


Analysis of Triphenylmethane Dye Residues in Fish

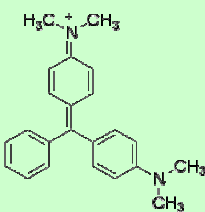

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Malachite Green (MG)

- Triphenylmethane dye
- Effective fungicide
- Common treatment for ornamental fish
- Not approved for use in aquaculture

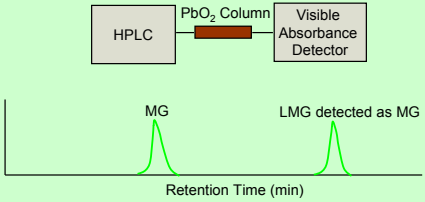



Fate and Toxicity of MG

- MG is used as a water bath treatment and is readily absorbed by fish.
- MG is metabolized to colorless leucomalachite green (LMG) in fish.
- LMG is lipophilic and can last in tissues for 40+ days.
- Both MG and LMG are potential mutagens.

Detection of MG and LMG

- Previous method for catfish (Roybal 1995) required conversion of LMG to MG using post column derivatization.



- Lead oxide columns are disadvantageous due to difficult preparation, rapid depletion, and peak broadening.

New Concerns


Salmon - problems with Chilean farm-raised salmon
 Eel - MG residues found in exports from China

Lower levels of concern- based work in Canada and EU (1-2 ng/g)

Need more sensitive and rugged analytical methods

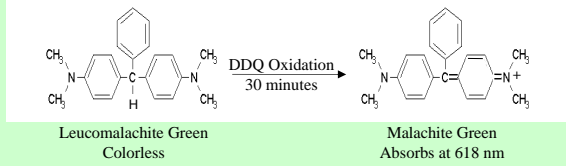
Banned in Hong Kong Malachite Green Found in Chinook Salmon
 By F&D
 Jun 4, 2005, 8:49

H.K. bans carcinogenic chemical after it was found in eels
 ASIAN ECONOMIC NEWS, August 22, 2006



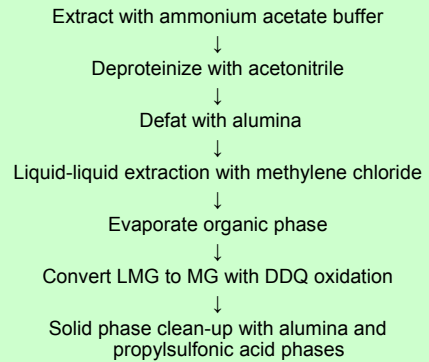
Conversion of LMG to MG

- New method uses 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to oxidize LMG during the extraction



- Results in the detection of a single analyte, which represents the sum of MG and LMG residues present in fish tissue

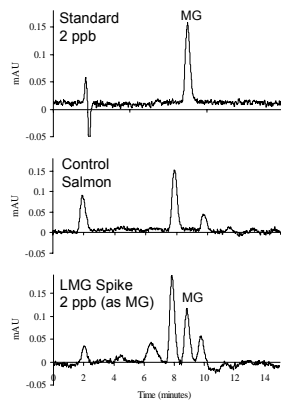
Extraction



LC-VIS Chromatogram

LC-
C18 (4.6 x 150mm) LC Column
47.5% Acetate buffer/52.5% ACN
@ 1.0 mL/min

Diode Array-
monitor 618 nm



Confirmation of MG by LC-MSⁿ

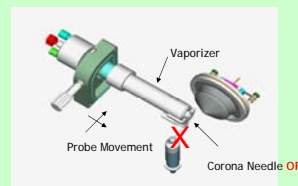
- A complete regulatory package needs positive identification of residue.
- CVM guidelines (Guidance to Industry #118) indicate product ion spectra of unknown needs to match standard with 2 or more structurally significant product ions monitored.
- Analysis of LC-VIS extracts on ion trap LC-MSⁿ instrument provide this information with 5 ions monitored.
- Different LC-MSⁿ ionization techniques (ESI and APCI with and without discharge current) were investigated for the detection of MG.

No-discharge APCI (ND- APCI)

Characteristics:

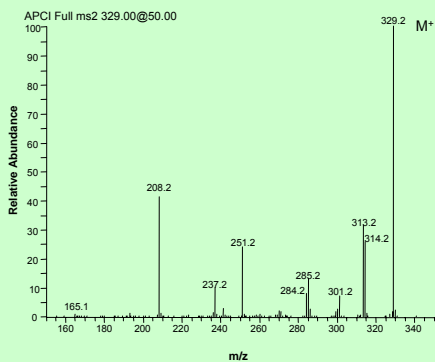
Using APCI with corona discharge OFF (no current)

- Works well for compounds already ionized in solution
- Some characteristics in common with thermospray & sonic spray ionization
- Spectra obtained similar to electrospray



Precedence for using this technique to monitor triphenylmethane dyes –
Doerge et al. *Rapid Commun. Mass Spectrom.* 1996, 1998

Product Ion Spectra of MG (M⁺) with ND-APCI LC-MSⁿ

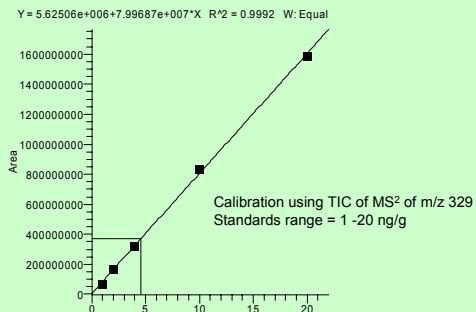


Example of LC-MSⁿ Qualitative Data (Trout)

Type sample	N	Ret. Time	% Rel Abund. m/z 329	% Rel Abund. m/z 313-315	% Rel Abund. m/z 284-286	% Rel Abund. m/z 251	% Rel Abund. m/z 208
Standards	9	4.98 ± 0.06	100	70 ± 9	25 ± 3	29 ± 3	48 ± 4
1 ng/g fortified	5	4.98 ± 0.01	100	68 ± 8	22 ± 3	25 ± 4	43 ± 6
2 ng/g fortified	5	5.03 ± 0.02	100	71 ± 4	25 ± 3	29 ± 3	50 ± 6
4 ng/g fortified	5	4.99 ± 0.02	100	73 ± 5	26 ± 2	30 ± 3	48 ± 4
10 ng/g fortified	5	4.95 ± 0.02	100	67 ± 4	24 ± 2	28 ± 2	45 ± 4

Confirmation limit for LC-MSⁿ (determined in salmon) = 0.25 ng/g

LC-MSⁿ Quantitative Data



Good overall agreement between LC-VIS and LC-MSⁿ methods

Analysis of Incurred Fish

Incurred Fish	Depuration Time	LMG/MG Found by LC-VIS	LMG/MG Found by LC-MS ⁿ
Catfish	16 hours	32.2 ng/g (6.8 % RSD)	31.3 ng/g (8.7 % RSD)
Trout	16.5 hours	27.1 ng/g (5.2 % RSD)	28.6 ng/g (3.8 % RSD)
Tilapia	16.25 hours	1.9 ng/g (7.2 % RSD)	2.1 ng/g (14.2 % RSD)
Salmon	24 hours	26.4 ng/g (3.2 % RSD)	27.4 ng/g (7.3 % RSD)
Basa	*	64.3 ng/g (5.8 % RSD)	64.7 ng/g (11.3 % RSD)

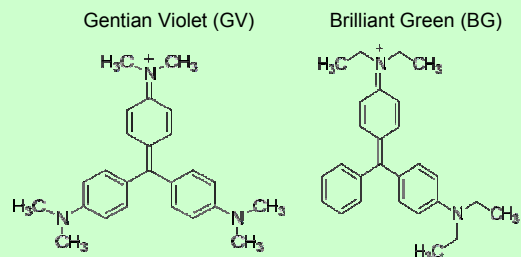
All fish except basa were exposed to 10 µg/L MG in water for 1 hour. MG residues found in basa were from store-bought (untreated) fish.

Denver Regulatory Laboratory Results

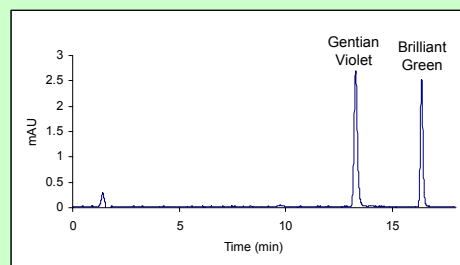
- Over 100 samples analyzed (Jan- June 2006)
- Any sample found positive by LC-VIS was confirmed by LC-MSⁿ
- Basa- over 18 samples found violative (many OCI samples)
- Eel - ~85% of samples (n = 20) found violative (several > 100 ng/g)
- LMG/MG residues have also been found in several regulatory samples each of catfish and tilapia (*but not salmon*)

FDA has announced increased sampling of fish for MG

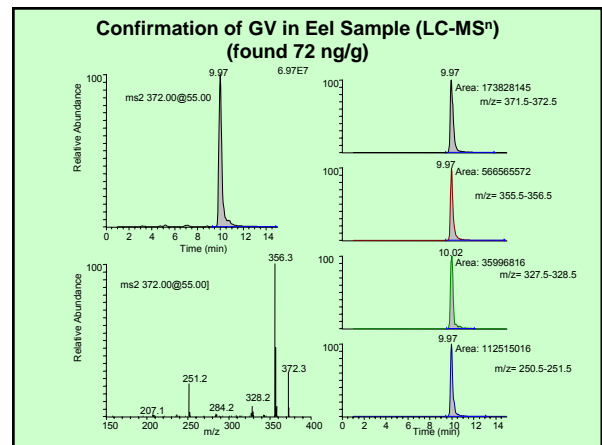
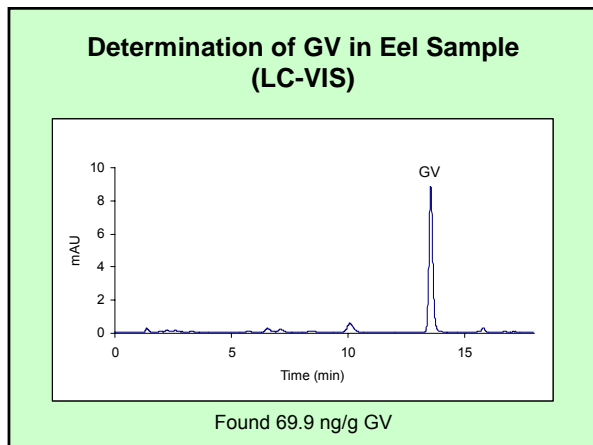
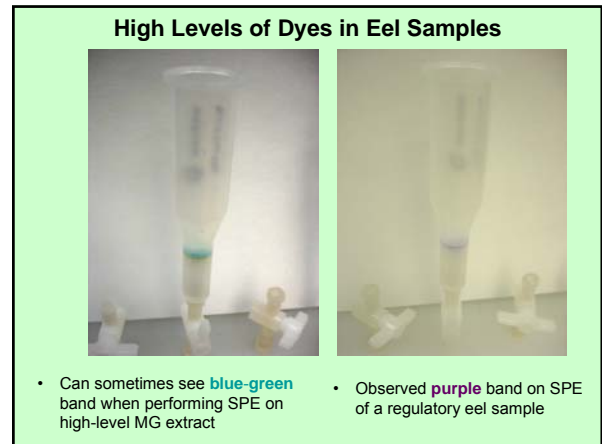
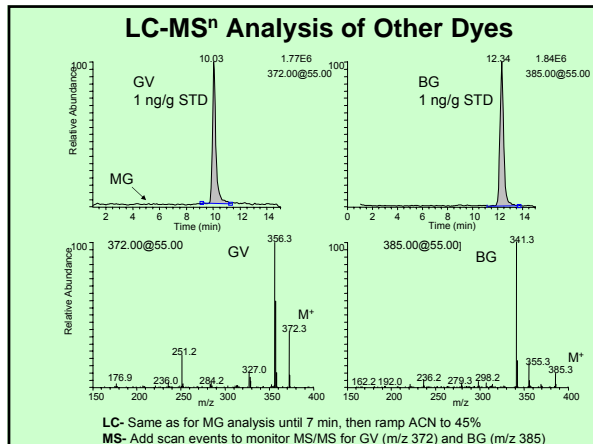
Extending Method to Other Dyes



LC-VIS Analysis of other dyes (20 ng/g Standard)



LC- Same as for MG analysis until 8 min, then ramp ACN to 50% over 7 min
Diode Array- Add channels to monitor for GV (588 nm) and BG (627 nm)



- ### Conclusions
- Methods allow for sensitive analysis of MG residues in fish by LC-VIS & LC-MSⁿ w/ no-discharge APCI.
 - Methods were developed and validated for salmon, catfish, trout, tilapia, basa, shrimp, and eel.
 - Same extract can be used for both methods.
 - Good quantitative agreement between LC-VIS & LC-MSⁿ.
 - LC-MSⁿ provides sufficient structural ions for confirmation of residue.
 - Methods are being used in regulatory laboratory to monitor for MG in aquacultured products.
 - Methods have been extended to monitor for other dyes.

- ### References
- US FDA Laboratory Information Bulletins #4363, 4334, 4333
 - Andersen, Roybal, Turnipseed *J. AOAC Int.* **88** (2005) 1292.
 - Turnipseed, Andersen, Roybal *J. AOAC Int.* **88** (2005) 1312.
 - Andersen, Turnipseed *J. Ag. Food Chem.* **54** (2006) 4517.

Acknowledgements

- Denver Veterinary Drugs Section
- Denver Laboratory Management
- Center for Veterinary Research/Office of Research