Routine Monitoring of Shellfish Biotoxins at Cefas

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Food Safety Group

Cefas, Weymouth

Cefas

Centre for Environment, Fisheries and Aquaculture Science (Cefas)

- Established over 100 years
- UK Government's foremost source of marine evidence and applied science
- >500 professional staff
- Two UK laboratories + research vessel
- >160 peer reviewed papers each year
- Fisheries
- Aquaculture
- International government capability development
- Marine and coastal infrastructure
- Nuclear energy
- Offshore renewable energy
- Oil and gas
- Shipping

Lowestoft: 400 Colleagues

Weymouth: 140 Colleagues

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World Class Science for the Marine and Freshwater Environment

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Groups at Weymouth

- Aquatic Animal Disease
  - Epidemiology
  - Non Native Species
- Environment and Animal Health
  - Ecotox, welfare, endocrine disruption, pollution
- Food Safety
  - Shellfish: viruses, bacteria, Harmful Algal Blooms (biotoxins)

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Overview

- Introduction
  - What we Deliver?
  - How we deliver?
- Biotoxin monitoring programme
  - What We Test for?
  - How we do the tests?
- PSP analysis at Cefas
  - Implementation strategy
  - Developments
  - Alternatives

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How Biotoxin Programme is managed

Political

Dictates Programme of work

European Union

Food Standards Agency
&
Food Standards Scotland
(Competent Authorities)

Technical

Control of methodologies

European Union

European Reference Lab (EURL MB)
(Vigo, Spain,)

Reference lab
(AFBI N Ireland)

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Official Control Monitoring

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Biotoxin Monitoring Programme at Cefas

Shellfish monitoring programme

Shellfish are collected from pre-determined monitoring points and posted to Cefas

Phytoplankton monitoring programme

Water samples are collected from pre-determined sites enumeration of potentially Toxic Phytoplankton

2 Contracts with (competent authorities)

Food Standards Agency
&
Food Standards Scotland

- >200 sites
- >3,500 samples per year
- Covering all of GB
- 5-55 per day

Overview of the programme

- Samples received at Cefas daily
- Shellfish shucked, >100g tissue homogenised
- Samples aliquoted for each test
- Extraction, clean-up, derivatisation (PSP)
- Analysis overnight
- Results reported next day (PSP +ve 2 days)
  - Results > Maximum Permitted Limit = shellfish beds closed for harvest
  - Two consecutive <Regulatory Limit to re-open

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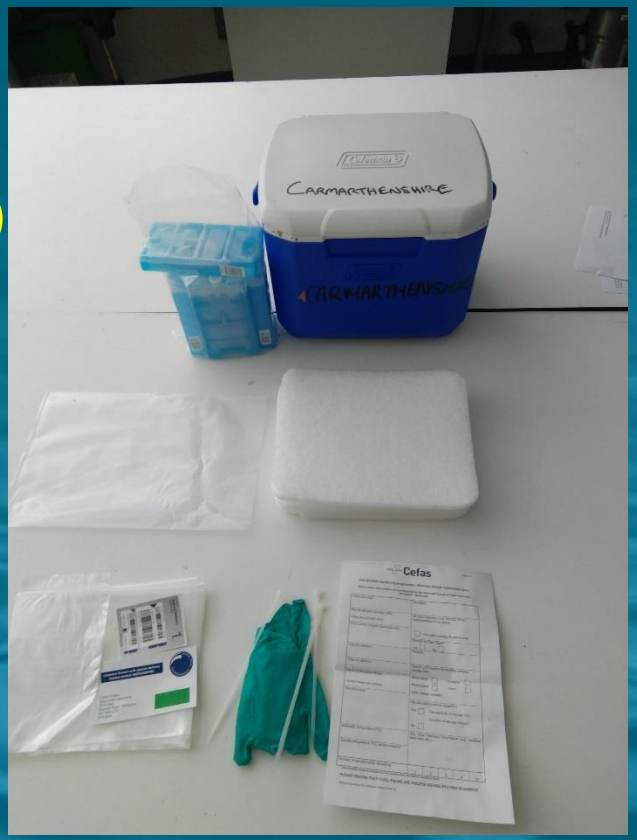
Sample Logistics

- Collected and dispatched by Local Authorities
- Postage via Royal Mail
- Usually received within 24 hrs of dispatch
- “Coleman” picnic boxes used
  - 6 ice packs and foam spacers required
- Validated for temp control (samples must be live)
- Posted back to sampling officers
- Each box contains a sampling kit
  - Gloves
  - Paper Work
  - Plastic Bag
  - Zip ties
  - Cool packs (to be frozen)
  - Foam insulation spacers



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Laboratories

- Post Mortem room
  - ($50m^2$)
  - Shucking
- Laboratories x2
  - ($50m^2$ x2)
  - Sample processing
- Robot cupboard
  ($10m^2$) SPE clean-up
- Analytical suite ($50m^2$)

Approximate total floor space:
$200m^2$

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Analytical Hardware

- 3x Gilson Aspec (SPE and fractionation)
  - Sample clean-up
  - PSP (C18, COOH and de-salting methods)
- 4x Agilent 1200
  - PSP & ASP analysis
- 2x Waters Acquity & MS/MS Xevo TQ/TQs
  - LT analysis
  - Upgraded auto sampler
- 1x Agilent 1290 Infinity 2 & 6495B MS/MS
- 3-4 instruments used for routine monitoring daily
- Other instruments are contingency and used for method refinement, research or QC investigations

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Staff requirements

Team of 20ish staff

Project manager	x1
Principal Chemist	x1
Senior Chemist	x1
Project Co-ordinators:	x2
Analytical Chemists:	x5
Laboratory analysts:	x4
Shucking assistants:	x3
Students:	x2-3

- Most staff are not working full time in the team
- Minimum of 5 full time staff can deliver contractual obligations

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Cefas current methods

- ASP Amnesico
  - HPLC-UV
  - Used since 2001
- PSP Paralizante
  - Lawrence, AOAC 2005.06 (PreCOX)
  - HPLC-FLD
  - Used since 2006
- DSP Diarreico
  - LCMS/MS EU reference method Used since 2011

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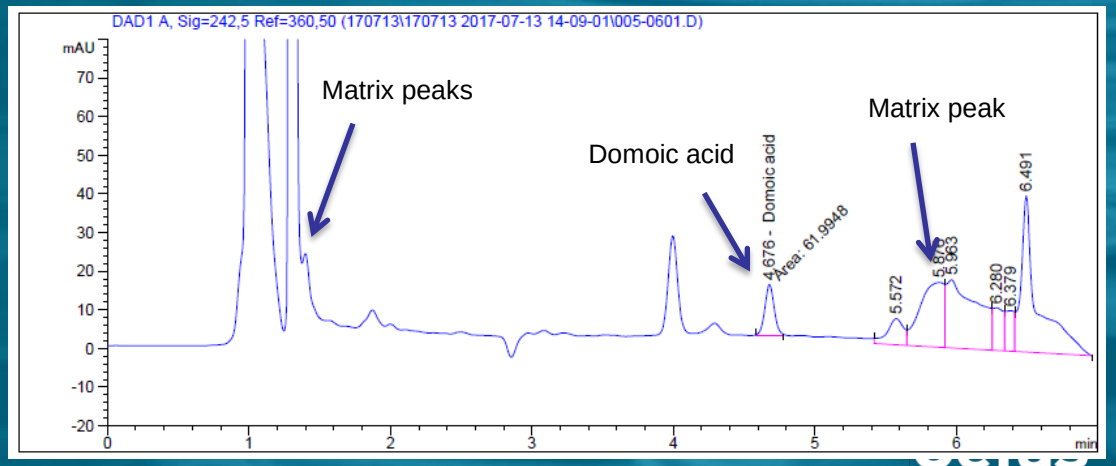
ASP

- 2 Compounds only
  - Domoic acid
  - Epi-domoic acid
- EU reference method: HPLC-UV
- Shellfish + 50% Methanol extraction
- No SPE clean-up
- Simple and reliable
- 7 min cycle

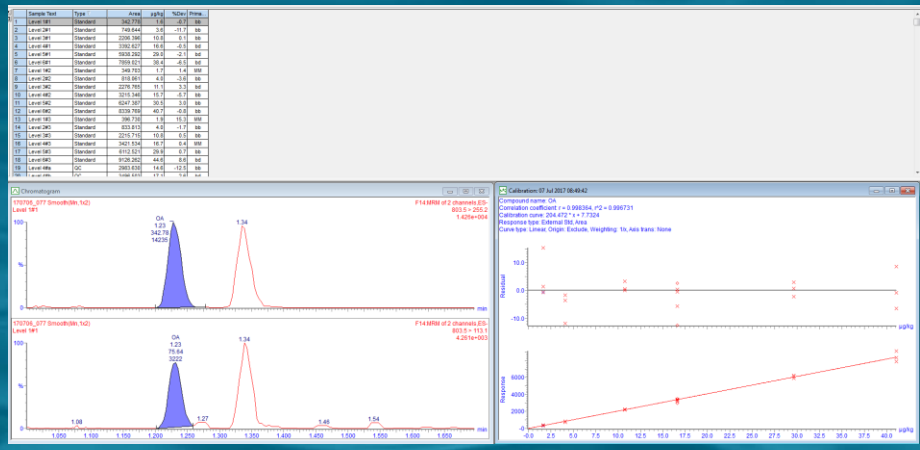
HPLC-UV Chromatogram



LC-MS/MS for Lipophilic Toxins

From 1st July 2011

- EU Reference Method
- Alkaline method
  - 5 min cycle
  - 2 injections per sample
    - Hydrolysed & Unhydrolyzed
  - 3 groups of toxins
    - OA, AZA, YTX
  - Can be challenging
    - Standard peak area drift
    - Instrument Reliability
    - Chromatographic issues
    - Poor performance in –ve mode



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PSP Analysis At Cefas

Only 2 Toxic species present in GB waters

1: Alexandrium tamarense:

- Usually Scotland (recently observed in Wales)
- Spreads from East to west in early summer
- Toxins: GTX1&4, NeoSTX, C1&2, GTX2&3 and STX
- Requires fractionation prior to analysis

2: Alexandrium minutum:

- *South west England*
- *Occurs later in the summer*
- *GTX2&3 and STX*
- *Does not require fractionation*

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Pre 2006:
MBA (Mouse Bio Assay)

- *Also known as AOAC 959.08*
- Used for all samples tested for PSP
- 2-3 tests per sample
- 1000 samples per year
  - 90% requiring PSP analysis
  - >3000 animals

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Why Stop Using The MBA?

- Methodological issues:
  - No toxin profile data
  - Potential for False Positives
  - Poor reproducibility
  - High Limit of Detection
- Ethical Issues
- Governmental pressure
  - In 2006 demanded immediate reduction in MBA
  - Threat of withdrawal of animal testing licence

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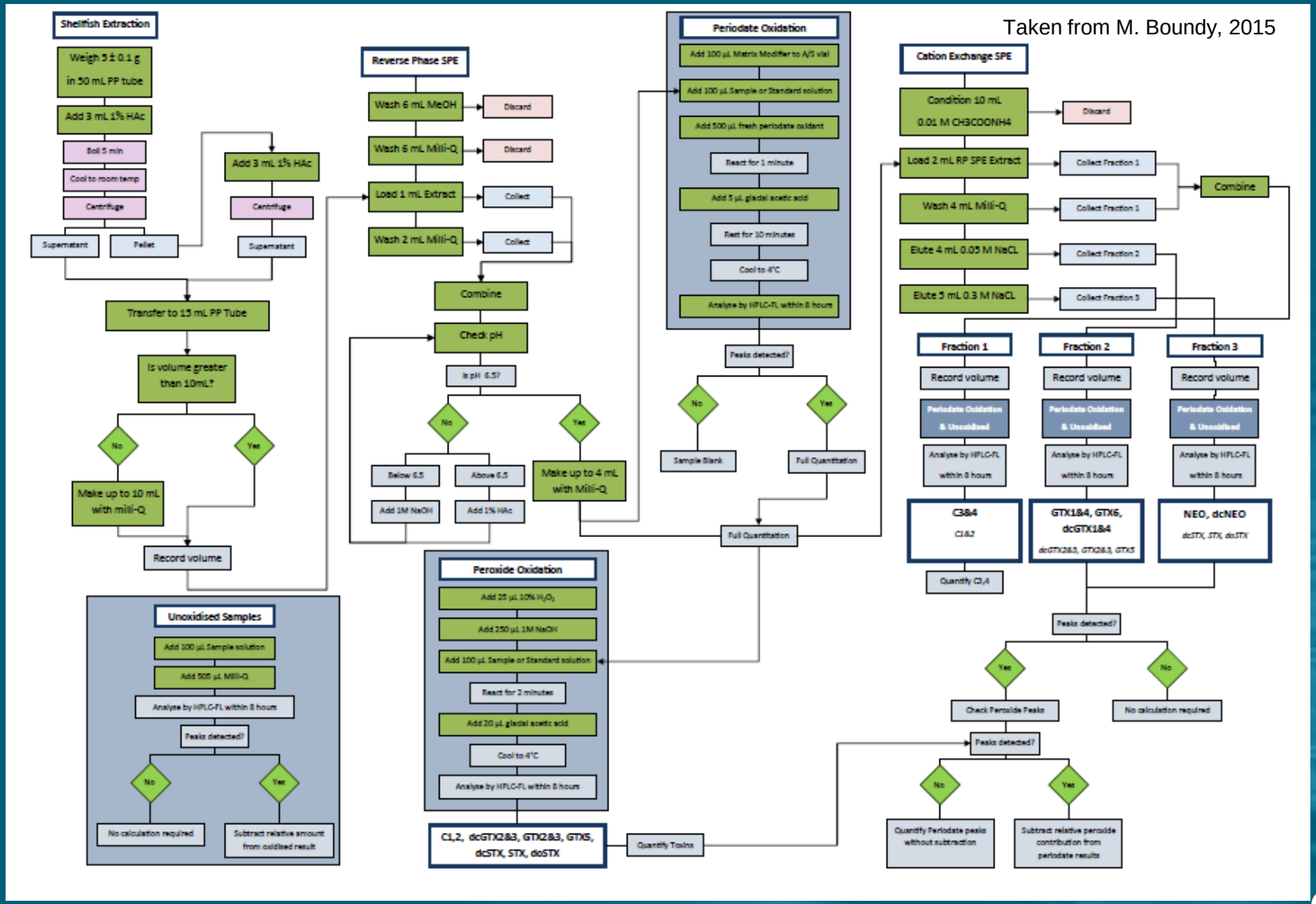
The Lawrence Method



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Validation of Methods

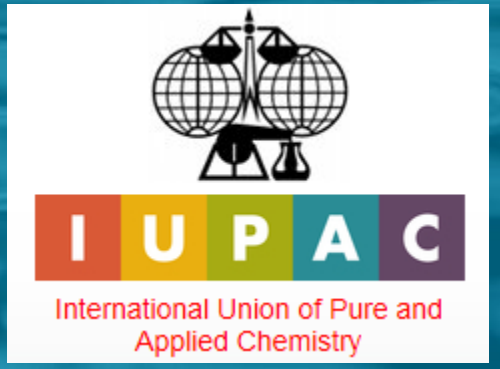
Not an easy, quick or cheap process:

- IUPAC and EC guidelines
- Initial testing of method
- In-house validation to define performance →
- Comparison with other methods
- Assessment of issues
- Resolve practical issues and pitfalls
- Define implementation approaches
- Implement

Validation

Selectivity
LOD/LOQ (screen & quant)
Linearity and range
Accuracy (CRM)
Toxin recovery
Precision (short, medium, long term)
Ruggedness
Uncertainty of measurement

To be done for each species



Strategic Phases of Validation

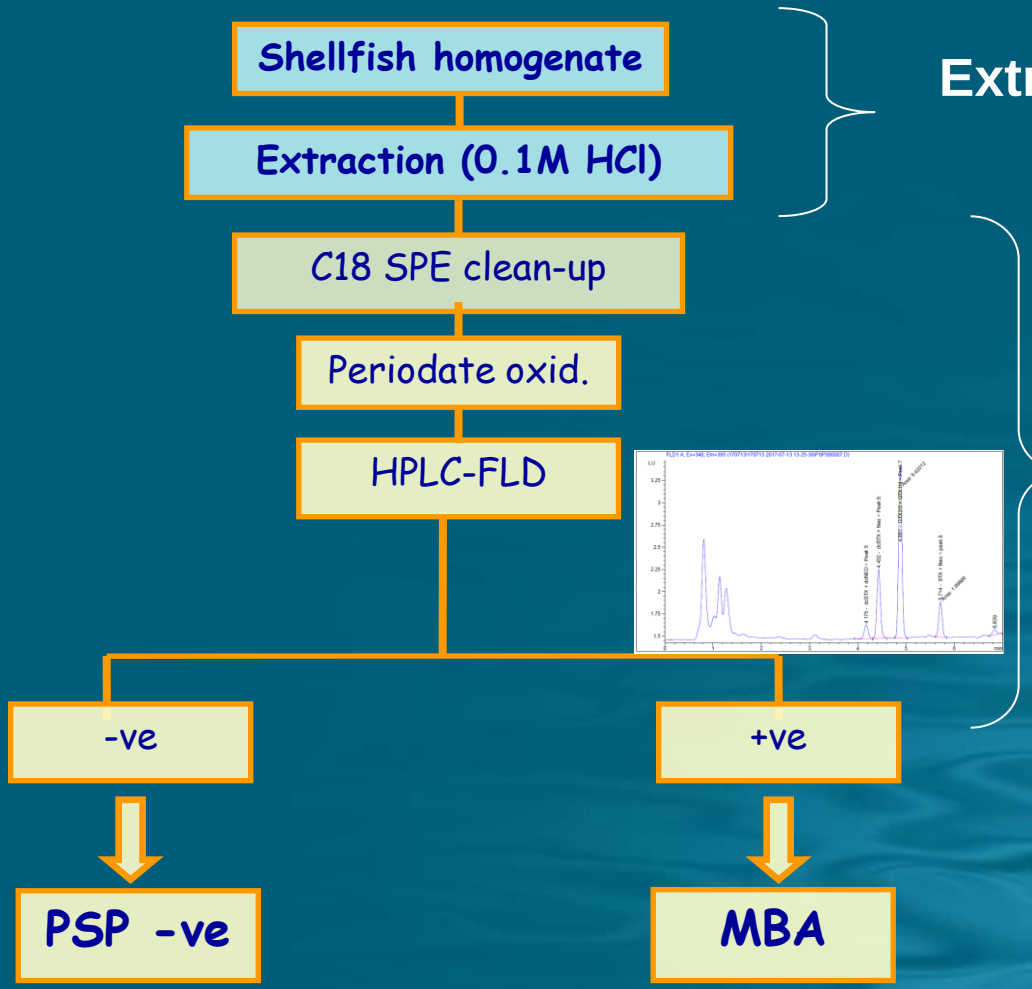
- Phase 1: Screen
  - +ve samples forwarded to MBA
- Phase 2: Quant Validation of Mussels
  - Mussels was chosen as most commonly tested organism
- Phase 3: Quant Validation of other organisms (non scallops)
- Phase 4: Quant method for Scallops

Phase 1:

HPLC Screen

- Validation of screen started in Nov 2005
- Identified issues with Scallop
- Used MBA extraction method
- Run in parallel in summer of 2006
- Implementation in October 2006
- Only HPLC +ve samples forwarded to MBA
- Toxin peak above 3 x signal to noise was regarded as +ve

Screening Decision Tree



Extraction as per AOAC method 959.08

AOAC HPLC method 2005.06

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Phase 2:

Quantitation validation for Mussels

- Implemented in 2008
- Used AOAC 2005.06 acetic acid extraction
- Mice no longer used for any Mussel samples

Phase 3:

Quant Validation of other species

- Implemented 2010
- Clams, Cockles, Oysters, Razor shells each being validated individually.

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Phase 4:

Quant Validation of Scallop species

- Implemented in 2011
- Periodate oxidation suppression caused sensitivity issues
- Changes included:
  - Increased sample volume for SPE (50%)
  - Higher concentration of periodic acid in periodate.
  - Use of Scallop matrix to suppress the standard oxidation

Approximately 5-6 years for complete removal of MBA

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Schedule of work

- Day 1
  - Shuck, homogenise, extract, perform SPE and oxidation. Run HPLC over night.
- Day 2
  - Take results identify positive samples and if they require fractionation, release negative results.
  - Peroxide oxidise the C18 extract and periodate Fraction 2 and 3, also prepare an unoxidized aliquot of the C18.
  - HPLC analysis of standards (21 injections) and samples (up to 4 each) over night.
- Day 3
  - Process data, complete reports and double checked.
  - Forward results to customer.

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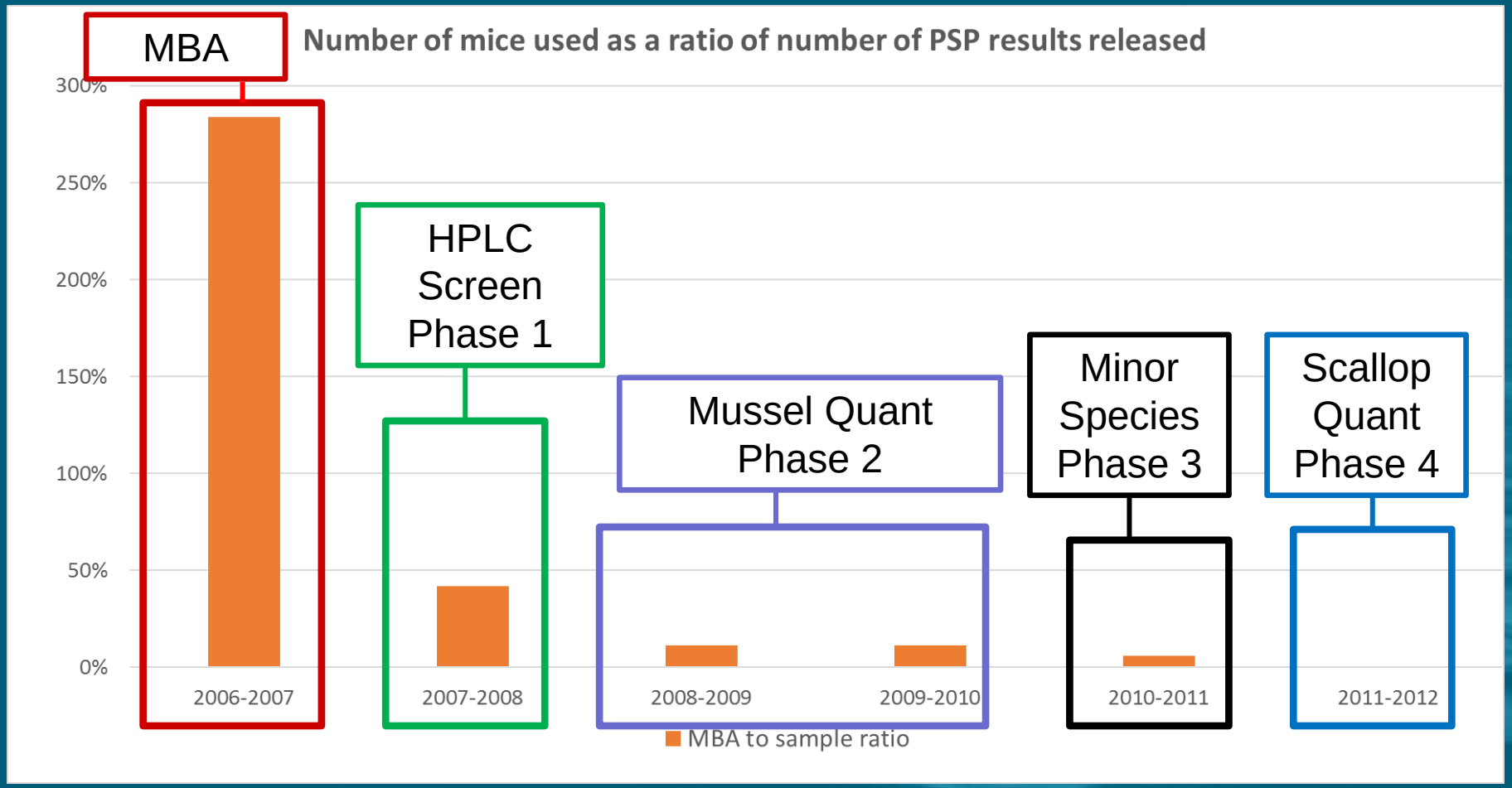
Time line of MBA replacement



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Developments since Implementation

Semi Quant replaced Screen

- Same method as Screen
- Applied Semi Quant to historic data
  - checked against quant result
- Uses 2 point calibration
- Gives estimated toxicity
- Over estimates by factor of >2
  - Dependant on Profile
- Threshold set at 400 µg STX eq/kg
- Reduced number of samples requiring quantification

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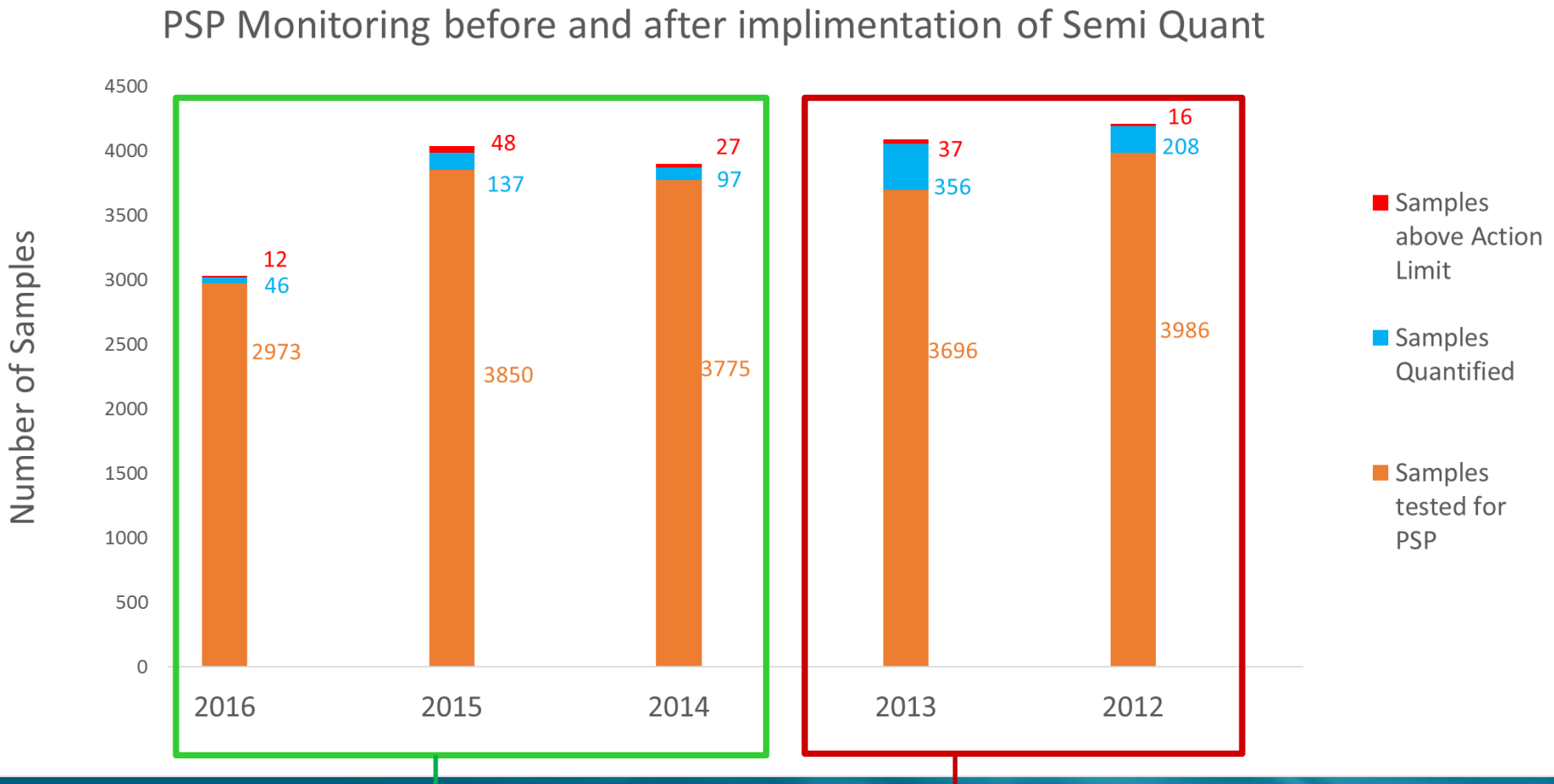
Benefits



Semi Quant: 2-4% of samples required Quant

Screen: 5-10% of samples required Quant

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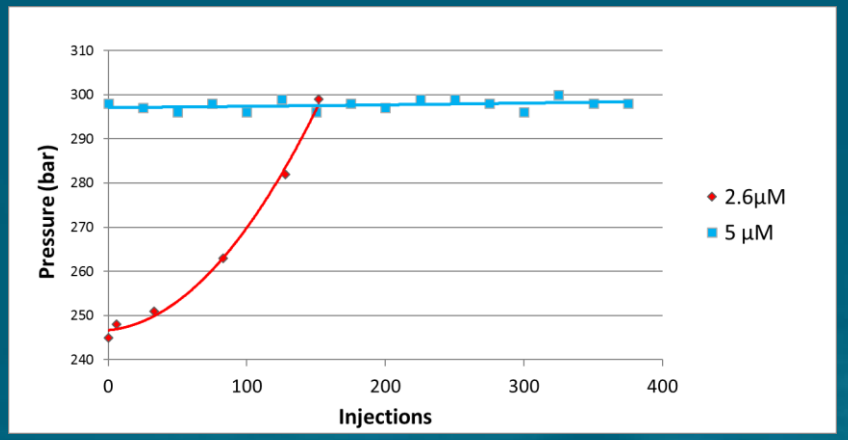
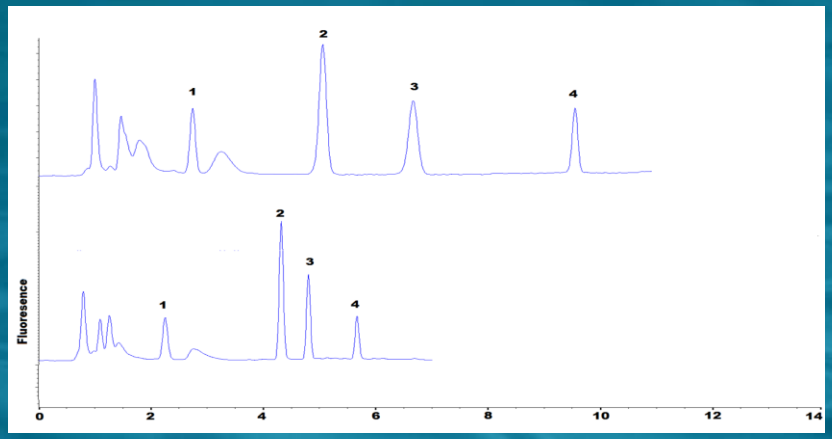
Fast HPLC

- 2.6µM 100x4.6mm fused core column
  - Standard HPLC hardware
  - Reduced Cycle time (halved)
  - Poor column life (<500 cycles)



- 5 µm; 150 x 4.6 mm fused core column
  - Standard HPLC hardware
  - Reduced Cycle time (halved)
  - Extended life (>3000 cycles)



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Benefits:

- More sensitive
  - Up to 275% for GTX2&3
- Faster sample analysis
  - Analysis time halved
- Reduced instrumental demands
  - Single instrument can do screen and quant
- >99% of results released on time
- No additional cost $
- No Notable drawbacks yet identified

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UPLC analysis of PSP

- >800 bar pressure
- <3µm & <2µm columns tested
- Fused core & porous columns tested
- Different manufacturers
  - Phenomonex, Agilent, Waters and more.
- 4 minute cycle time
- Poor life expectancy
  - Drop in efficiency & peak shape

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Alternative Methods

- PCOX: Post Column Oxidation (AOAC 2011.02) - HPLC-FLD
- Ion Pair Chromatography
- Single Laboratory Validation 2009
- Inter-lab validation completed in 2011
- Cefas collaboration
- Not included in EU legislation
- Used in Canada for routine monitoring

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PCOX The Good The Bad and the Ugly!

The good :

- Separates isometric pairs, (e.g., GTX1&4)
- No C18 clean up required.
- No manual oxidation required

The Bad:

- 2 separate methods and columns
  - STX & GTX on a C18 column and C toxins on C8 column
- Post column derivatising hardware
- 25 min cycle time
- Poor column life

The Ugly!

- The chromatography!

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The Future

- Cefas developed LC-MS/MS Method (Turner & Boundy)
  - HILIC chromatography
  - Uses porous Graphite SPE for salt removal
  - Single lab validated 2014
  - Inter-lab validation currently underway including participation from Chile
  - 11 min cycle time
  - 1 injection per sample for full quant, including additional analogues (doSTX, GTX6, dcGTX1&4, M toxins)
  - Able to detect and quantify analogues which PCOX cannot determine (above + dcNEO)
  - Especially important for *G. catenatum* profiles

To Conclude

- Validating the Lawrence for full Quant is Lengthy, complicated and Expensive!
- Consider validating the Screen/Semi-quant
  - Test historic extracts and run in parallel with routine samples
  - Assess performance and adjust if necessary
- Use the screen to select samples to be forwarded to MBA
- By doing this you can achieve high throughput
  - 50-100 samples per day 1 instrument.
- Wait for LCMS method or do full Lawrence in future

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I would like to thank:

- Andrew Turner: Cefas
- Lorena Delgado: Instituto de Salud Pública de Chile& Andrea Rivera: Departamento de Nutrición y Alimentos
- Marcelo Campos Larraín: Acuasesorias
- The FSA and FSS
- The Fishing and Aquaculture Vice-Ministry for funding this visit

Questions?

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Practical Application of Methods

Key Points

- ISO 17025
  - Multiple QC checks at each stage
- Highly trained analysts
- Robust instrumentation
- Automated processes
- Risk awareness, mitigation and contingency
- Availability of reference materials
- QA, Inter-lab, proficiency tests

ISO17025:2005 accreditation

- Quality system
- Training and management
- Quality control & proficiency testing
- Suppliers
- Calibration and validation
- Equipment
- Improvements

QC/QA Checklist?

•HPLC performance / system suitability / preventative maintenance
•SPE instrument checks
•LRM prep / homogeneity / stability
•Tube tolerances
•Proper calibration of all equipment
•Standardisation of timings / parameters
•Checks on std prep (new vs. old)
•Shewhart charts for all RMs
•Full, traceable reagent prep
•Reagent performance testing
•Collection volume verification
•Verification of labelling, tube positioning and instrument sequences
•pH checks
•Full 5/6 point calibrations (r^2 / LOQ check)
•System and procedural blanks
•Regular continuing calibration checks
•System flushes
•Regular calculation template checks
•Inter-lab tests
•Multiple analyst tests when possible

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Extraction

Exhaustive

- 5g Homogenate
- 3ml HAC
- Vortex
- Boil 5 min
- Cool 5 minutes
- Vortex
- Centrifuge
- Collect supernatant
- Add 3 ml HAC
- Vortex
- Centrifuge
- Collect supernatant
- Make up to 10 ml

Dispersive

- 5g Homogenate
- 5ml HAC
- VortexBoil 5 min
- Cool 5 minutes
- Vortex
- Centrifuge
- Collect supernatant

Not EURL Method

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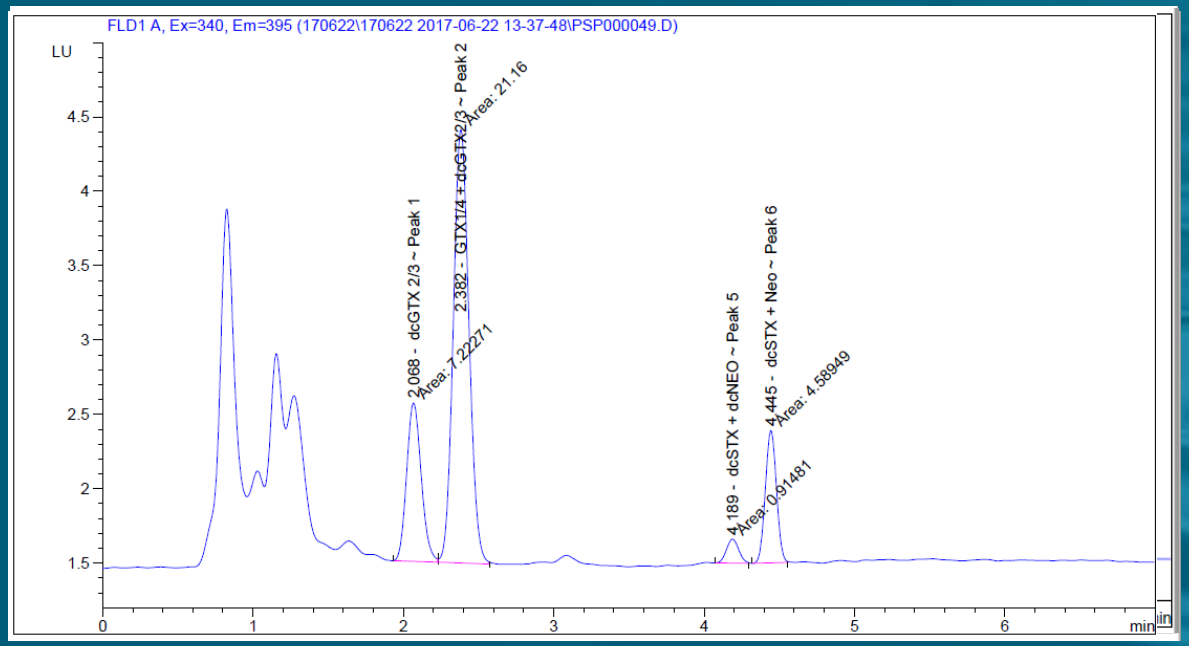
PSP toxin Standards and AOAC 2005.06

- 10 Toxins routinely tested at Cefas
- For the purposes of this method in 2 defined groups:
- N-Hydroxylated
  - Mix 1: NEO, GTX1&4 dcNEO
- Non-N-Hydroxylated
  - Mix 2: dcSTX, GTX2&3, GTX5, STX,
  - Mix 3: dcGTX2&4, C1&2

Thankfully: PSTs commonly occurring in naturally contaminated shellfish in UK/EU are available as standards **and** most have fairly well described TEFs

Not the case for M toxin!



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Why not Use PCOX

- AOAC 2011.02 – PCOX LC-FLD:
  - Not in EU legislation
  - Requires at least 2 columns/systems to run each sample
  - Use of ion-pairing chromatography
  - Very short column lifetime
  - Prone to matrix effects – false +/-
- Tested for UK samples
  - Complex interpretation
  - Difficult to run daily – NOT USED

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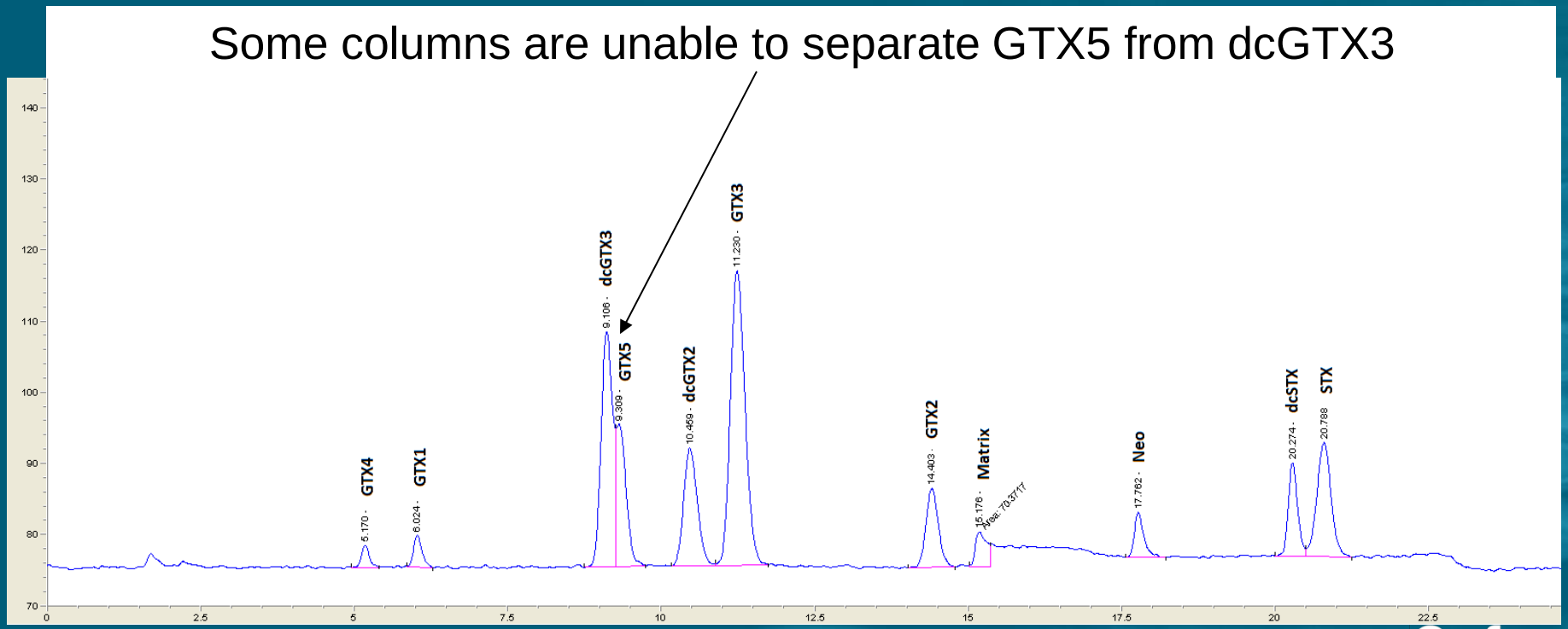
PCOX chromatographic issues

Example of PCOX standard Chromatogram with no shellfish matrix present



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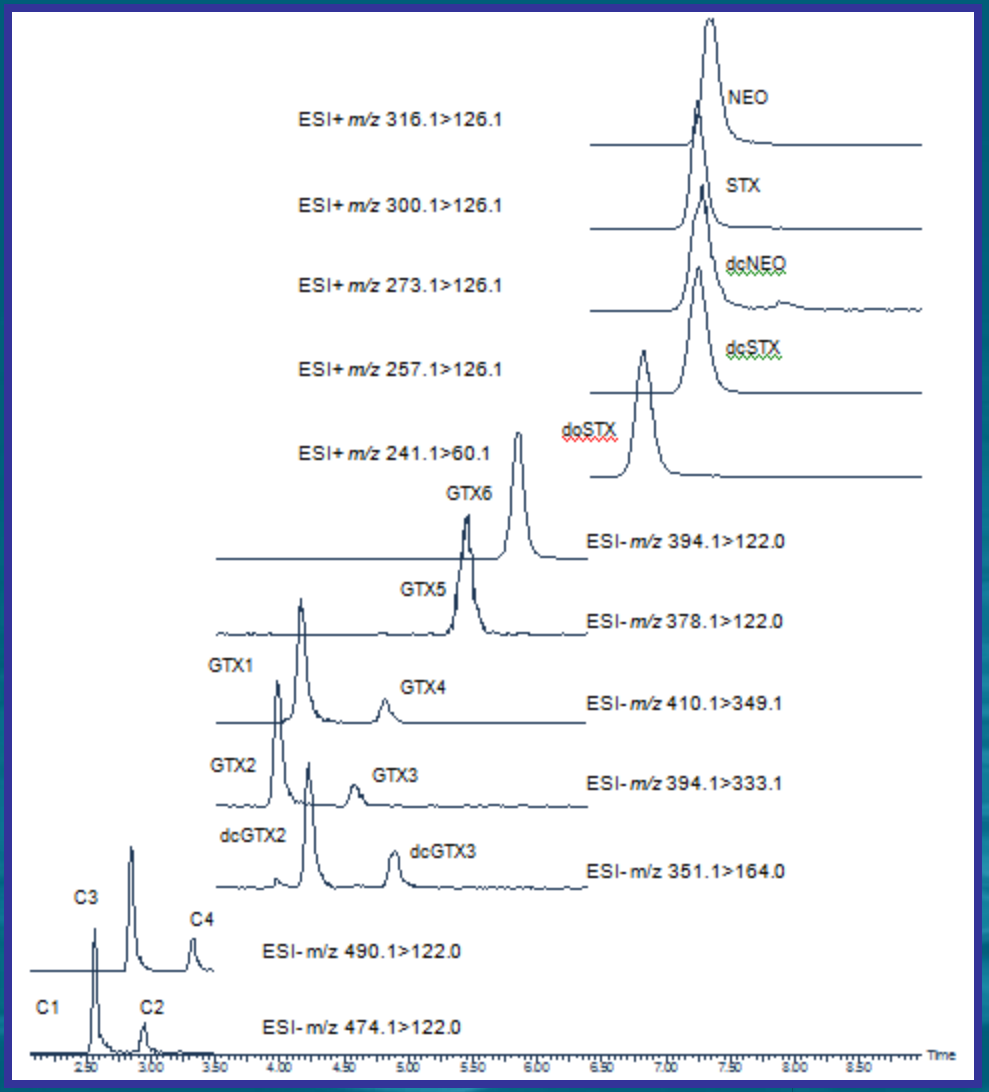
HILIC separation

- Waters BEH Amide HILIC
- +/- switching
- Full separation of critical pairs, including epimers
- Total cycle time of 11.5 min for all PSTs



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