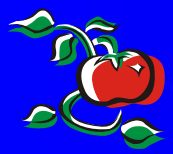
Determination of Emamectin Benzoate and other Avermectin Residues in Fish Tissues Using LC/MS

Dayue Shang, Angelo Di Cicco, Nicole Gibbons, Monica Dyck and Helen Nicolidakis

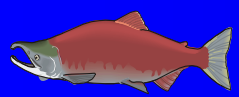
Health Canada, HPFB, Western Region Laboratory, Burnaby, B.C. V5G 4P2

Avermectins

- What are they?



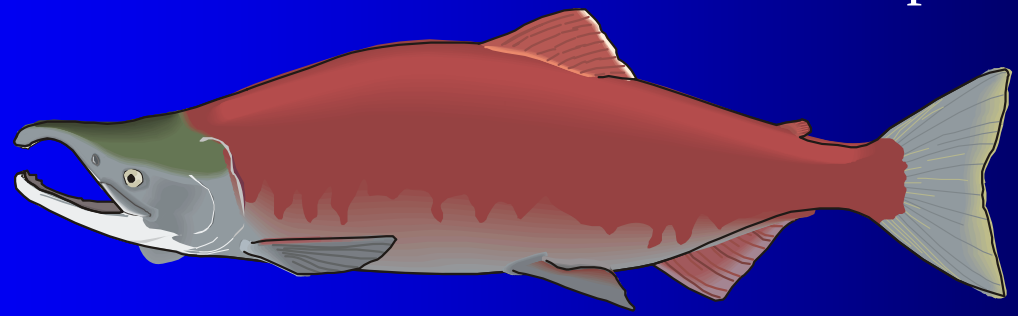
  - A group of compounds used as acaricides or parasiticides for animals or plants.



  - In aquaculture – emamectin and ivermectin are used to control sea lice in farmed salmon and trout.

The Sea Lice Issue

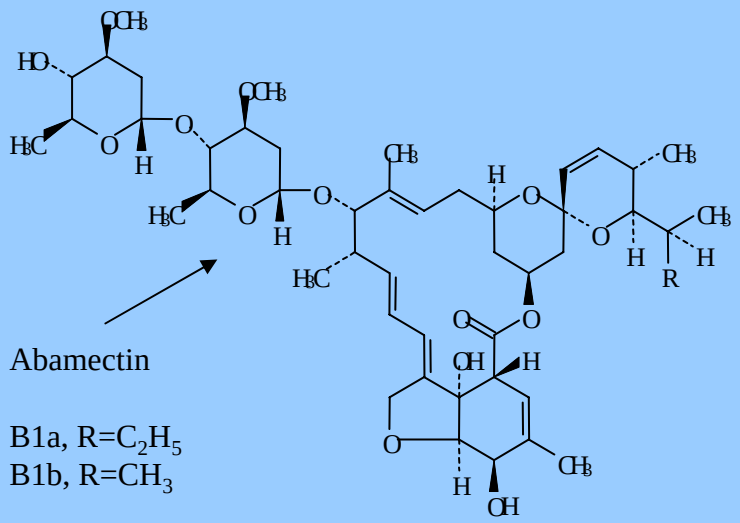
- Sea lice produce larvae which attach to salmon and feed on blood and mucous, causing serious damage and death to fish.
- Farmed fish in unnatural and crowded conditions may lead to more sea lice, which could have an impact on wild salmon.
- Ivermectin has been approved by VDD to treat sea lice infestation. Emamectin is still being evaluated by VDD, but can be used under the Emergency Drug Release program on a case by case basis.
- Human health and environmental impacts are being investigated.
- CFIA conducts routine tests of about 100 samples per year.



Health Canada's Concern

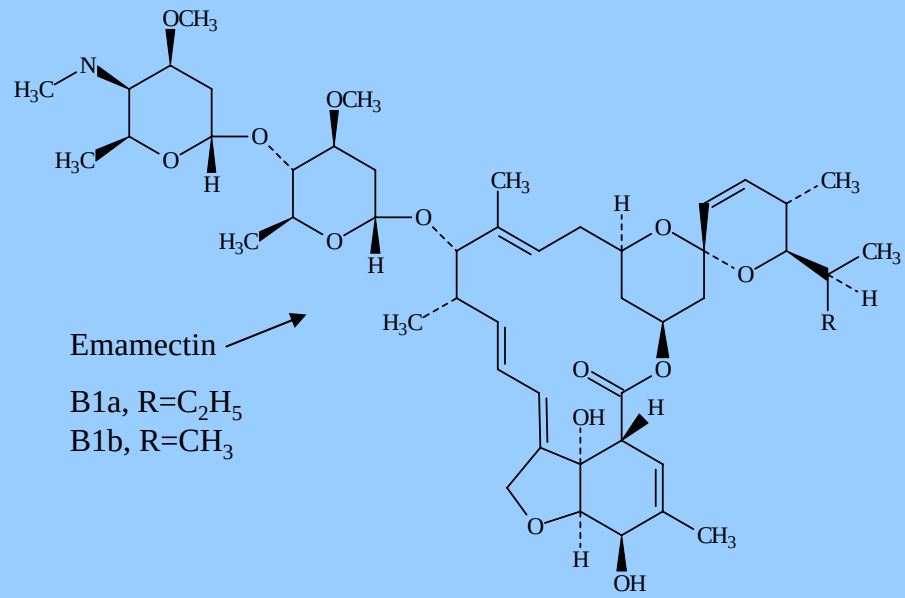
- Health Canada's concerns around avermectins:
  - Accumulation of residues over time
  - Long term effects, especially in people whose diet consists of considerable fish consumption
  - Effects on most vulnerable individuals, e.g. children and people with compromised immune systems

Chemical Structures



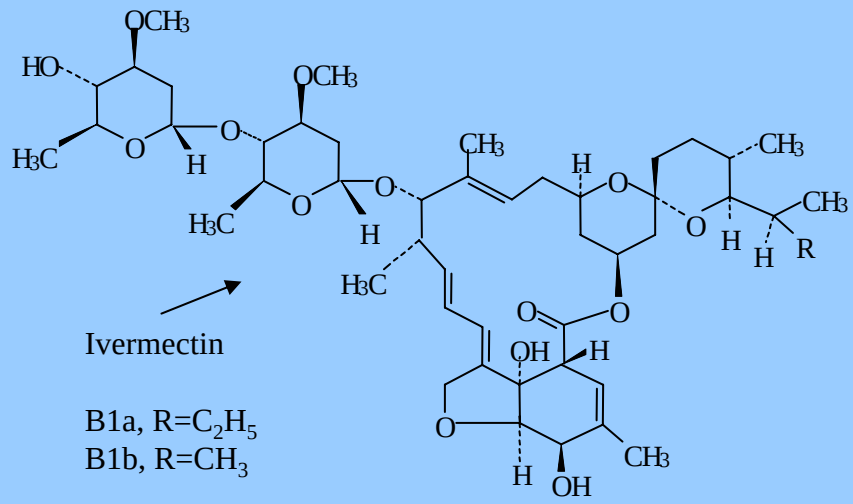
Abamectin

B1a, R=C_2H_5
B1b, R=CH_3



Emamectin

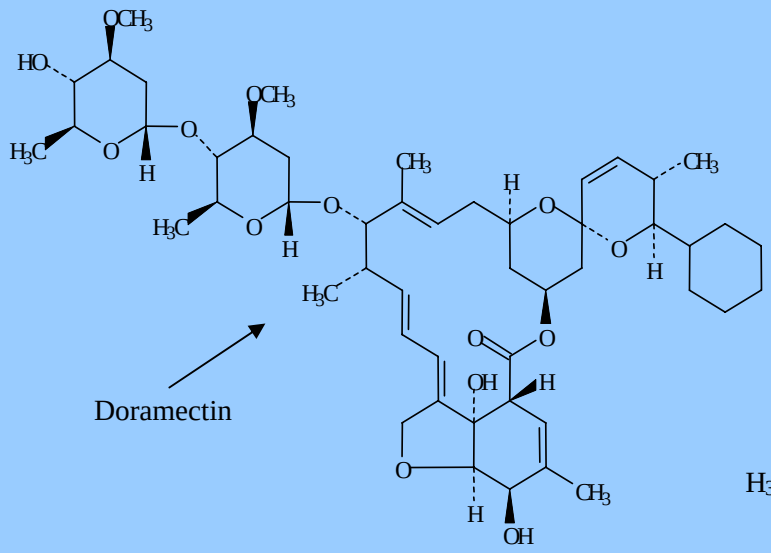
B1a, R=C_2H_5
B1b, R=CH_3



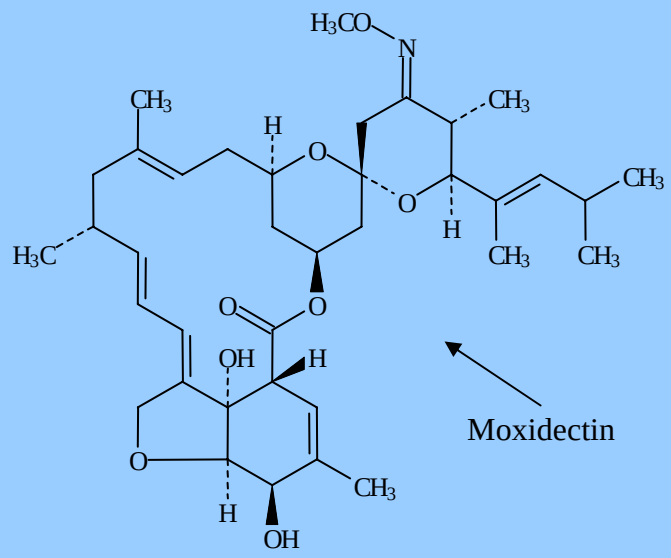
Ivermectin

B1a, R=C_2H_5
B1b, R=CH_3

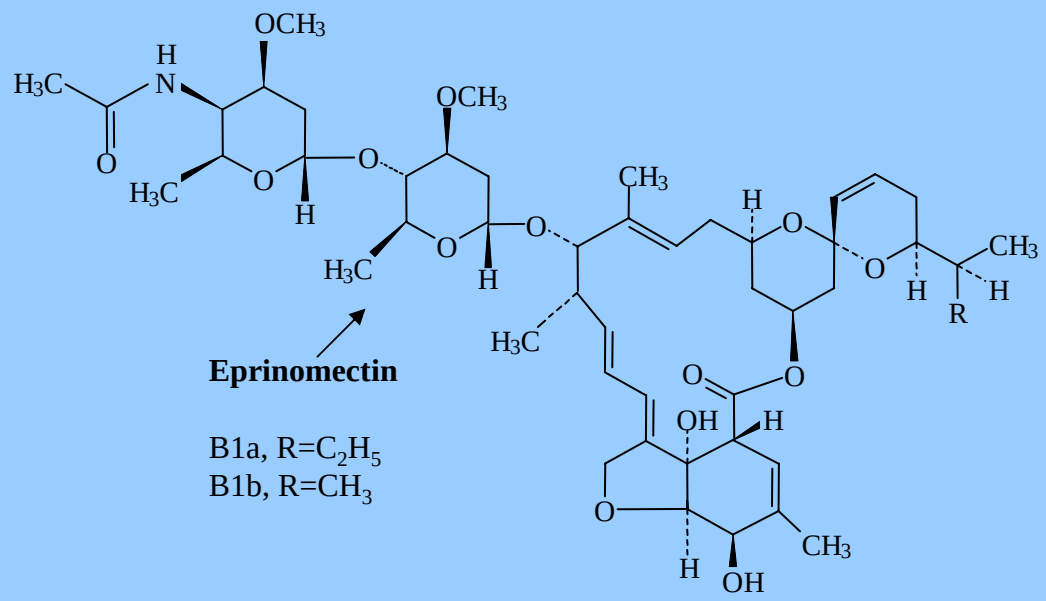
Chemical Structures



Doramectin



Moxidectin



Eprinomectin

B1a, R=C_2H_5
B1b, R=CH_3

Avermectins

- Some characteristics:
  - Very hydrophobic
  - Low toxicity
  - Unknown cumulative effects

Sample Preparation

- Urgent need to develop a so-called “universal” sample preparation method for emergency situations.
- More than two-thirds of the analysis time is spent on sample preparation.
- Approximately 30% of the analytical error stems from the sample preparation step.
- Optimizing the sample preparation procedure is crucial to the development of robust, reproducible, and accurate analytical methods.

Myth No. 1: SPE clean-up and concentration is always needed for a good LC/MS/MS method

- Fact: an SPE cartridge normally has 3 to 5 plates while a HPLC column has a plate number of about 10,000.
- Fact: it is often simply too much for a small SPE column to deal with the demand of a multi-residue method.
- Solution: avoid SPE as much as possible when developing a multi-residue method. Instead, use a solvent removal unit to concentrate samples, followed by a good chromatographic separation.

Myth No. 2: Only ASE, MSE, SFE, etc. can efficiently extract analytes from solid matrices

- Fact: many of the new apparatus require elaborate preparation steps, e.g. packing of 24 columns for ASE process.
- Fact: tubing and re-used cartridges are potential sources of cross contamination.
- Solution: design a method to test multiple solvents to find a good solvent system for the job. For trace analysis, disposable containers are recommended for sample preparation.

A review of sample preparation techniques

- From several review papers, the techniques used for the detection of over 100 pharmaceutical compounds in biological and environment matrices (100% = all cases):
  - 98 % liquid-liquid (L-L)
  - 77 % protein precipitation
  - 45 % SPE
  - 26 % on-line and automated SPE
  - 8 % MSPE
  - 3 % ASE
  - 2 % Microwave
  - 1 % Other (MSPD, SFE, etc)
- Your best bet: ---------

Myth No. 3: Liquid-liquid extraction has limited use when dealing with solid matrices

- Fact: solid matrices usually can be liquefied with the help of homogenizers, and liquid samples may be laden with solid particulates.
- Fact: L-L has been used in the determination of trace organics in solid samples for many years, eg. PCBs and Dioxins.
- Solution: design a method to test multiple solvents and buffers to find a good solvent system; miniaturize the L-L process using disposable centrifuge tubes.

Factors to consider in developing a L-L extraction method

- All these factors have to be considered together:
  - Distribution coefficient
  - Solvent miscibility
  - pH adjustment
  - Temperature
  - Fatty substance
  - Solvent toxicity
  - Agitation
  - Solvent removal

A Systematic Approach

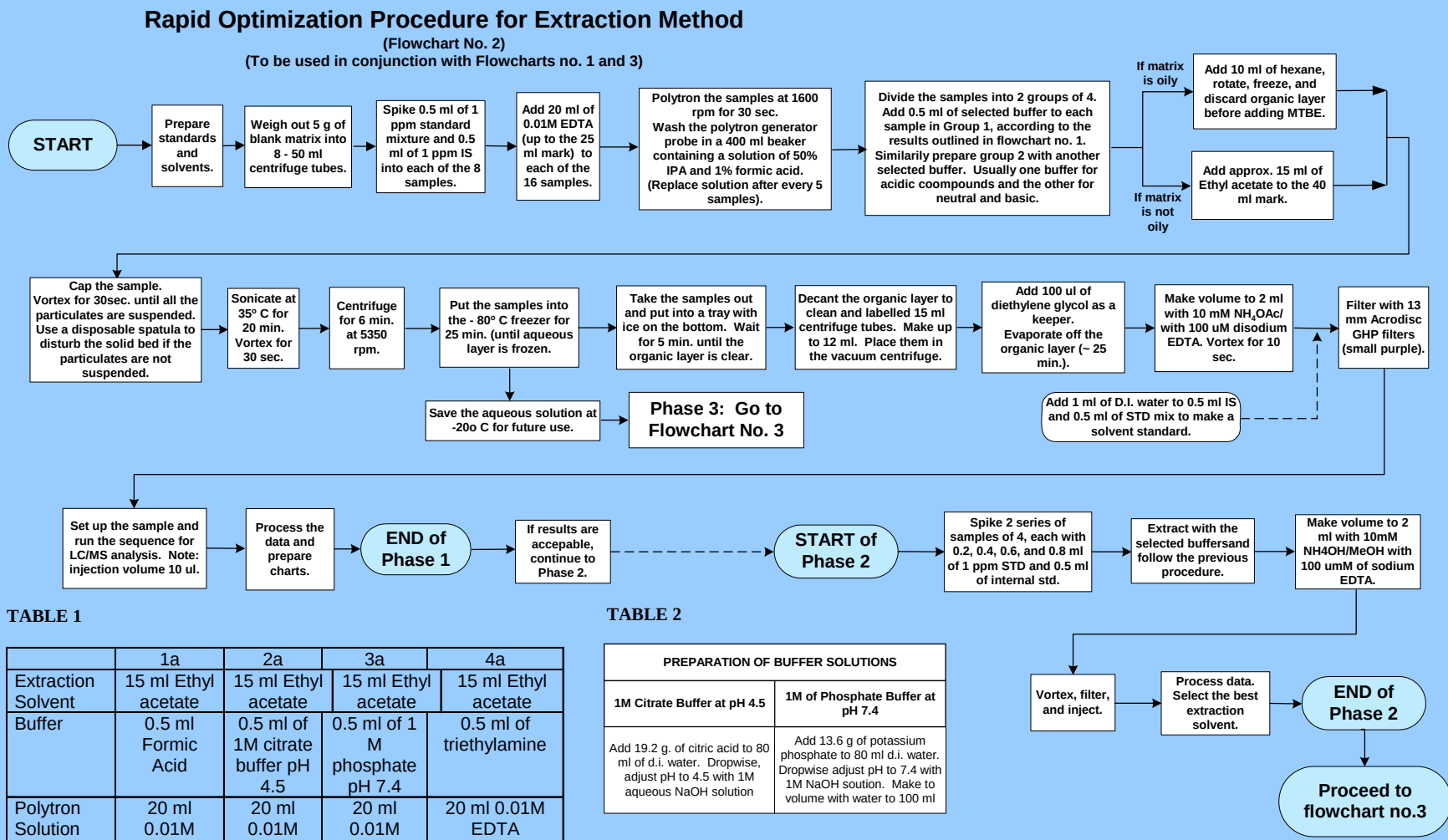


TABLE 1

	1a	2a	3a	4a
Extraction Solvent	15 ml Ethyl acetate	15 ml Ethyl acetate	15 ml Ethyl acetate	15 ml Ethyl acetate
Buffer	0.5 ml Formic Acid	0.5 ml of 1M citrate buffer pH 4.5	0.5 ml of 1 M phosphate pH 7.4	0.5 ml of triethylamine
Polytron Solution	20 ml 0.01M EDTA	20 ml 0.01M EDTA	20 ml 0.01M EDTA	20 ml 0.01M EDTA

TABLE 2

PREPARATION OF BUFFER SOLUTIONS	
1M Citrate Buffer at pH 4.5	**1M of Phosphate Buffer at pH 7.4**
Add 19.2 g. of citric acid to 80 ml of d.i. water. Dropwise, adjust pH to 4.5 with 1M aqueous NaOH solution	Add 13.6 g of potassium phosphate to 80 ml d.i. water. Dropwise adjust pH to 7.4 with 1M NaOH soution. Make to volume with water to 100 ml

Buffers Rule the L-L Extraction

	1a	2a	3a	4a
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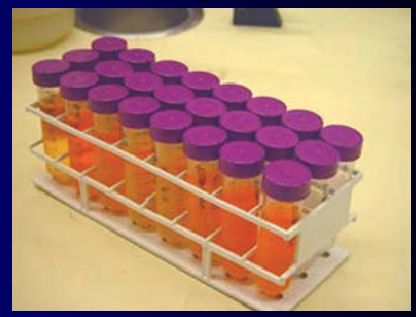
Buffer	0.5 ml Formic Acid	0.5 ml of 1M citrate buffer pH 4.5	0.5 ml of 1 M phosphate pH 7.4	0.5 ml of triethylamine
Polytron Solution	20 ml 0.01M EDTA	20 ml 0.01M EDTA	20 ml 0.01M EDTA	20 ml 0.01M EDTA

Sample Preparation

- Weigh 5 g of sample into 50 ml centrifuge tube
- Add 30 ml of 50% acetonitrile with 10 mM EDTA, then add 0.5 mL of 1 M citrate buffer (pH 4.5)
- Polytron 30 seconds
- Sonicate sample for 20 minutes at 35 °C, vortex 30 seconds
- Centrifuge for 4 minutes at 5000 rpm
- Transfer supernatant to another 50 ml centrifuge tube.

Sample Preparation (cont'd)

- Reduce the extract to 10 mL with SpeedVac
- Add 15 mL of ethyl acetate. Vortex 30 seconds
- Freeze the sample at – 80°C for 25 minutes
- Decant the upper layer to a 15 mL centrifuge tube
- Reduce the extract to near dryness. Make up to 4 mL with 50% ACN, vortex 10 seconds
- Filter 1.5 mL of sample with 0.2 micron GHP Acrodisc filter into an autosampler vial
- Note: no SPE is used in the sample prep, which saves a lot of time. Up to 32 samples can be processed in one day by one chemist



Internal Standard

- Doramectin was used as an internal standard.
- Doramectin is also a member of the avermectins family but is seldom used in applications, which makes it ideal as an internal standard.
- Amount of doramectin spiked = 0.1 ppm
  - Good signal response
  - Good S/N
- All samples, blanks, and standards were spiked with IS at the beginning of the analysis.

Agilent 1100 HPLC Parematers

Mobile Phase A	H_2O + 0.1% formic acid
Mobile Phase B	ACN + 0.1% formic acid
Flow Rate	0.25 mL/min
Column Temperature	30°C
LC Column	Zorbax SB-C8, 2.1 x 100 mm, 3.5µm
Guard Column	Javelin Basic C8, 2.1 X 10 mm, 5µm
Injection Volume	60 µL

Gradient Time (min)	0	2.5	2.6	6.0	6.1	10
%B (ACN+0.1% formic acid)	5	90	95	95	5	5

Avermectin Ions

Compound	M+1	Na adduct
Emamectin	**886.3**	908.3
Eprinomectin	913.3	**936.3**
Doramectin (IS)	898.3	**921.3**
Ivermectin	874.3	**897.3**
Abamectin	872.3	**895.3**
Moxidectin	639.4	**662.4**

Quattro II MS Parameters (SIR)

Ion Mode	ESI +
Capillary	3.00 kV
Cone Voltage	50–80 V *
Ion Energy	2 eV
Source Temperature	120°C
Desolvation Temperature	400°C
Cone Gas	100 L/hr
Desolvation Gas	500 L/hr

* Compound specific

Batch Analysis for Validation and for Sample Analysis

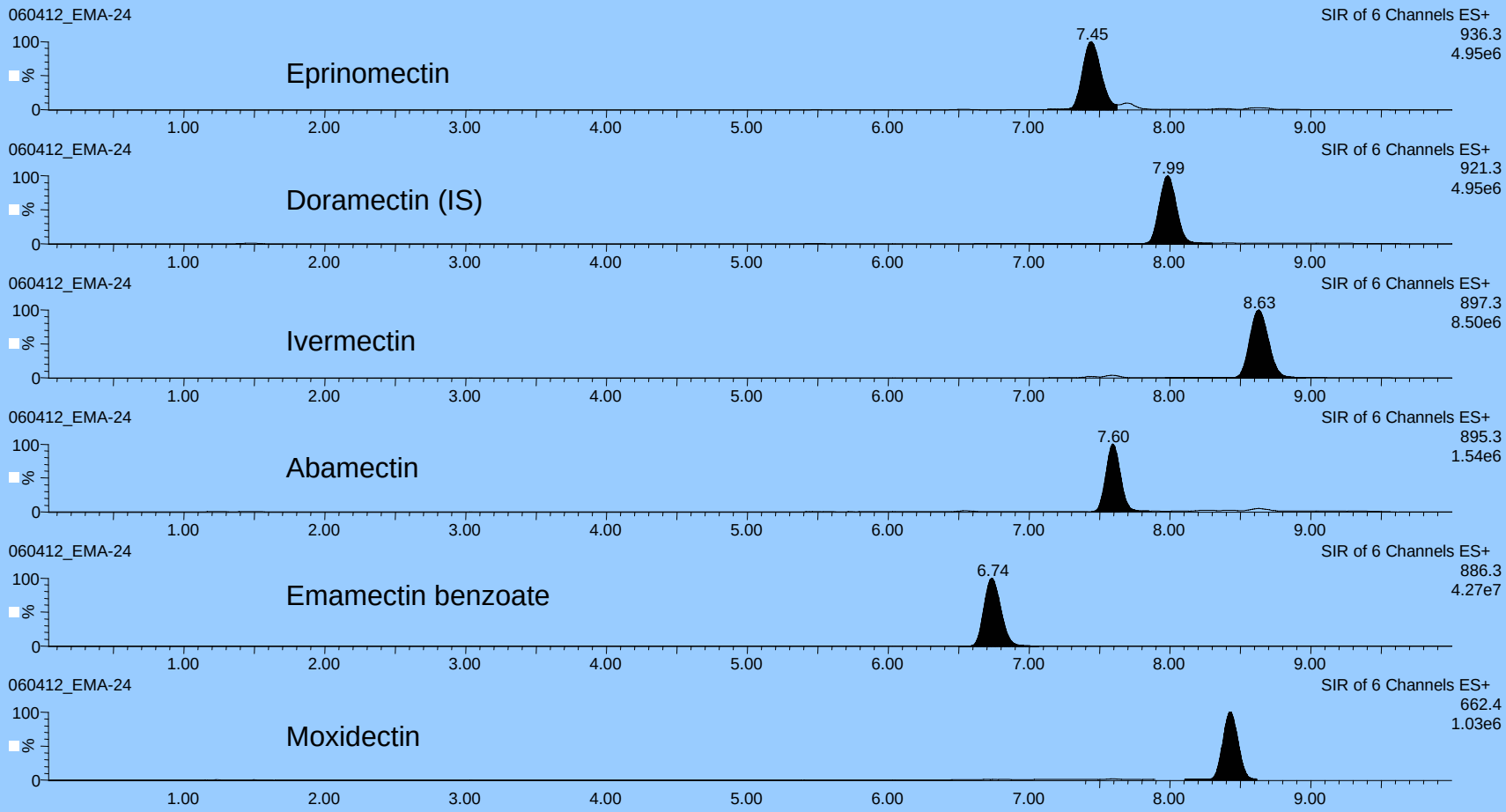
- 6 matrix standards at various levels ranging from 10 ppb to 400 ppb
- 3 QC spikes at 50 ppb
- 3 sample blanks
- 20 real world samples in each batch of samples run

By eliminating SPE, method robustness is improved, and it is possible to process 20 real world samples plus QA/QC samples in one batch.

Lower Limit of Quantitation, Precision, Recovery and Reproducibility

- Lower limit of quantitation (LLOQ) for all five avermectins was 10 ppb.
- The recovery of the avermectins was between 60 –140 %
- The relative standard deviation of the pre-validation run was within 20%.

Chromatography of Avermectins



Standard mix at 1 ppm

Summary

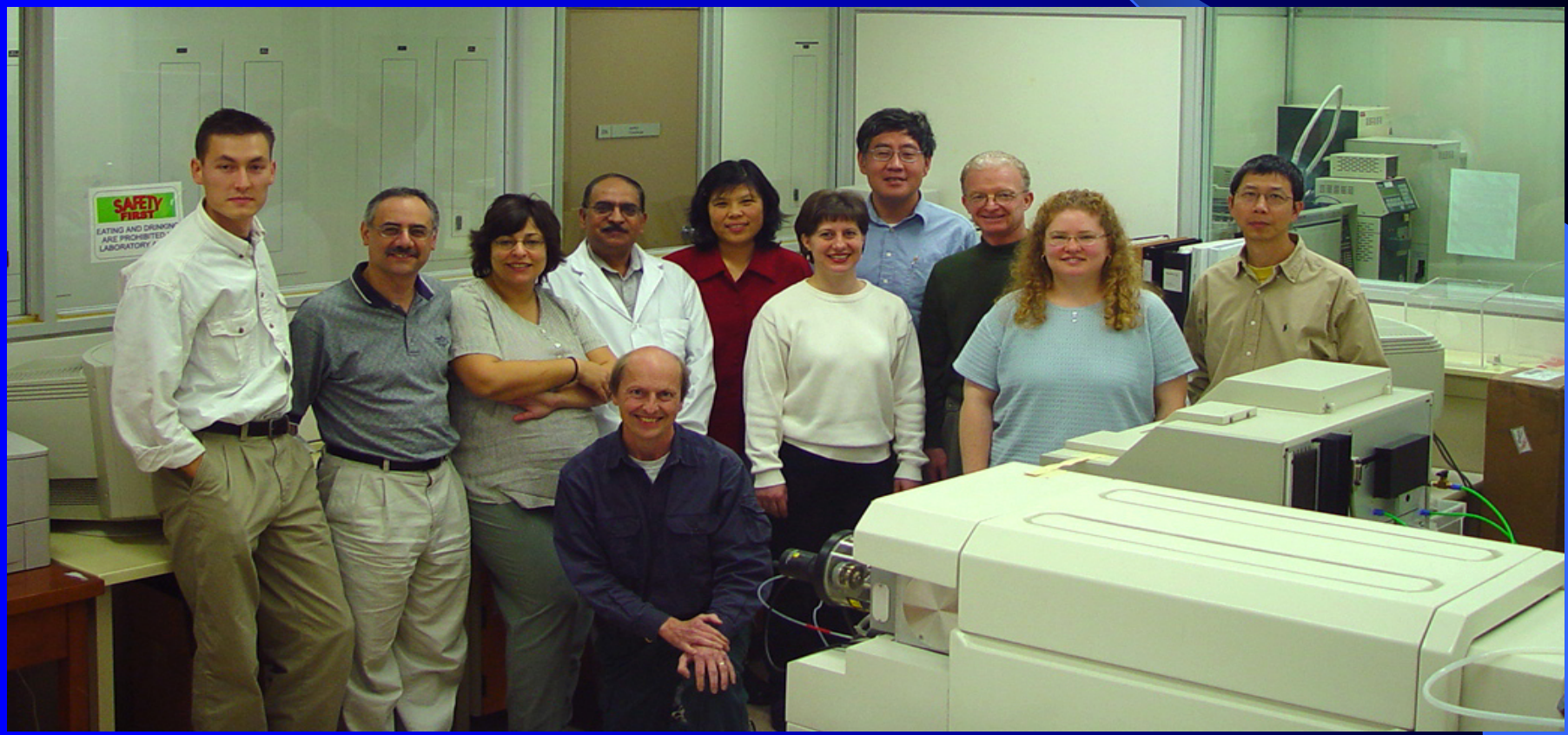
- A multi-residue LC/MS method was developed for the quantitative determination of 5 avermectins in fish tissues.
- Traditional liquid-liquid extraction technique compared favourably with some of the modern instrument based techniques.
- A systematic approach for optimization of extraction procedure was used, resulting in a simple and rapid method and allowing up to 20 real world samples to be processed by one chemist on a daily basis.
- Unlike most other similar methods, this method does not require solid phase extraction. In this way, a potential source of cross contamination is eliminated, sample turn out becomes much faster and more stringent quality control can be applied.
- A systematic method development strategy was implemented, significantly shortening the time for optimization of LC/MS parameters.

Future Work

- Further improve the LOD and LOQ of the method by using LC/MS/MS.
- Use Lithium in the mobile phase to produce Li adducts; preliminary results show Li adducts are stable, and that significant stable fragments can be produced.
- Extend the validation from salmon tissues to other animal tissues, vegetables, milk and fruits.
- Participate in proficiency tests for analysis of endectocides in animal tissue, organized by the CFIA.
- Discuss with stakeholders (CFIA, Kitasoo First Nation, and BCAFN) to decide on a sampling plan for a fish survey.

Acknowledgements

We gratefully acknowledge the invaluable support and input of our colleagues at the Western Region Laboratory of Health Products and Food Branch, Health Canada.



Next Generation of Chemists