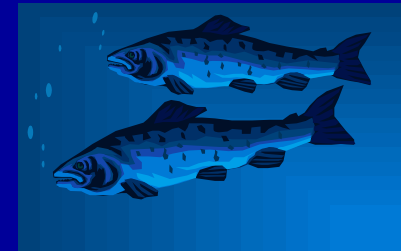
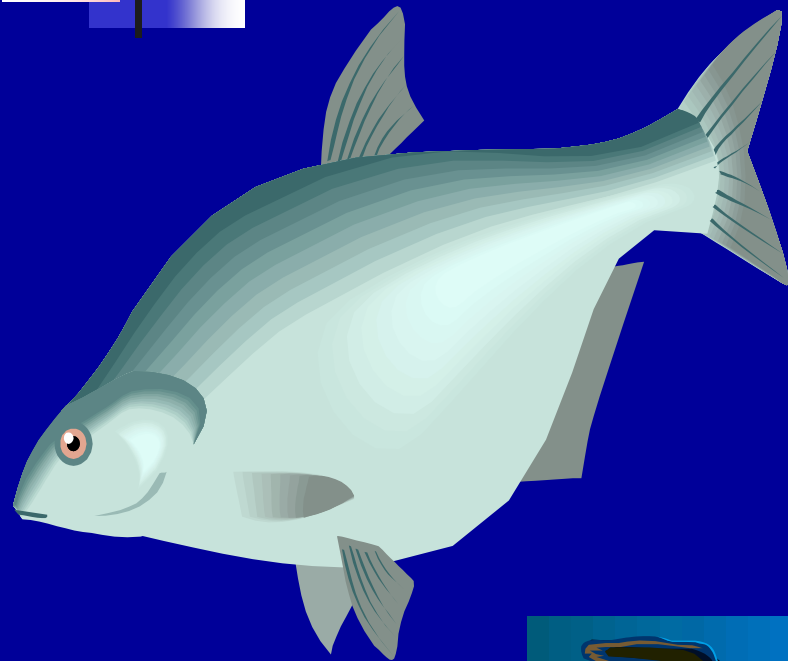


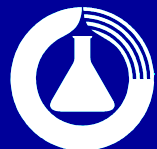
Analysis of Malachite Green in Fish



BY
Hubert PO Tang
Jacky Y Y CHOI

14 Sept 2005

HKSAR Government Laboratory





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

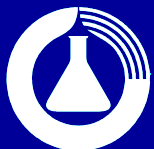




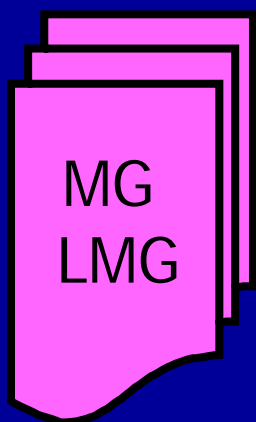
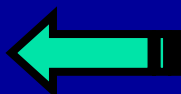
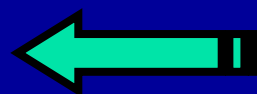
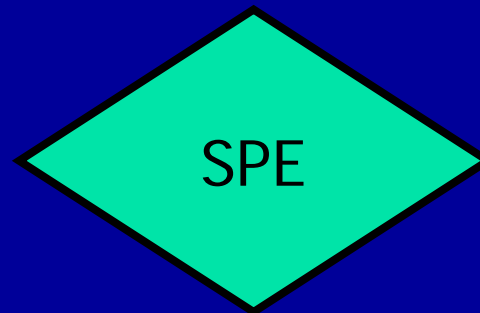
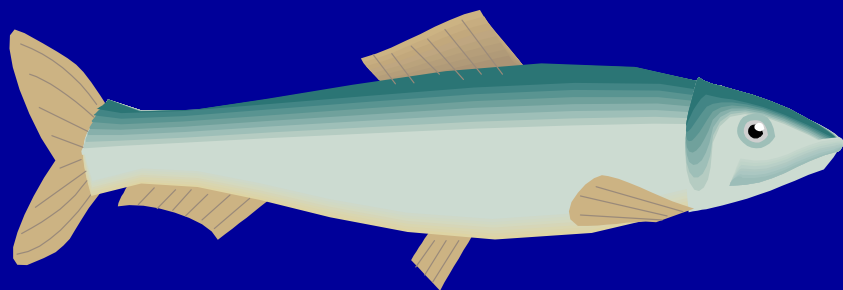
Useful References

- 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



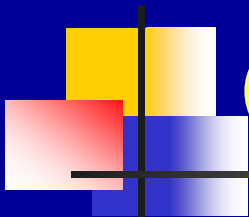
Sample Preparation Summary



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Consideration ?

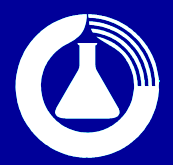
Analytical Time

HPLC UV

Sensitivity

HPLC MS

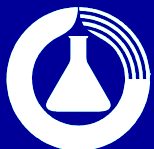
Selectivity





Pros and Cons

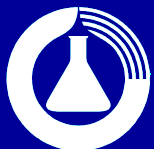
- GC-MS (SIM)
 - The most Common MS in laboratories
 - Relatively higher selectivity than common LC detectors e.g. UV, FLD, DAD.
 - Malachite green: Non-volatile, decompose in GC.
 - Only LMG analysis; no information on MG
 - (Relative higher detection limit: 5ng/g)





Pros and Cons

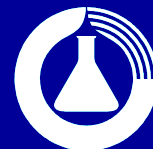
- LC-UV
 - Low cost
 - Relatively sensitive at wavelength maxima
 - Only single wavelength, not confirmative
 - LMG Maxima at 266nm – interference problem !!





Pros and Cons

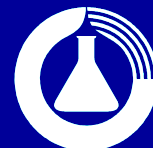
- LC-DAD
 - Multiple wavelength measurement
 - Peak purity information
 - Relatively less sensitive
 - Not confirmative enough compared with Tandem MS. Intensive sample purification required to prevent potentially interference from impurities, prevent co-elution.





Pros and Cons

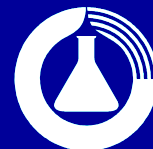
- LC-FLD
 - Higher sensitivity than UV / DAD detectors
 - Less background noise
 - Not confirmative enough
 - Intensive sample cleanup required
 - Prevent co-elution

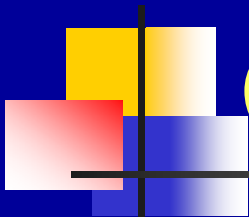




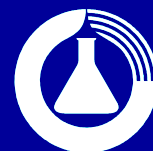
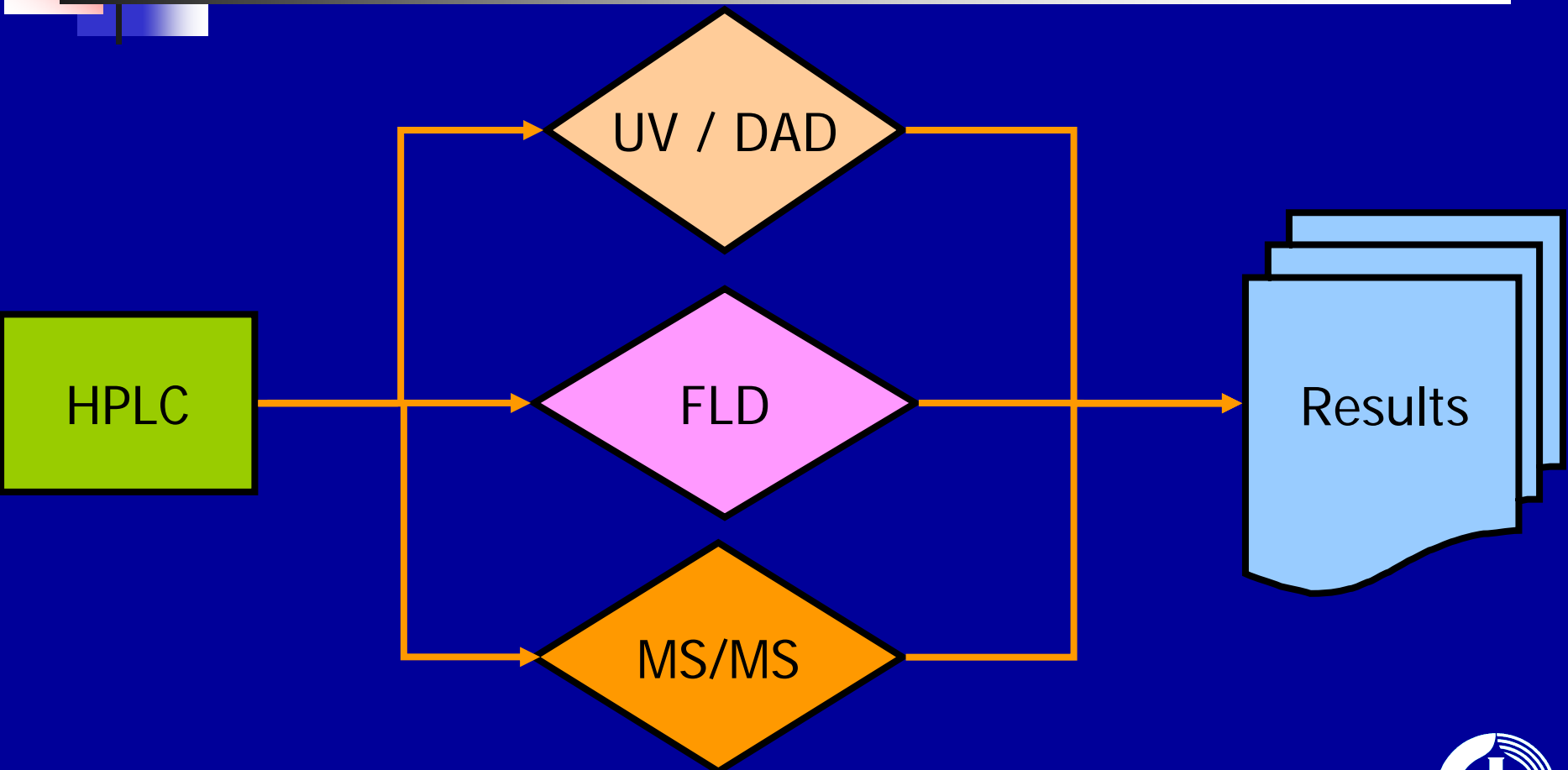
Pros and Cons

- 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:

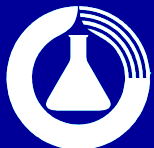
- Analytical time

- ... fast ... imported live



HPLC-MS/MS

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



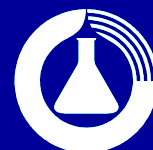
Analysis of Malachite green in HK SAR Government Laboratory

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



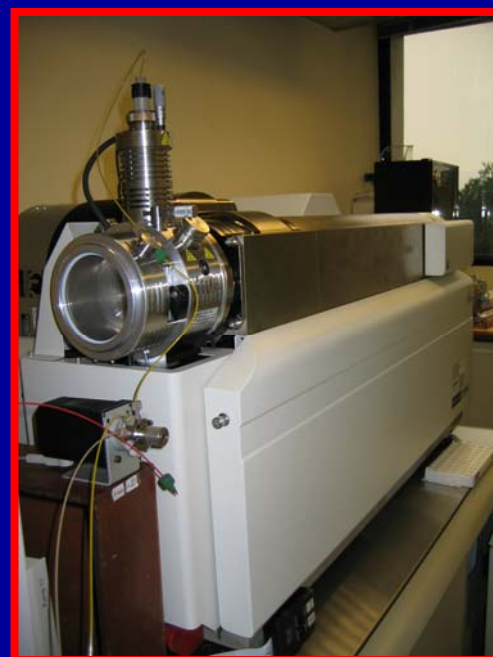
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Analysis of Malachite green in HK SAR Government Laboratory

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



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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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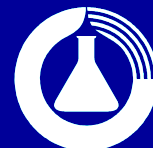
HKSAR Government Laboratory



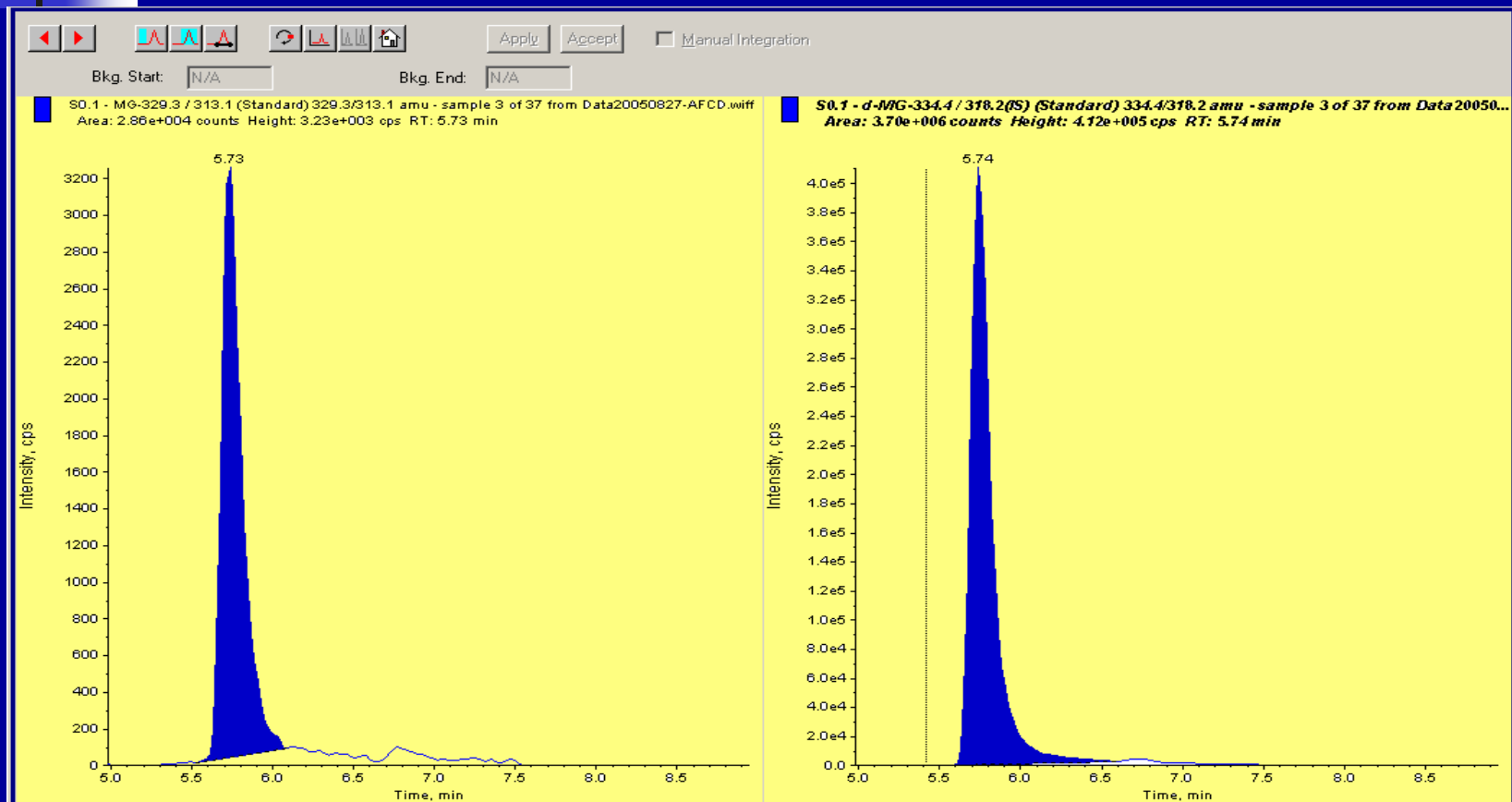


Tips on MS analysis

- 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

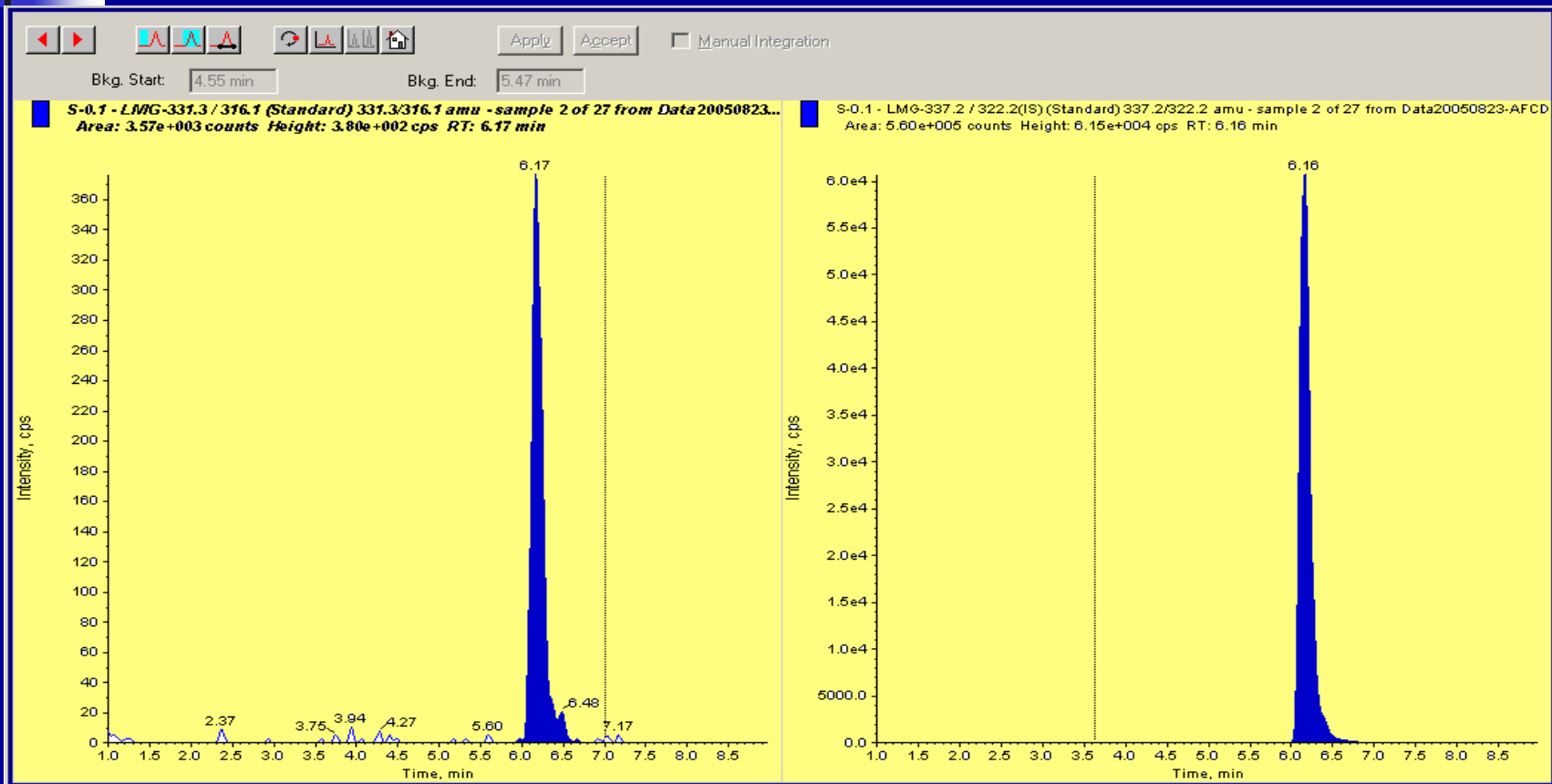


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0.1 ug/L standard solution – Leuco malachite green

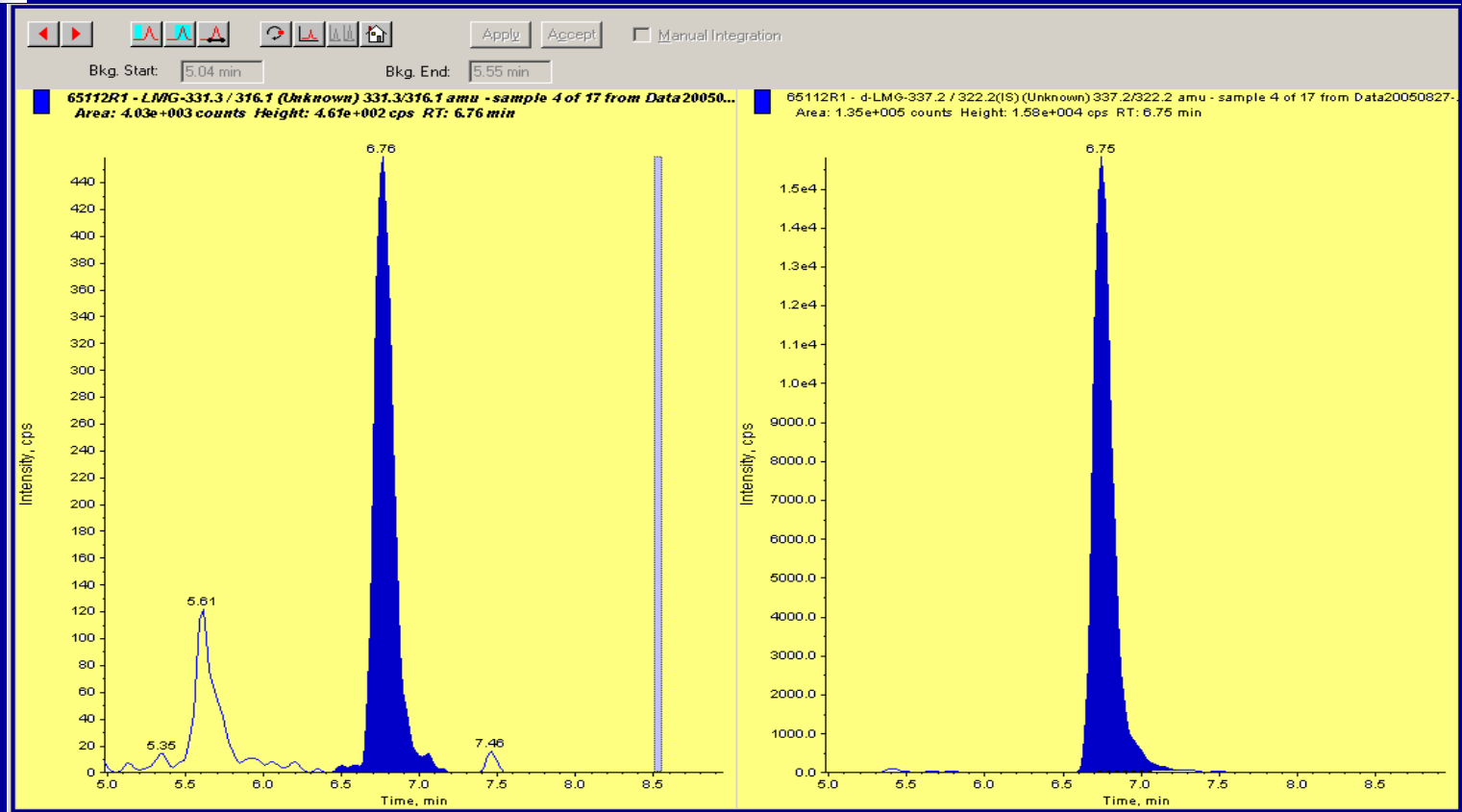


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0.5 ug/kg QC spike in fish sample - Leucomalachite green

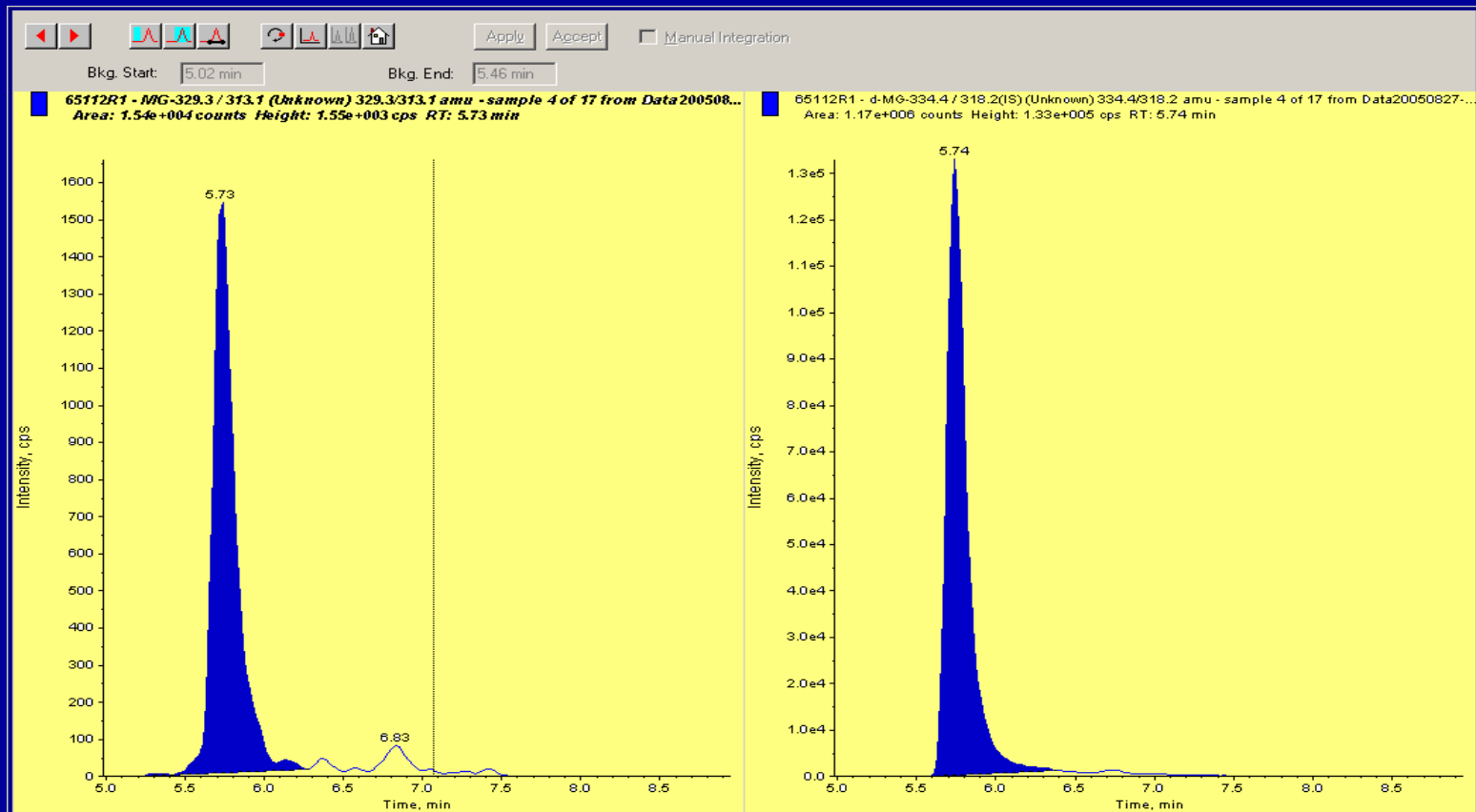


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0.5 ug/kg QC spike in fish sample - Malachite green

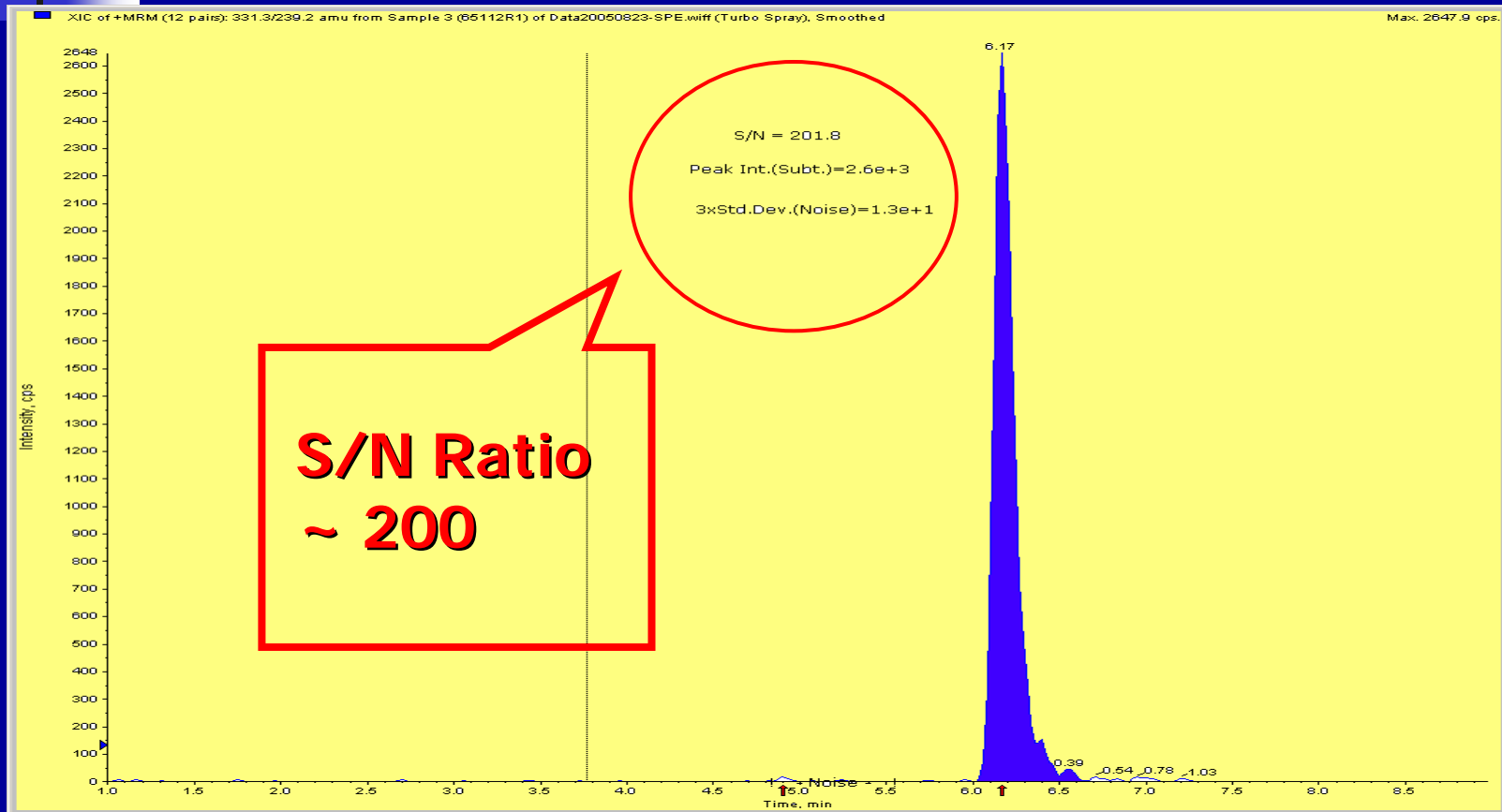


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0.5 ug/kg QC spike in fish sample – Leucomalachite green



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Requirements for Method Validation **Food (Veterinary drug residue)**

Codex CAC/GL16-1993

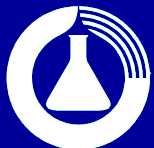
- **Codex Guidelines for the Establishment of a Regulatory Programme for Control of Veterinary Drug Residues in Foods**
- **Food and Drug Administration Center for Veterinary Medicine July 1999 Guidance for Industry 64, “Validation of Analytical Procedures: Methodology”**
- **Food and Drug Administration 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**

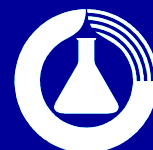




Method Types – Codex

Based on Intended use:

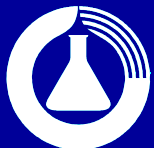
- **Level I: Confirmatory Methods**
 - unambiguously identifies the analyte and
 - may provides quantitative information
- **Level II: Determinative Methods**
 - quantitative results
- **Level III: Screening (semi-quantitative) Methods**





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



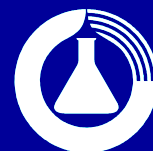


Requirements for Method Performance Confirmation (vet. drug)

■ **Chromatography separation**

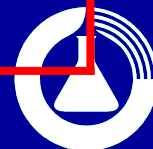
Attribute	EU
Retention time	2 times void volume
Relative retention time (with Internal standard I.S.*)	GC\pm0.5%, LC\pm2.5%
Relative retention time (without I.S.)	GC/LC\pm5%
ID Points	\geq 4

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



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

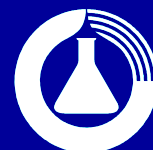
<i>Technique</i>	<i>No. of ions</i>	<i>I.D. points</i>
GC-MS (EI or CI)	N	n
GC-MS (EI or CI)	2 (EI) + 2 (CI)	4
GC-MS (EI or CI) 2 derivatives	2 derivatives A + 2 derivatives B	4
LC-MS	N	n
GC-MS-MS	1 parent ion, 2 product ions	4
LC-MS-MS	1 parent ion, 2 product ions	4
LC-MS-MS	2 parent ions, each 1 product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

<i>Attribute</i>	<i>AOAC</i>	<i>Codex</i>	<i>EU</i>
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

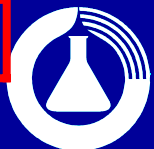


Requirements for Method Performance

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



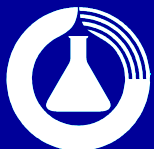


Requirements for Method Performance

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**

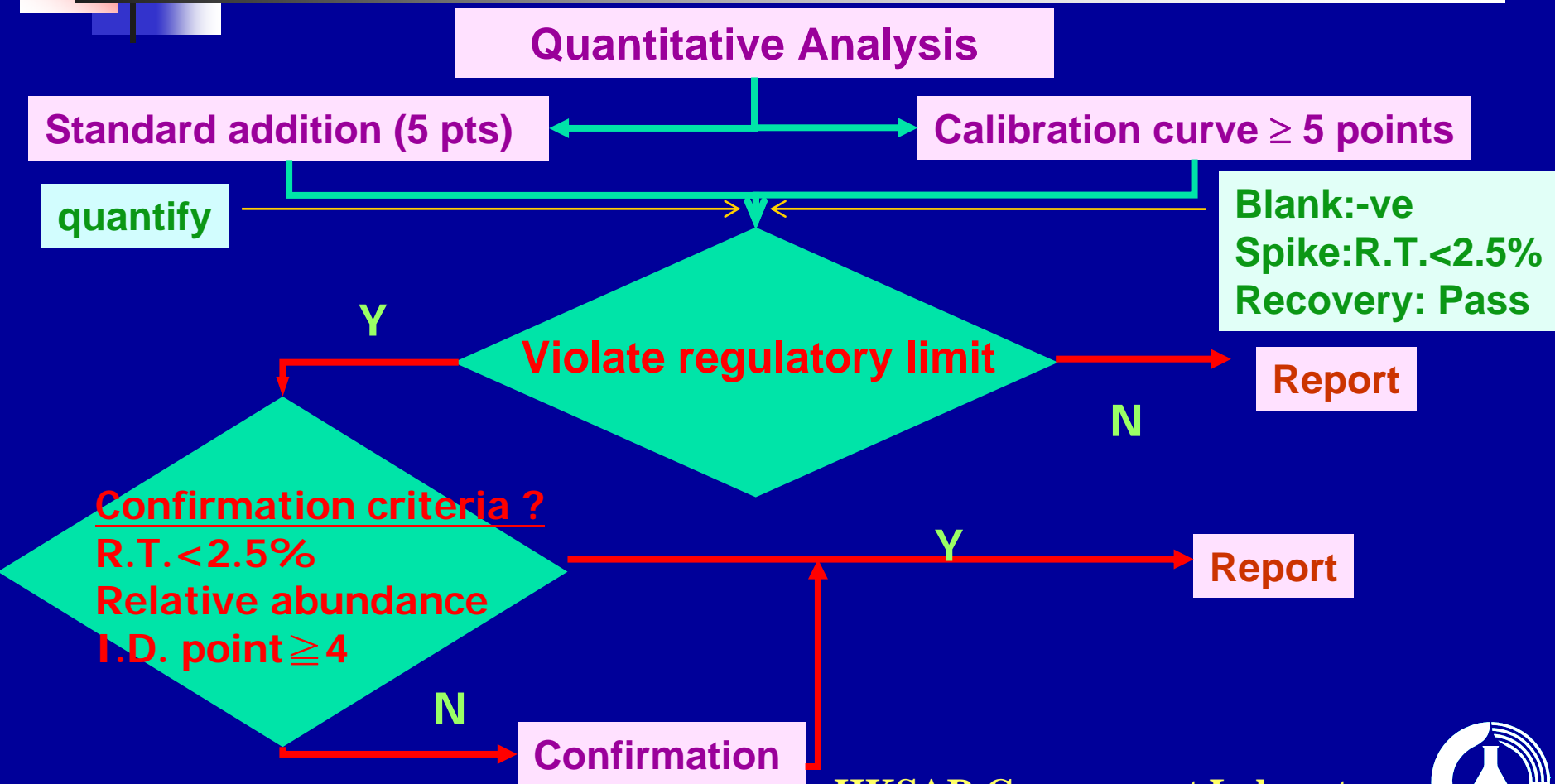
Analyte stability (vet. drug)

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



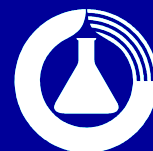
Analysis Workflow

Quantitative and Confirmatory analysis



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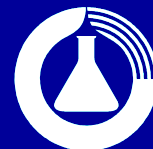


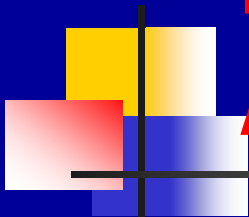


Examples

Quantitative & Confirmatory Method

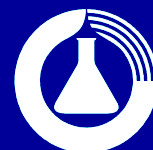
- LC-MS/MS
- Analyte: Chloramphenicol
 - A prohibited veterinary drug reported commonly used both agriculture and aquaculture industry



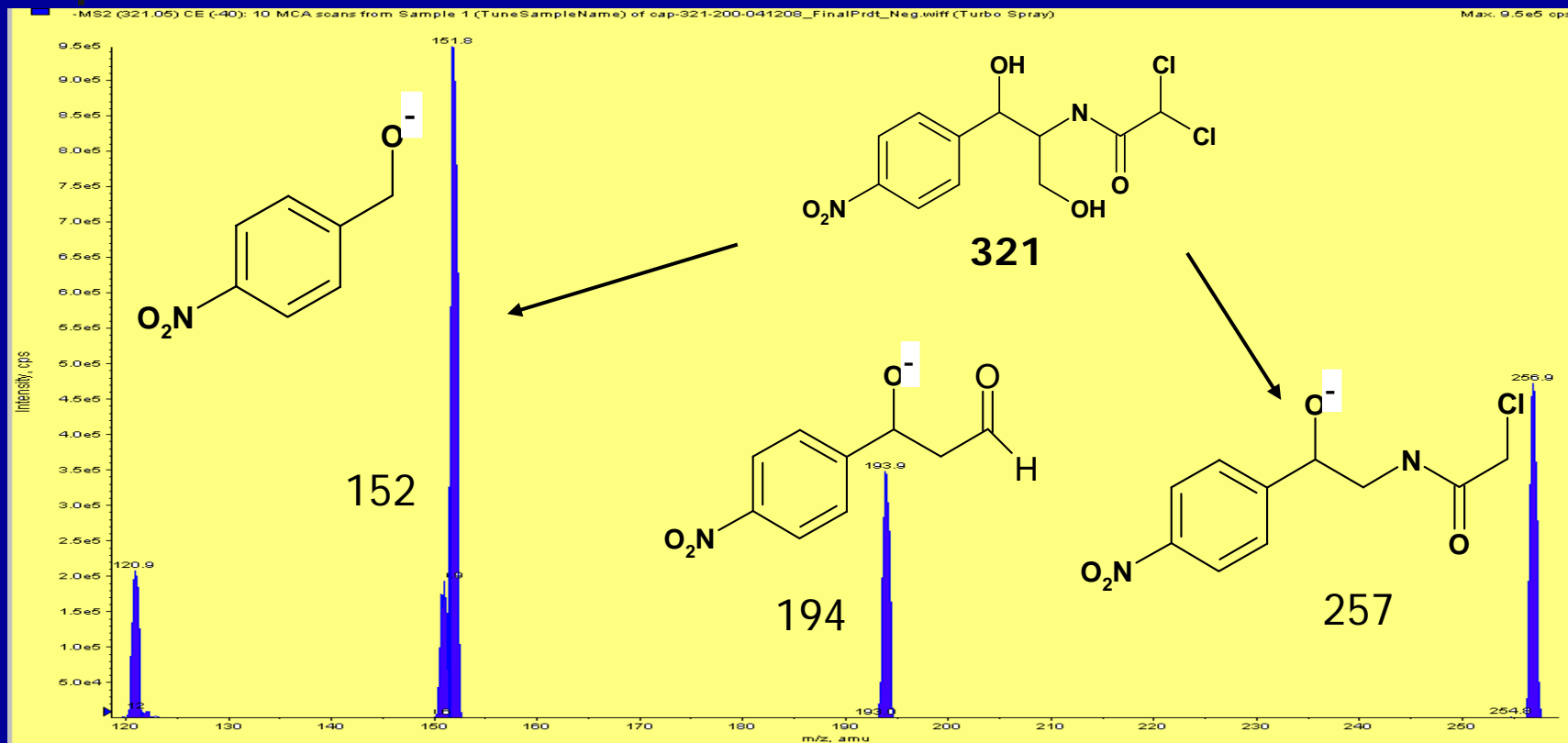


Determination of Chloramphenicol in Animal Tissue by LC-MS/MS

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



Chloramphenicol – Product ion scan

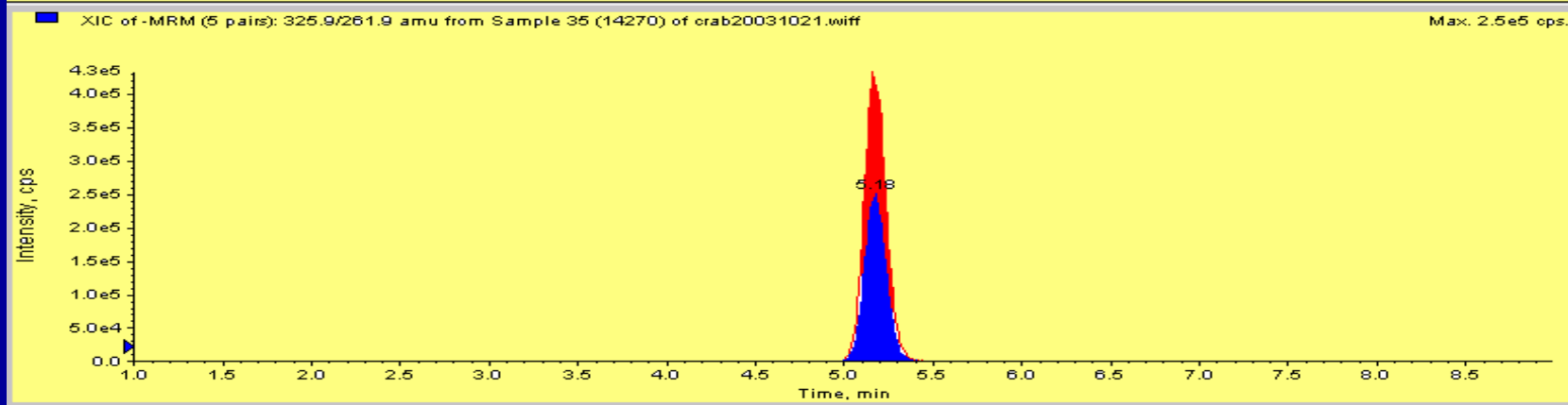
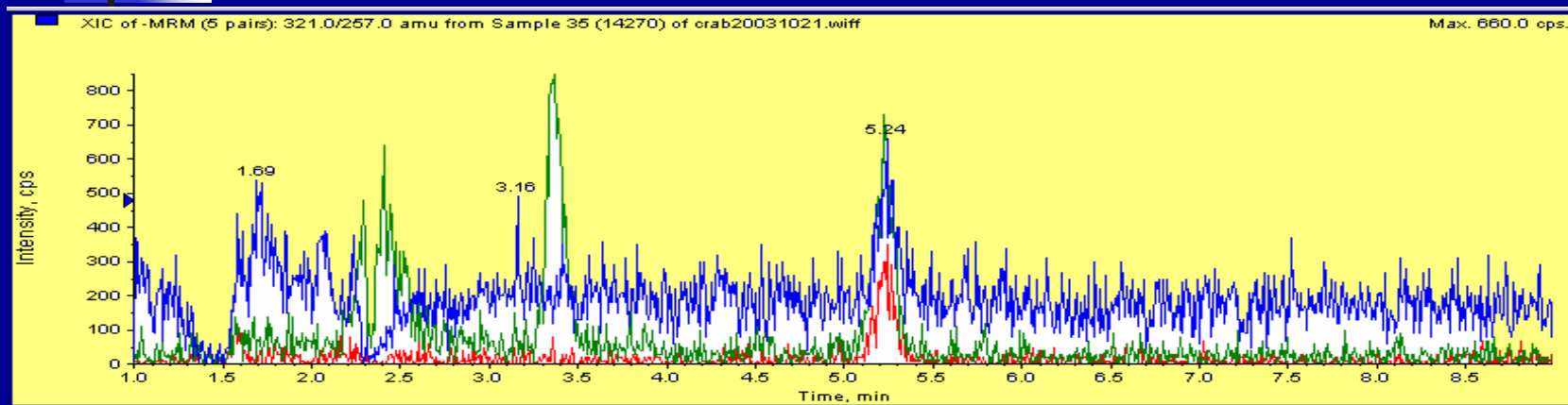


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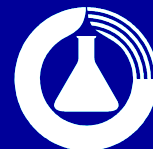


Chloramphenicol – Blk sample



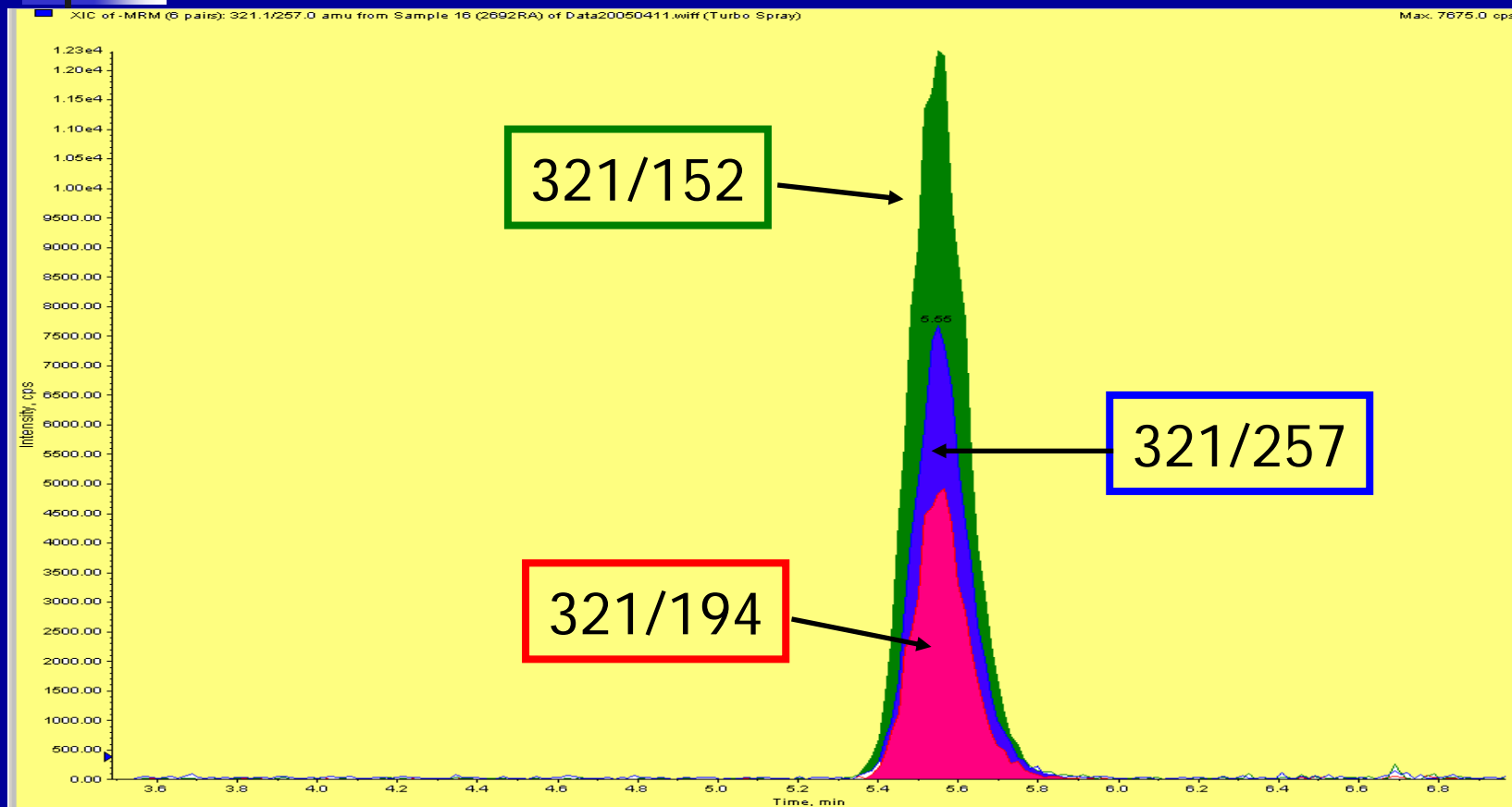
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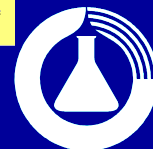
Method Validation

Chloramphenicol 1ug/kg Spike



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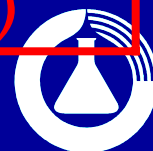
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QC Performance

<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 \leq LOQ</i> <i>Sample Blk \leq LOQ</i>
Stability		<i>1.5 years</i>
Method performance	QC spikes recovery	<i>Control Chart</i>





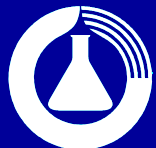
Proficiency Test - FAPAS

<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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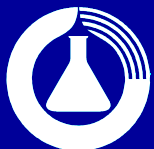


Malachite Green & Leucomalachite green method performance

- 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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