhydramine
- Fluoxetine
- Gemfibrozil
- Ibuprofen
- Metformin
- Naproxen
- Propranolol
- Ranitidine
- Sertraline
- Simvastatin
- Sotalol
- Valsartan
- Rifampin
- Aldicarb/Aldicarb sulfone/Aldicarb sulfoxide
- Methylene blue
- Acriflavine/Proflavine
- Rotenone
- Thiabendazole
- Sulfisoxazole
- Rifaximin
- Roxithromycin
- Marbofloxacin
- Orbifloxacin
- Baquilocprim
- Virginiamycin M1

- Initially ~ 60 additional compounds
- The majority worked well through the method, some were not detected, and others were detected only at higher levels
- Tested 4 different fish fortified at 100, 10 and 1 ng/g
- This increased the number of residues validated for our method and expands the scope of the type of contaminants we are monitoring for in aquaculture.

www.fda.gov

Food Addit. Contam. (2019)
Expanding method
Detection of additional chemical contaminants

Using HRMS screening method, several eel samples were initially presumptive positive for additional chemical contaminants. (HRMS identification criteria were met using non-targeted data acquisition)

• Further analysis (targeted MS² data acquisition, standard addition, analysis on separate QqQ method) confirmed thiabendazole (~ 6 ng/g) in one eel sample.

• Acriflavine was presumptive positive in many eel samples, but further analysis (targeted MS² data acquisition, standard addition) ruled out the presence of this compound.

• Trace levels (< 1 ng/g) of diltiazem were detected in another eel sample.
Expanding method
Acriflavine in Eel?

m/z 224.1182

Eel sample

Eel sample fortified at 10 ng/g

(10 ng/g std)

10 ng/g std

www.fda.gov
HRMS screening method for aquaculture

• HRMS screening method was able to identify test compounds in aquaculture at or below their target testing level.

• FDA Office of Foods and Vet Medicine guidance documents were followed to develop and validate methods.

• Detection and identification of other residues including metabolites demonstrated ability to expand screening in aquacultured products.

• Will begin to look at more nontargeted data analysis workflow

• Continue working to implement HRMS technology to improve enforcement of food safety.

www.fda.gov
HRMS methods for antibiotics and chemical contaminants in animal feed

“Analysis of veterinary drug and pesticide residues in animal feed by high-resolution mass spectrometry: comparison between time-of-flight and Orbitrap” (2015) Gómez-Pérez et al., Food Addit Contam A 32:1637

Table 4. Results obtained from the analysis of 18 feed samples. Concentrations expressed as µg kg⁻¹.

<table>
<thead>
<tr>
<th>Compound</th>
<th>M1</th>
<th>M2</th>
<th>M5</th>
<th>M11</th>
<th>M15</th>
<th>M16</th>
<th>M17</th>
<th>MRL ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos</td>
<td>52 (65)</td>
<td>18 (18)</td>
<td></td>
<td></td>
<td></td>
<td>75 (92)</td>
<td>148 (193)</td>
<td>5000 ²</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td></td>
<td></td>
<td>1053 (1114)</td>
<td>193 (217)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethoprim</td>
<td></td>
<td></td>
<td>311 (225)</td>
<td>157 (72)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Robenidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>36 (12)</td>
<td></td>
<td></td>
<td>6600</td>
</tr>
<tr>
<td>Monensin Na</td>
<td>144 (124)</td>
<td>715 (315)</td>
<td></td>
<td></td>
<td></td>
<td>142 (239)</td>
<td>141 (189)</td>
<td>1250</td>
</tr>
</tbody>
</table>

Notes: ¹ MRL, maximum residue level.
        ² Concentrations obtained with TOF are given in brackets.
        ³ Value provided for Codex Alimentarius for primary animal feed commodities.
        ⁴ EU MRL.

Similar strategies using HRMS have been used to monitor for chemical contaminants in animal feed
HRMS methods for antibiotics and chemical contaminants in animal feed


For post-target screening a customised theoretical database including the exact mass, the polarity of acquisition and the expected adducts was built and used for post-run retrospective screening. The analytical strategy was applied to 32 feed samples collected from farms of the Valencia Region (Spain). Florsenicol, zearalenone and atropine were identified and quantified at concentrations around 10 µg kg⁻¹. In the post-target screening of the real samples, Sulfadiazine, Thrimetoprin and Pir-imiphosmethyl were tentatively identified.

Another example of HRMS been used to monitor for chemical contaminants in animal feed
Acknowledgements

Co-authors (Animal Drugs Research Center/FDA Denver Laboratory)

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Thermo Scientific Application Scientists and Engineers
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