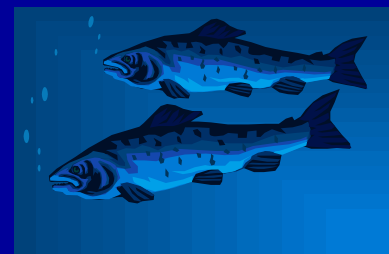
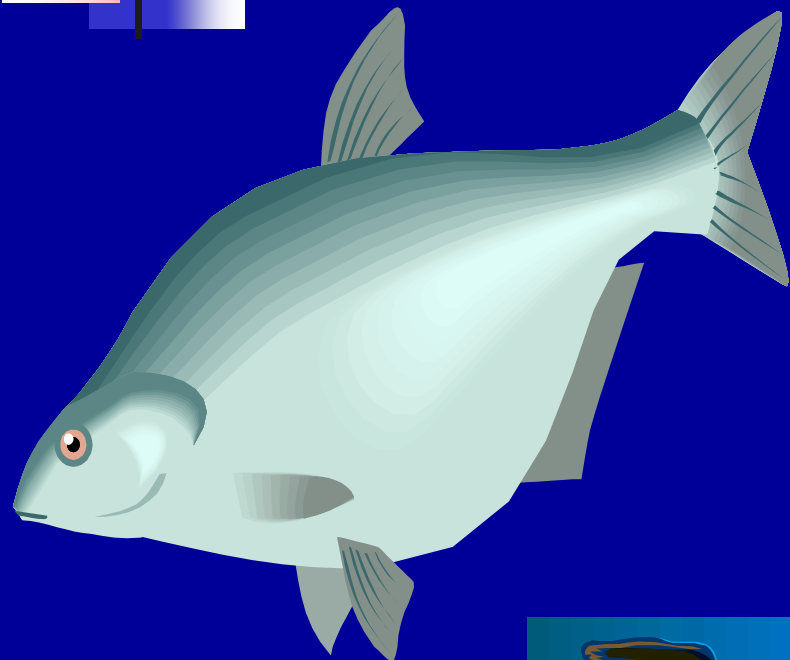


Analysis of Malachite Green in Fish



BY
Hubert PO Tang
Jacky Y Y CHOI

14 Sept 2005

HKSAR Government Laboratory



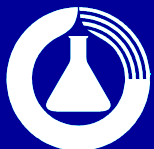


Analysis of Malachite Green in Fish – Part B

Instrumental Analysis & Quality Assurance

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Useful References

- GCMS
 - Journal of AOAC International, 78, 4, 1995, 971
- LC-UV / DAD / FLD
 - Journal of Chromatography B, 669, 1995, 219-227
 - Journal of Chromatography B, 688, 2, 1997, 325
 - Journal of Chromatography B, 700, 1-2, 1997, 223
 - Journal of Chromatography B, 700, 1997, 223-231
 - Journal of Chromatography A, 1089, 2005, 187-192

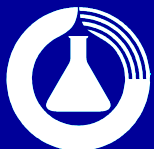




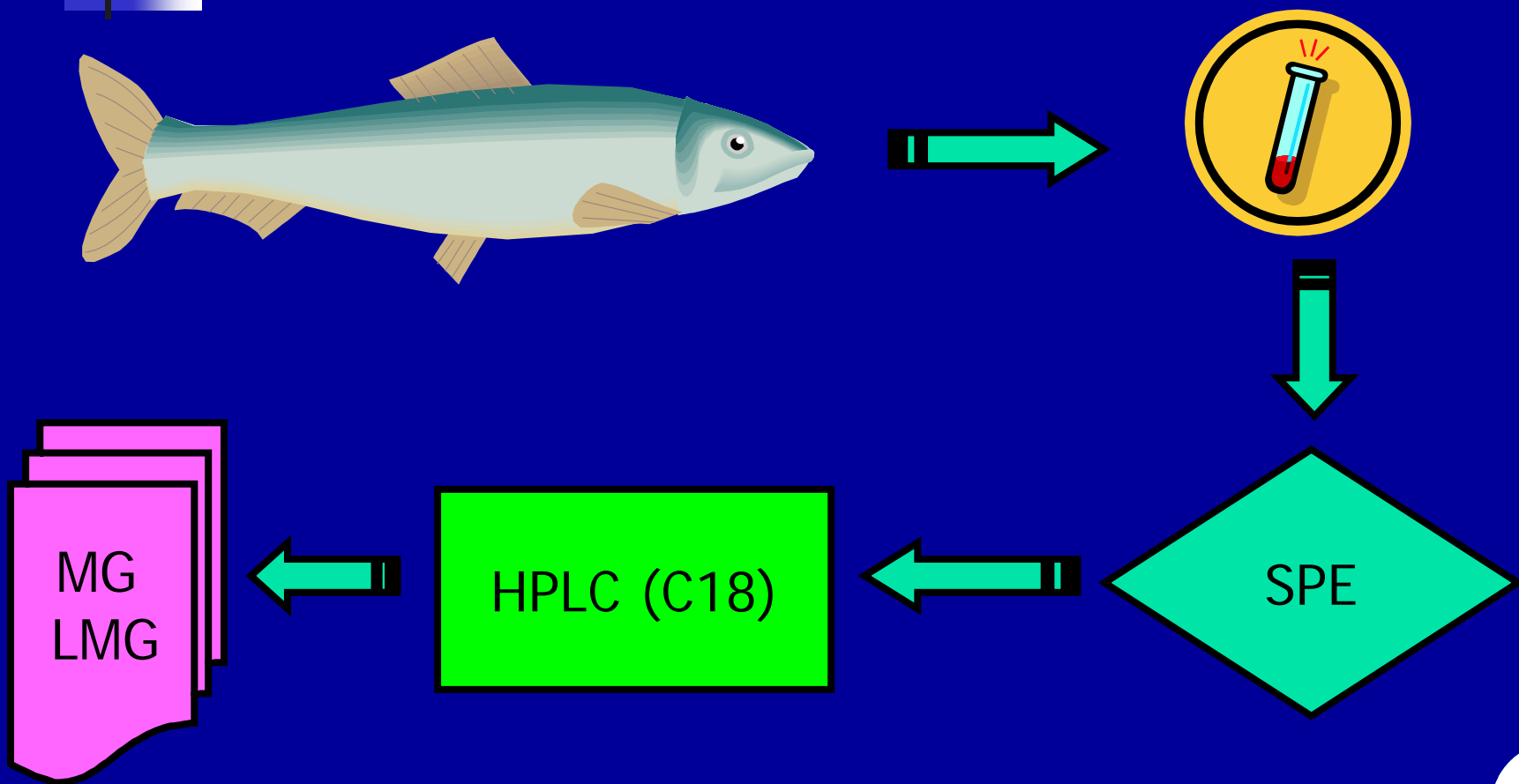
Useful References

- LC-MS/MS

- Rapid Communication in Mass spectrometry, 12,1998, 1625-1634
- Journal of Chromatography A, 1089, (2005), 187-192
- Journal of Chromatography B, 788, 2003, 351-359
- Analytical Chimica Acta, 529, 2005, 173-177
- Journal of AOAC International, 88, 3, 2005,745
- 3rd AOAC Europe, Eurachem Symposium, March 2005

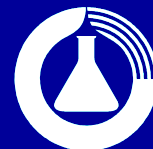


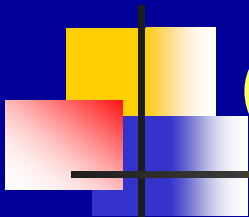
Sample Preparation Summary



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Consideration ?

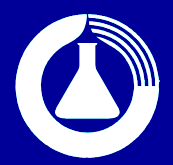
Analytical Time

HPLC UV

Sensitivity

HPLC MS

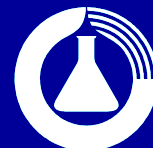
Selectivity





Pros and Cons

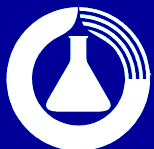
- GC-MS (SIM)
 - The most Common MS in laboratories
 - Relatively higher selectivity than common LC detectors e.g. UV, FLD, DAD.
 - Malachite green: Non-volatile, decompose in GC.
 - Only LMG analysis; no information on MG
 - (Relative higher detection limit: 5ng/g)





Pros and Cons

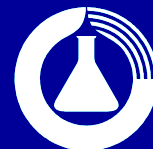
- LC-UV
 - Low cost
 - Relatively sensitive at wavelength maxima
 - Only single wavelength, not confirmative
 - LMG Maxima at 266nm – interference problem !!





Pros and Cons

- LC-DAD
 - Multiple wavelength measurement
 - Peak purity information
 - Relatively less sensitive
 - Not confirmative enough compared with Tandem MS. Intensive sample purification required to prevent potentially interference from impurities, prevent co-elution.





Pros and Cons

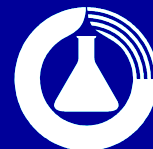
- LC-FLD
 - Higher sensitivity than UV / DAD detectors
 - Less background noise
 - Not confirmative enough
 - Intensive sample cleanup required
 - Prevent co-elution

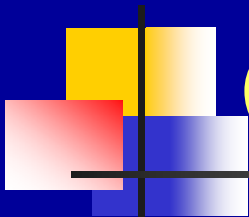




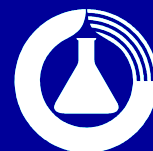
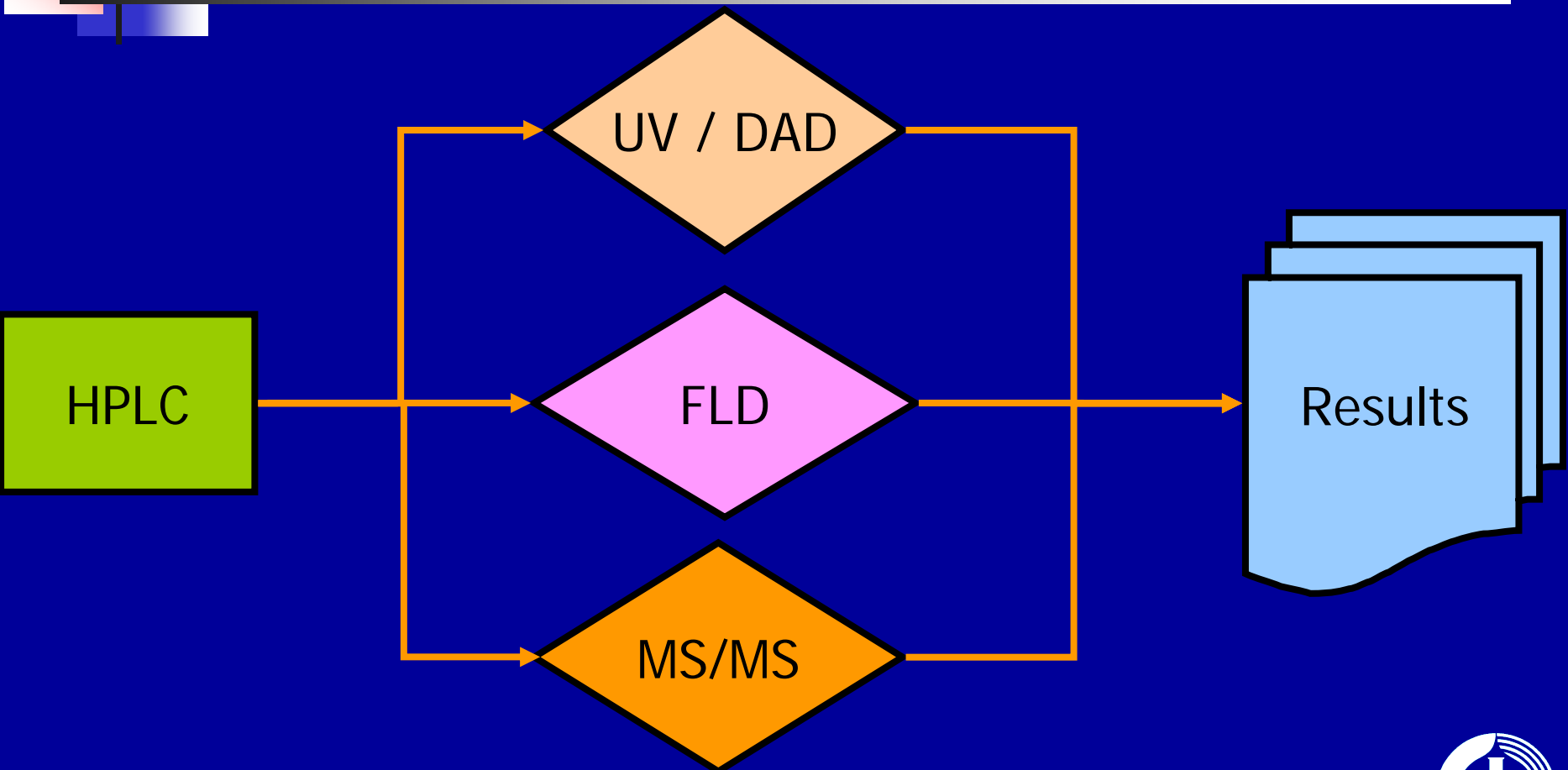
Pros and Cons

- LC-MS/MS
 - Very high sensitivity
 - Excellent selectivity
 - Fast analytical time, allow co-elution
 - Confirmative information
 - Expensive
 - Experienced personnel required for system maintenance and results interpretation





Choice of Techniques





Pros and Cons

- Taking into consideration:

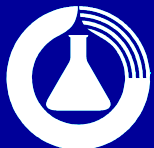
- Analytical time

- ... fast ... imported live



HPLC-MS/MS

- High ... unnecessary repeat of analysis to keep low mortality rate of live fish



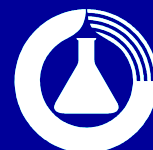
Analysis of Malachite green in HK SAR Government Laboratory

- One Ultra high sensitive LC-MSⁿ Ion Trap Mass Spectrometer for rapid preliminary determination



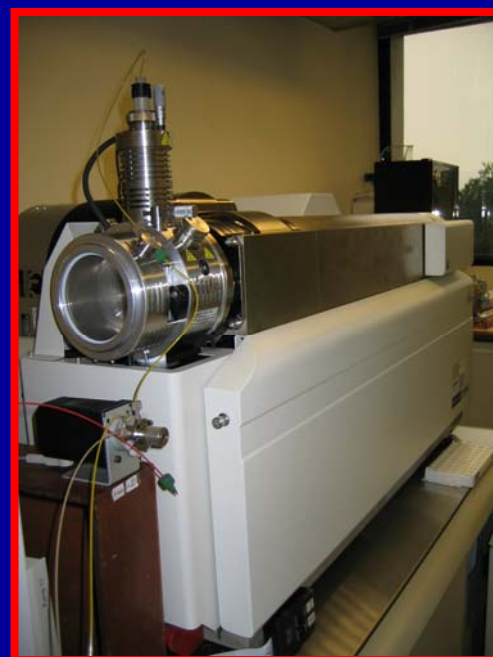
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Analysis of Malachite green in HK SAR Government Laboratory

- Two HPLC Triple quadrupole Tandem MS for accurate quantification and confirmation



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HK SAR Government Laboratory



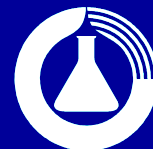


Mass Transitions monitored

Analyte	Q1 Mass	Q3 Mass	Analyte	Q1 Mass	Q3 Mass
Malachite Green	329	208	Leucomalachite Green	331	239
	329	313		331	223
	329	241		331	316
d5-Malachite Green	334	318	d6-Leucomalachite green	337	240
	334	213		337	278

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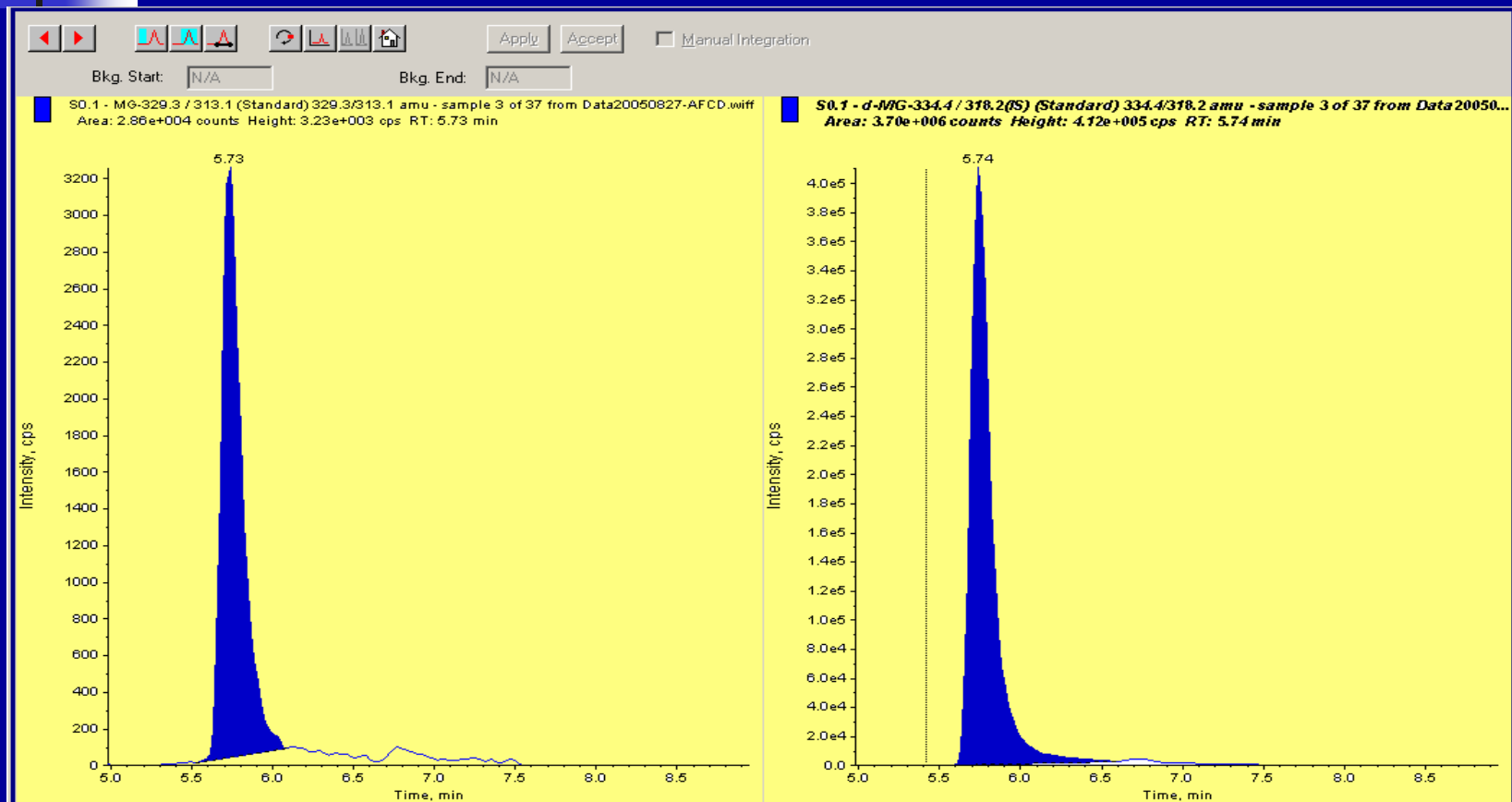


Tips on MS analysis

- Isotope labelled internal standard
 - Adv
 - Account ion suppression & matrix effect (esp. Ion Trap MS – *automatic gain control*)
 - Account effects on sample preparation
 - Disadv
 - Expensive
 - Source of isotope labelled standard



0.1 ug/L standard solution - Malachite green

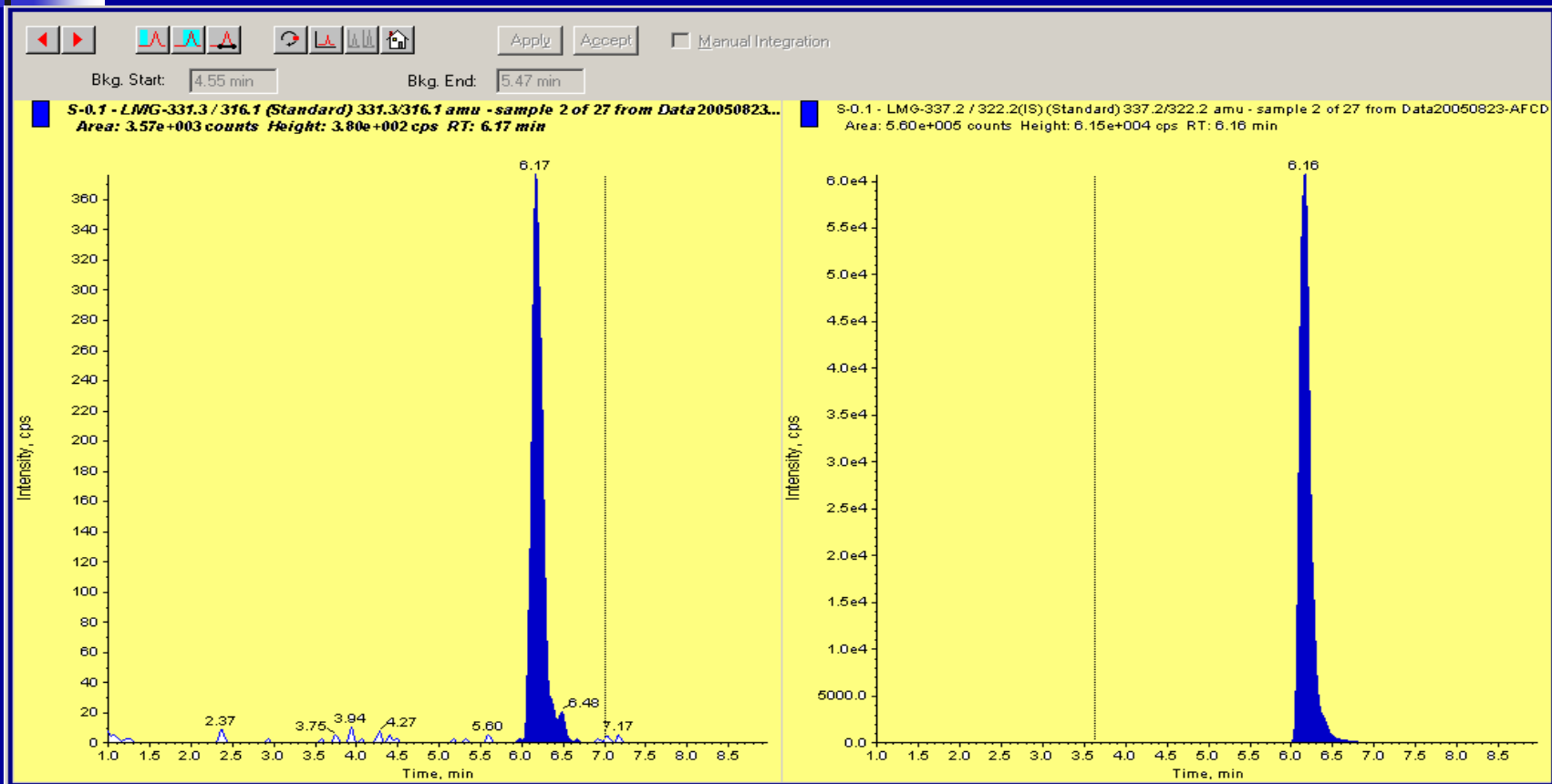


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0.1 ug/L standard solution – Leuco malachite green

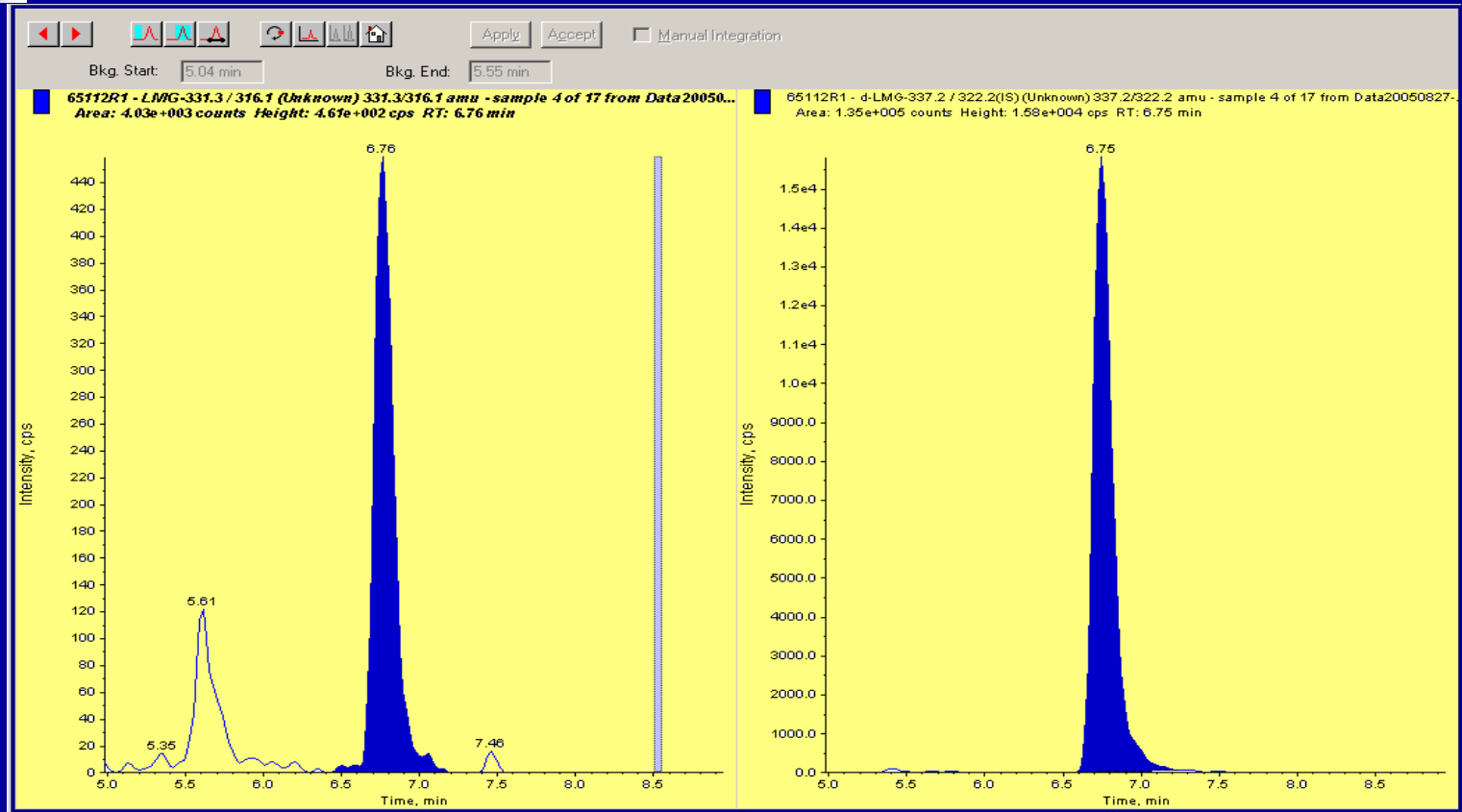


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0.5 ug/kg QC spike in fish sample - Leucomalachite green

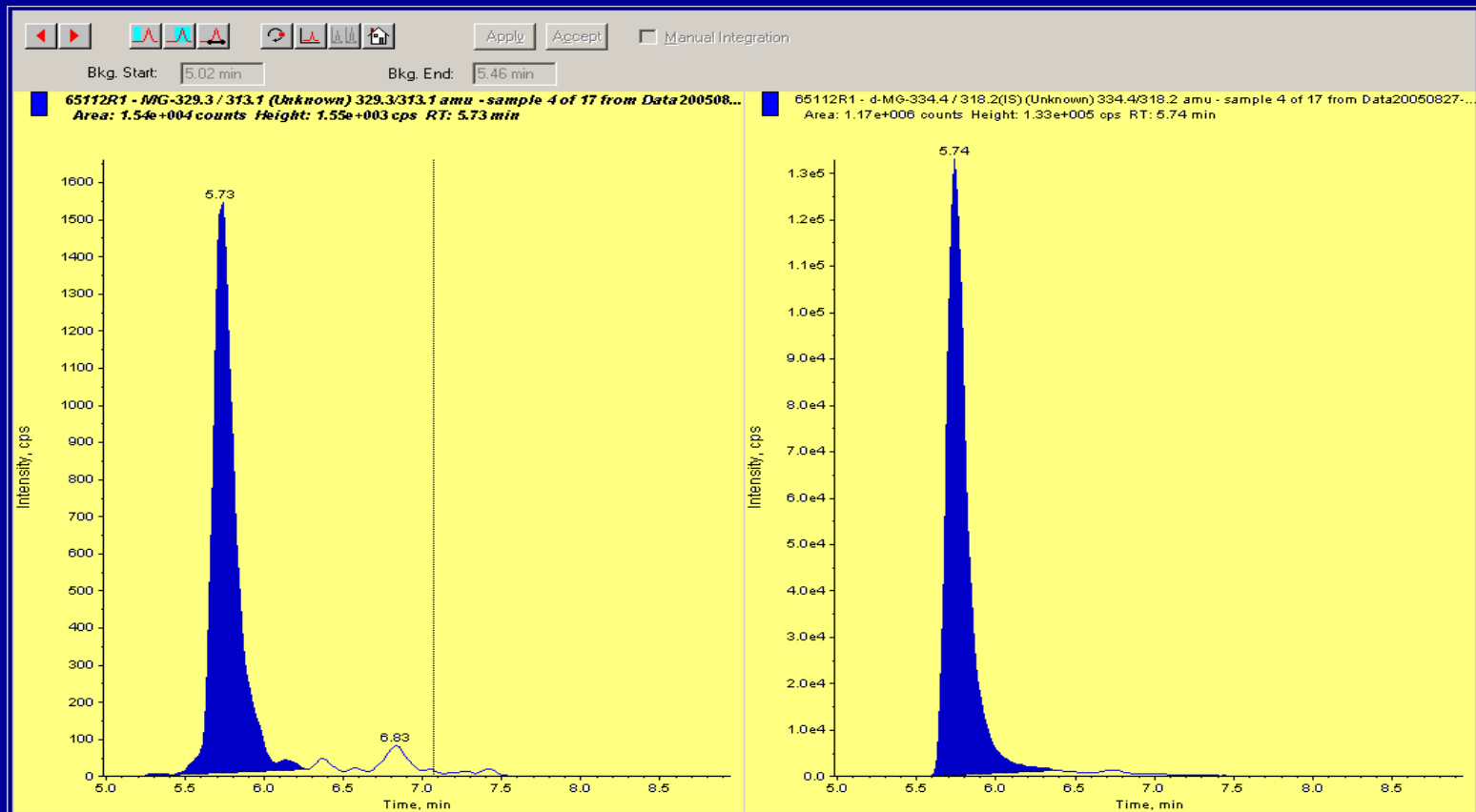


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0.5 ug/kg QC spike in fish sample - Malachite green

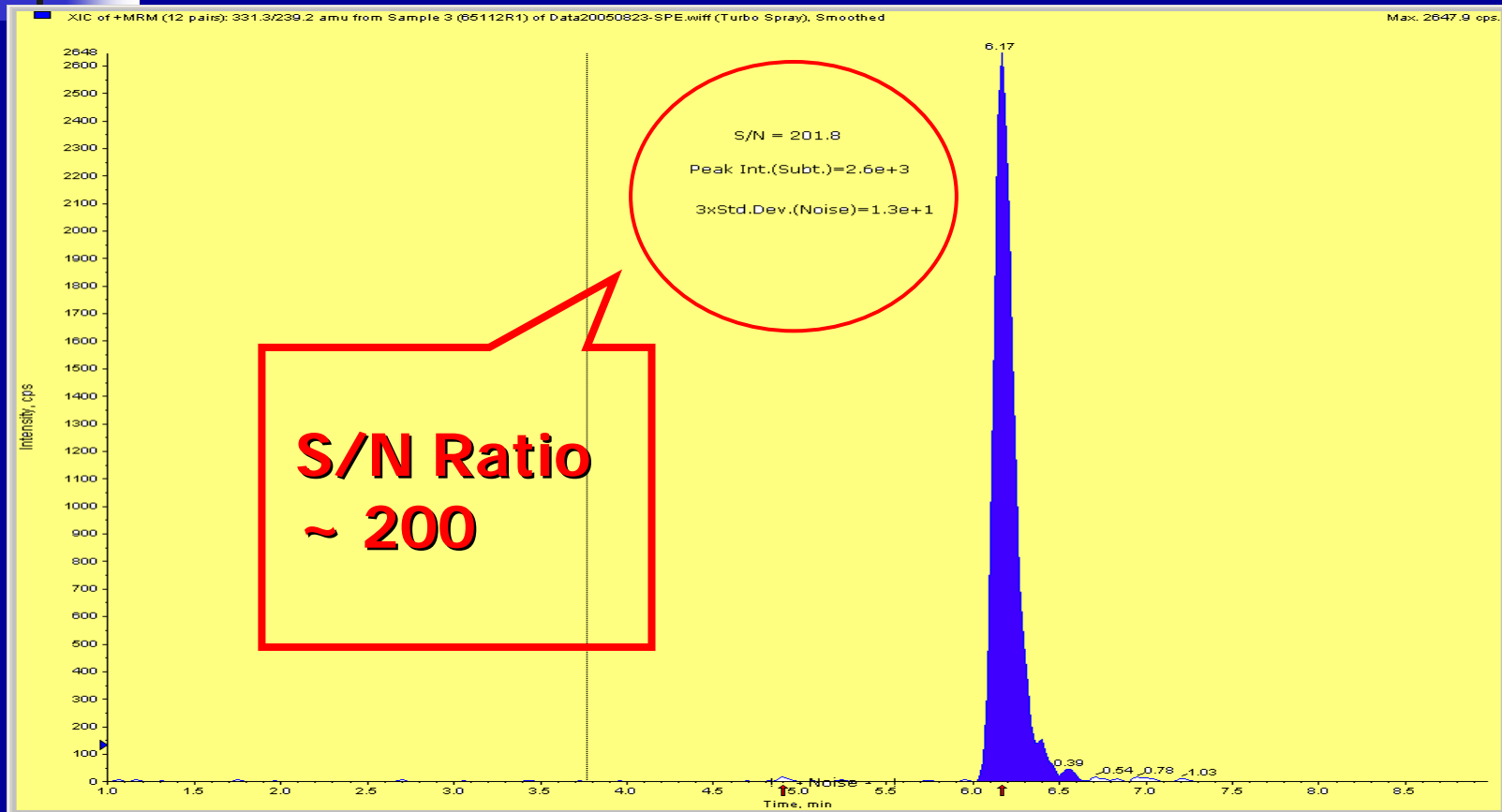


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0.5 ug/kg QC spike in fish sample – Leucomalachite green



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Requirements for Method Validation Food (Veterinary drug residue)

Codex CAC/GL16-1993

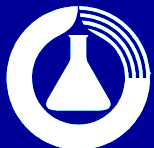
- Codex Guidelines for the Establishment of a Regulatory Programme for Control of Veterinary Drug Residues in Foods
- Food and Drug Administration Center for Veterinary Medicine July 1999 Guidance for Industry 64, "Validation of Analytical Procedures: Methodology"
- Food and Drug Administration Center for Veterinary Medicine May 2003 Guidance for Industry 118, "Mass Spectrometry for confirmation of the identity of animal drug residues"

Commission of the European Communities (EU)

- 2002/657/EC, Official journal of the European Communities.

National Registration Authority (NRA)

- NRA Residue Guideline No. 26 - Veterinary drug residue analytical methods

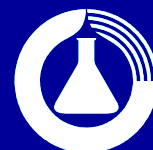




Method Types – Codex

Based on Intended use:

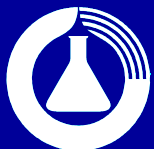
- **Level I: Confirmatory Methods**
 - unambiguously identifies the analyte and
 - may provides quantitative information
- **Level II: Determinative Methods**
 - quantitative results
- **Level III: Screening (semi-quantitative) Methods**





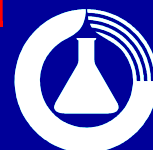
Additional Method performance characteristics for quantitative methods

- 1. Calibration, linearity and sensitivity**
- 2. Recovery**
- 3. Limit of quantitation**
- 4. Precision**
 - **Repeatability (intralaboratory)**
 - within batch (same operator, similar conditions)
 - **Intermediate precision**
 - between batch (different operators, various reagents, standards, environmental and instrumental conditions)
 - **Reproducibility (inter-laboratories)**
- 5. Ruggedness (robustness)**
- 6. Uncertainty**



Requirements for Method Performance Confirmation (vet. drug)

Technique	Criteria of EU
GC-ECD	Two columns of different polarity
LC-DAD	Peak maxima $\leq 2\text{nm}$ Relative absorbance $\leq 10\%$
LC-UV	Two columns/two detection systems
LC-FLD	Parent or derivative possess fluorescence
LC-MS/MS	Fulfil specific requirements



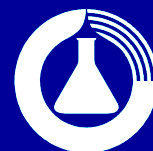


Requirements for Method Performance Confirmation (vet. drug)

■ **Chromatography separation**

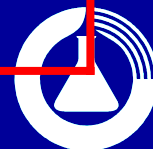
Attribute	EU
Retention time	2 times void volume
Relative retention time (with Internal standard I.S.*)	GC±0.5%, LC±2.5%
Relative retention time (without I.S.)	GC/LC±5%
ID Points	≥ 4

**IS should be used in all LC method if available – EU requirement*



Requirements for Method Performance Confirmation by MS (vet. drug)

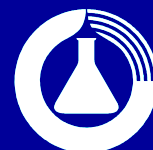
<i>Technique</i>	<i>No. of ions</i>	<i>I.D. points</i>
GC-MS (EI or CI)	N	n
GC-MS (EI or CI)	2 (EI) + 2 (CI)	4
GC-MS (EI or CI) 2 derivatives	2 derivatives A + 2 derivatives B	4
LC-MS	N	n
GC-MS-MS	1 parent ion, 2 product ions	4
LC-MS-MS	1 parent ion, 2 product ions	4
LC-MS-MS	2 parent ions, each 1 product ion	5
GC-MS and LC- MS	2 + 2	4



Requirements for Method Performance Confirmation by MS (vet. drug)

Relative intensity*	EU	
	EI-GC-MS	CI-GC-MS, LC-MS, LC-MS/MS
> 50 %	± 10 %	± 20 %
> 20 to 50 %	± 15 %	± 25 %
> 10 to 20 %	± 20 %	± 30 %
≤ 10 %	± 50 %	± 50 %

**Relative intensity requirement for both analyte and internal standard*



Requirements for Method Performance Confirmation by MS (vet. drug)

- **Confirmation sequence**

- EU - reagent blank, sample blank, suspected positive samples, sample blank (or -ve control), spike samples

- **Example:**

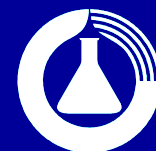
Stds
....
Stds
Reagent blk
Sample blk
+ve sample
-ve control
....
QC spikes
...
Std Check



Requirements for Method Performance

Quantitative methods (Food)

Attribute	AOAC	NATA	Codex (vet)	EU (vet)
LOD	Blank +3 S.D.	S/N>3 7blank/matrix 3S.D.	S/N>3 3 S.D. blank	3S.D. 20blank/spike standard error of y-intercept
LOQ	6 spike	10 LOD	10S.D. blank	LOD+1.64S.D. 20spike
<i>r</i>	>0.9995			
Calibration Levels	5 levels (not zero)	5 levels		5 levels



Requirements for Method Performance

Quantitative methods (Food)

Attribute	AOAC	Codex	EU
Recovery	6x3 levels per matrix	3 levels	6x3 levels per matrix (0.5 to 2 MRL)
Precision	5x4 levels (0-200%)		6x3 levels x3 occasions
Selectivity	Blank < 3%		20 blank

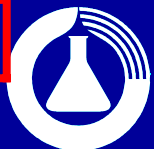


Requirements for Method Performance

Quantitative methods (Food)

Recovery (%)

Conc.	AOAC	EU	Codex
$\geq 10\%$	98-102		
$\geq 1\%$	97-103		
$\geq 0.1\%$	95-105		
100 ppm	90-107		
10 ppm	80-110		
> 1 ppm	80-110		
> 100 ppb	80-110		80-110
> 10 ppb	60-115	80-110	70-110
> 1 ppb	40-120	70-110	60-120
< 1 ppb		50-120	50-120



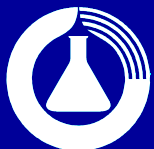


Requirements for Method Performance

Precision

Conc. ($\mu\text{g}/\text{kg}$)	Codex (%)	EU (%)
< 1	35	*
> 1~10	30	*
>10~100	20	23#
>100~1000	15	16#

By Horwitz Eq: $CV=2^{(1-0.5\log C)}$





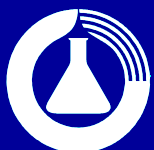
Requirements for Method Performance

Measurement uncertainty

- **EURACHEM/CITAC document
“Quantifying Uncertainty in Analytical Measurement”**
- **VAM Project 3.2.1 January 2000
“Development and Harmonisation of
Measurement Uncertainty Principles**

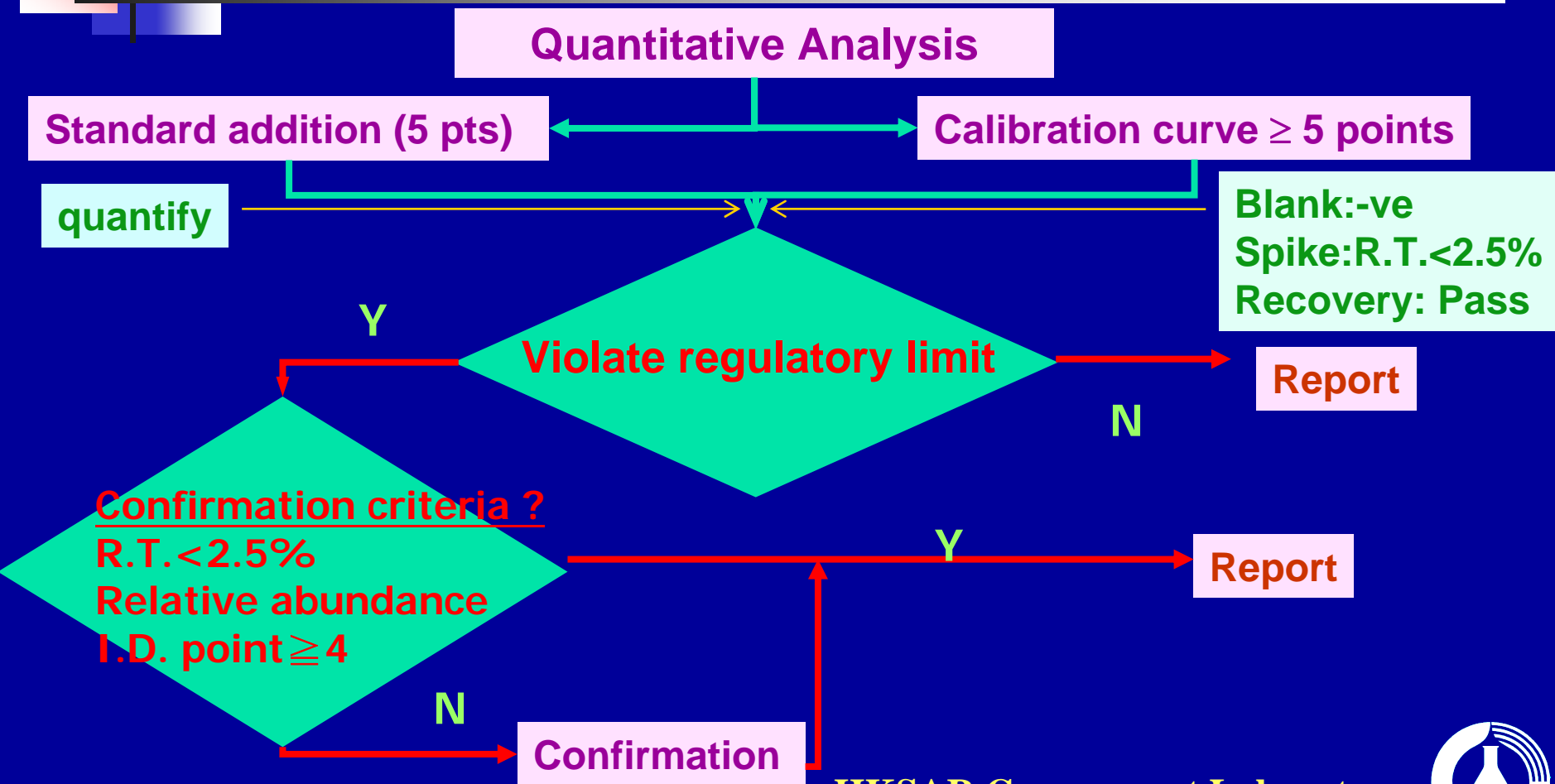
Analyte stability (vet. drug)

- **EU: stability check at normal storage
condition (E.g. -20 °C or 4 °C)**



Analysis Workflow

Quantitative and Confirmatory analysis



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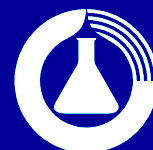


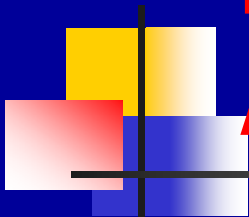


Examples

Quantitative & Confirmatory Method

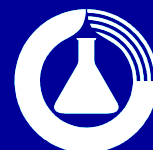
- LC-MS/MS
- Analyte: Chloramphenicol
 - A prohibited veterinary drug reported commonly used both agriculture and aquaculture industry



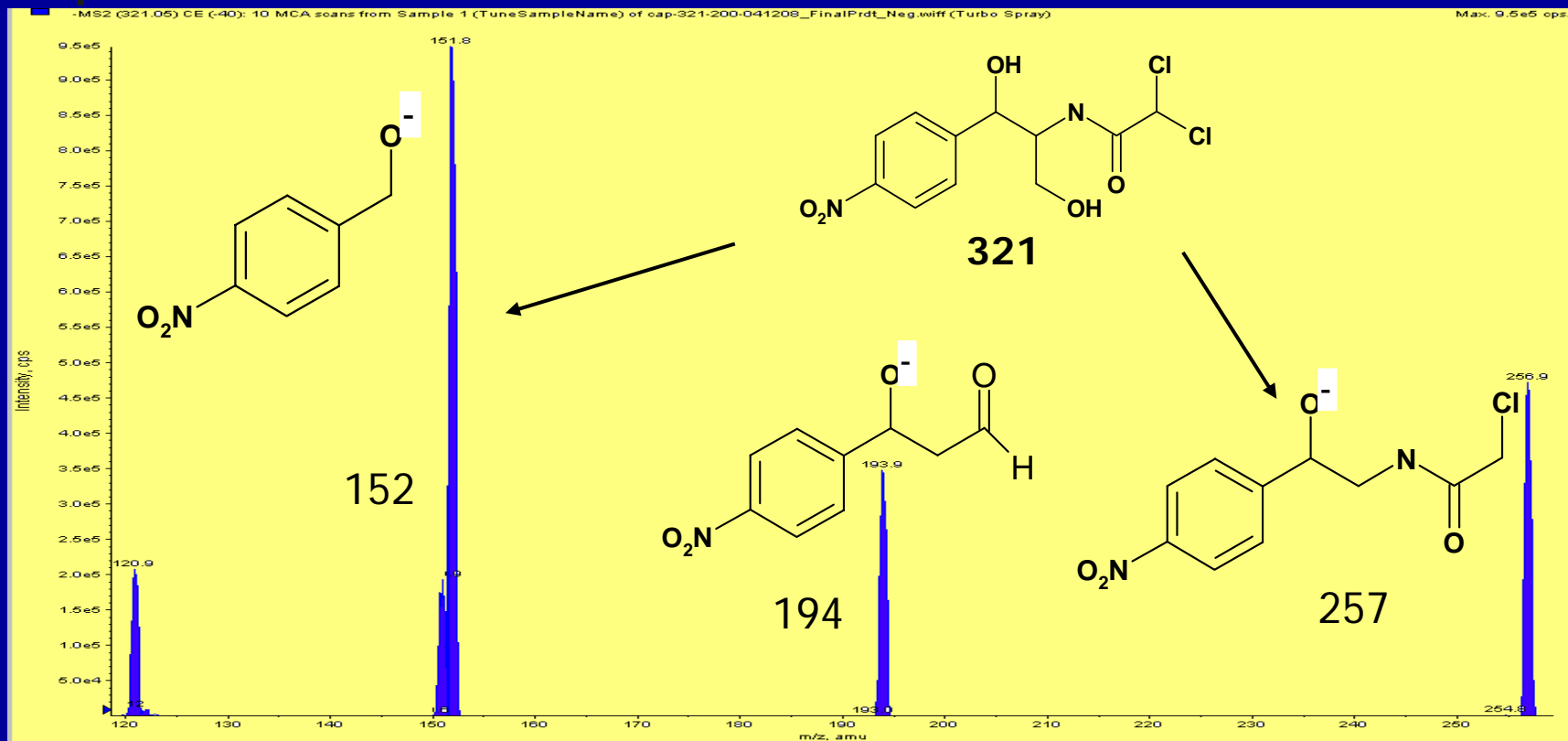


Determination of Chloramphenicol in Animal Tissue by LC-MS/MS

- Prohibited drug
- Isocratic HPLC program (~10 min)
- Three pair of MRMs (ID points >>4)

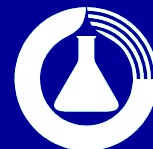


Chloramphenicol – Product ion scan

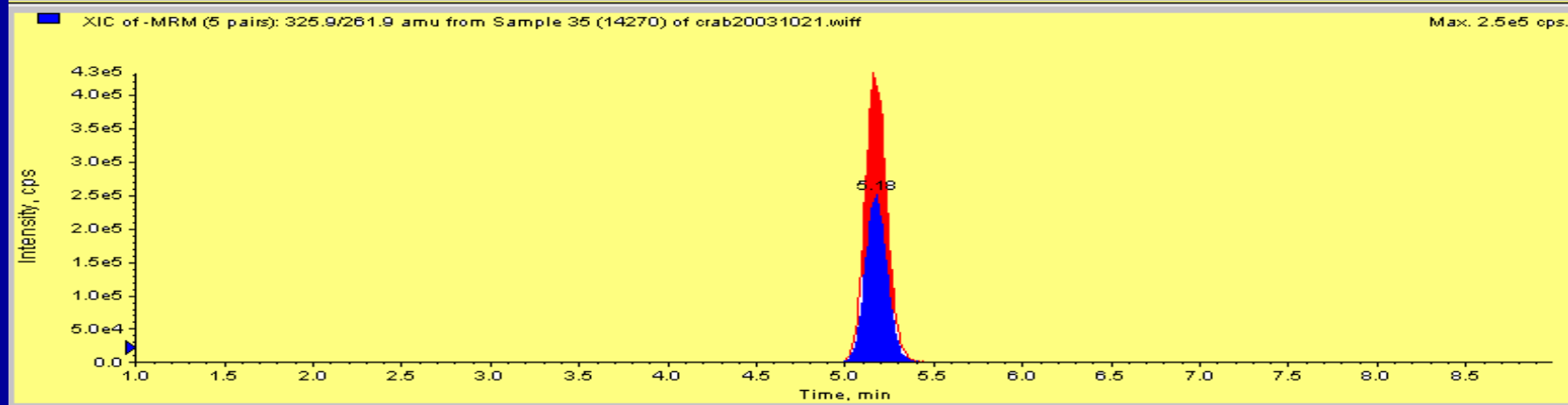
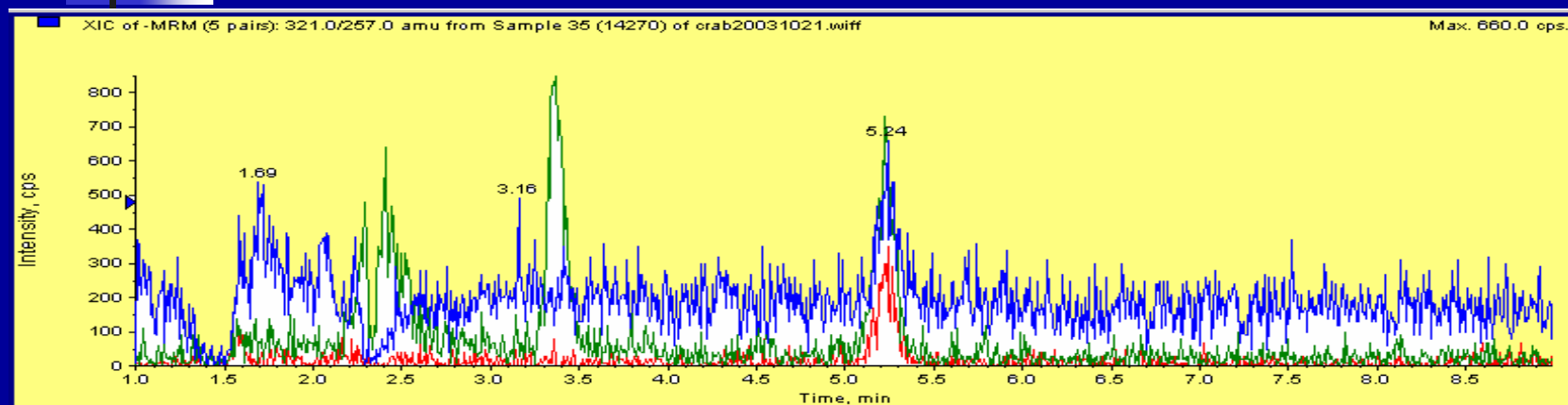


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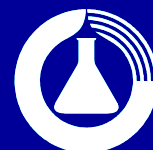


Chloramphenicol – Blk sample



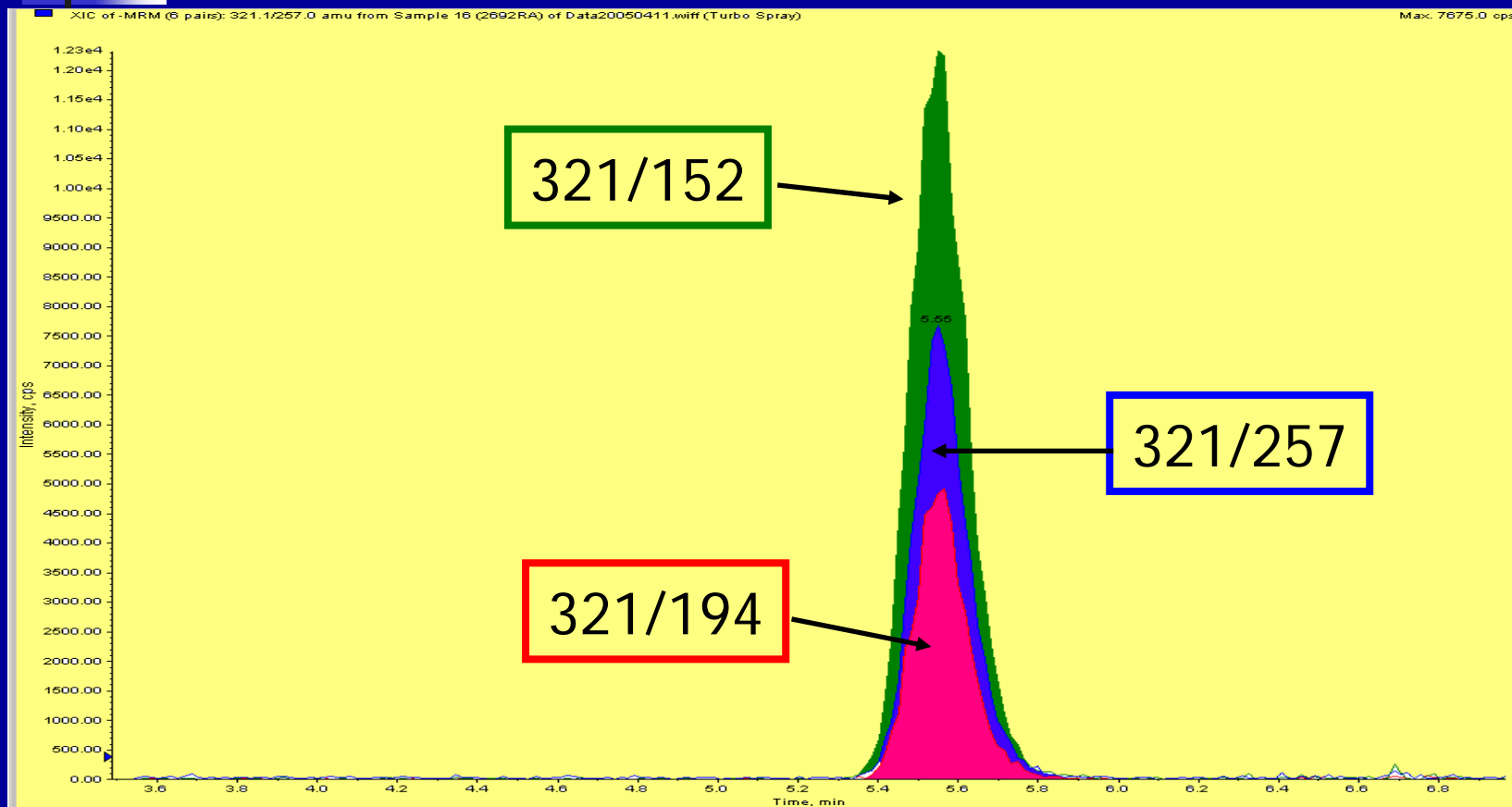
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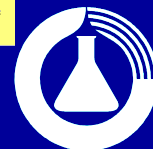
Method Validation

Chloramphenicol 1ug/kg Spike



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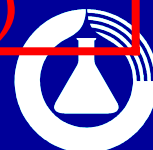
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QC Performance

<i>Attribute</i>	<i>Requirements</i>	<i>Outcome</i>
Chromatographic separation (with internal standard)	LC \pm 2.5 %	<i>Satisfactory</i> <i>(Labelled internal std)</i>
Identification points	≥ 4 (MRM ≥ 2 pairs)	<i>ID point = 5.5</i> <i>(3 MRM @ 1.5, parent 1)</i>
Relative abundance	EU	<i>Satisfactory</i> <i>(Compared with in batch standard solutions)</i>





QC Performance

<i>Attribute</i>	<i>Requirements</i>	<i>Outcome</i>
Linearity (working range)	Up to 2 MRPL	1 – 50 $\mu\text{g}/\text{kg}$
Precision (RSD%) @ MRPL	N=6	≤ 20
Recovery % @ MRPL	N=6	50-120%





QC Performance

<i>Attribute</i>	<i>Requirements</i>	<i>Outcome</i>
Trueness (By BCR 445: 8.9 ± 0.9)	Within certified range N=6	8.8 ± 0.3 $n = 7$
Selectivity / specificity	< LOQ or $\frac{1}{2}$ x reporting limit	<i>Reagent Blk</i> \leq LOQ <i>Sample Blk</i> \leq LOQ
Stability		1.5 years
Method performance	QC spikes recovery	<i>Control Chart</i>





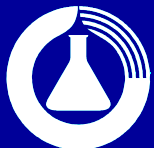
Proficiency Test - FAPAS

<i>Matrix</i>	<i>Number of Labs</i>	<i>Z-score</i>	<i>Year</i>
Milk powder	72	0	2003
Honey	59	-0.3	2005
Prawns	73	0.8	2005

Note: The coming proficiency test on Malachite green is scheduled in March 2006

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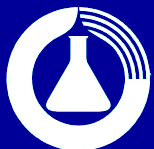


Malachite Green & Leucomalachite green method performance

- LOD* in fish samples

Analytes	LOD* (ug/kg)
Malachite green	0.14
Leucomalachite green	0.20

** Based on three times of the standard deviation of six determinations of fish sample spikes at 0.5 ug/kg.*





Malachite Green & Leucomalachite green method performance

- Recoveries study

Analytes	Spike Lvl (ug/kg)	n	Average Recovery (%)
Malachite green	0.5-1	25	94
Leucomalachite green	0.5-1	25	104



Analysis of Malachite Green in Fish



Thanks !



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