IDENTIFICATION OF METABOLITES OF BRILLIANT GREEN IN TROUTS USING LTQ-ORBITRAP

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INTRODUCTION

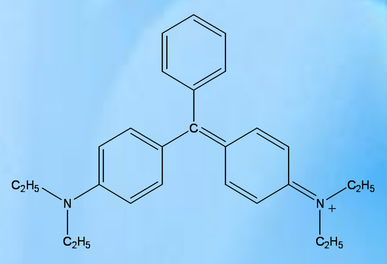
- The triphenylmethane dyes, Malachite Green (MG) and Crystal Violet (CV) are sometimes used illegaly as antimicrobial, antifungal and antiparasitic agents in aquaculture. These compounds are not authorized in food animal production because of their toxic effects on humans (mutagenic, carcinogenic). In vivo, MG and CV are rapidely metabolized in treated fish in their leuco forms by reduction. Another triphenylmethane compound, **the Brilliant Green (BG)**, also called Malachite Green G, displays a similar chemical structure as MG and CV, and therefore might hold the same toxic effects. There is no such literature data on the metabolism of BG as for MG and CV, but it is presumed to metabolize in its leuco form in vivo in treated fish as well.
- The present study was intended to identify possible metabolites of brilliant green in trout tissues by **high resolution mass spectrometry measurement using an LTQ-Orbitrap** instrumentation. Two complementary approaches were conducted in order to confirm the presence of the **leuco brilliant green form** and to identify others possible metabolites of brilliant green from treated trouts :
  - **A targeted approach** : Based on the extraction of the accurate mass of targeted compounds on full-scan LC-HRMS ion chromatogram.
  - **An untargeted approach** : Based on the comparison of full-scan LC-HRMS acquisitions of blank samples and positive samples using SIEVE software for identification of statistically significant differences .

ANIMAL EXPERIMENT

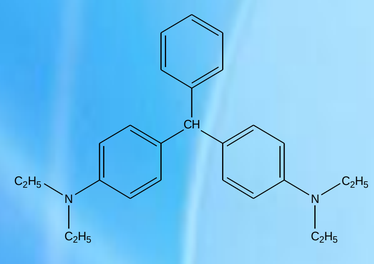
Aim : Obtain BG treated trouts and non-treated trouts

1 trout without treatment
3 trouts treated :
water bath with a concentration of BG of 100 ng/ml during 1 hour . Then depuration in clean water and slaughtering after 0, 1 and 2 hours.

TARGETED APPROACH : Extracted Ion Chromatogram (EIC)

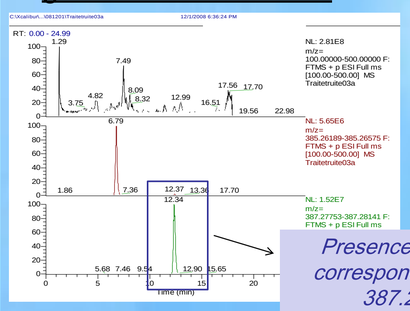


Brilliant green (BG) : $C_{27}H_{33}N_2^+$
Theoretical mass M^+: 385,26382



Leuco Brilliant green (LBG) : $C_{27}H_{34}N_2$
Theoretical mass $[M+H]^+$: 387,27947

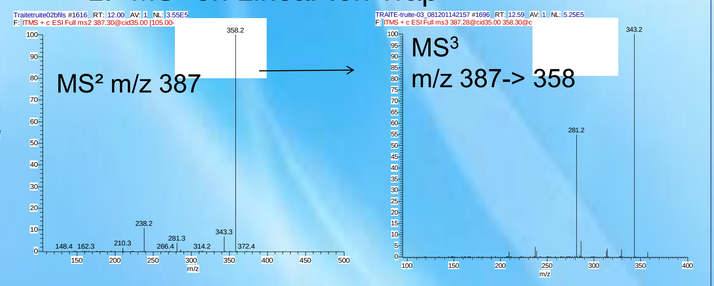
▪ Extracted ion chromatograms of theoretical masses (5 ppm) of Brillant green and leucobrillant green in a treated fish



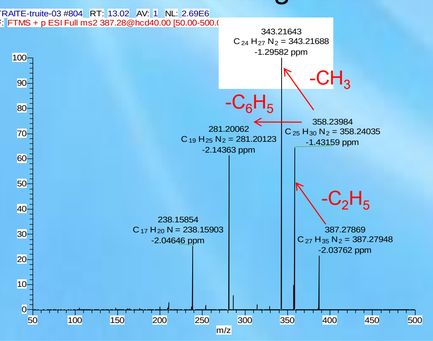
Presence of a peak corresponding to m/z 387.27902 (Δ ppm = -1.17, $C_{27}H_{35}N_2$)

▪ Structural identification using different fragmentation experiments

1. MS^n on Linear Ion Trap



2. MS^2 m/z 387.279 with HCD fragmentation on Orbitrap



▪ Validation of the identification of leuco brilliant green

The identification of leuco brilliant green metabolite was confirmed by comparison with a synthetized reference standard of leucobrilliant green (made by Atlanchim, Nantes).

SAMPLE PREPARATION

To 2 g of trout muscle, 500 µl of hydroxylamine (20g/l) were added and the mixture was let to stand for 10 min. Then tissue was extracted by shaking for 10 min with acetonitrile. 1 g MgSO4 was added and the mixture was shaken for 1 min. The homogenate was centrifuged and 6 ml of the supernatant was evaporated to dryness under N_2 at 40 C. The residue was then reconstituted with 800 µl of acetonitrile and injected into the LC mass spectrometer.

LIQUID CHROMATOGRAPHY

Pump : Accela (Thermo)
Column : C18, Symmetry, 2.1 x 100, 3.5 µm (Waters)
Mobile phase : gradient mode, Ammonium acetate 0.05 M / ACN, flow rate 0.20ml/min

MASS SPECTROMETRY

MS : LTQ-Orbitrap
Acquisition : Full scan resolution 60000, positive detection.

UNTARGETED APPROACH : Metabolomic approach

LC/HRMS Analysis of blank control and incurred samples (6 blank / 6 treated fishes) → Data analysis by SIEVE software → Identification of peaks of interest

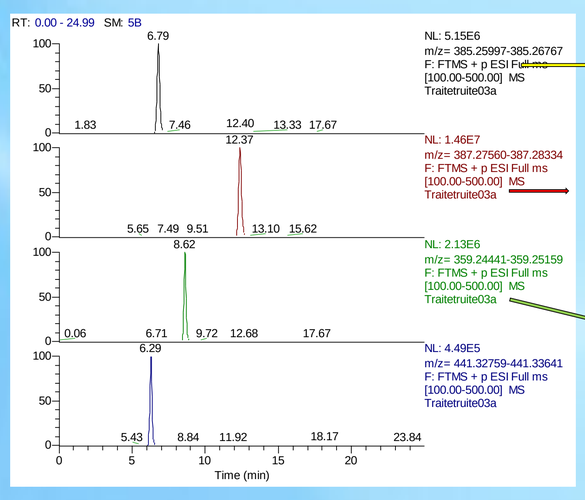
SIEVE table results

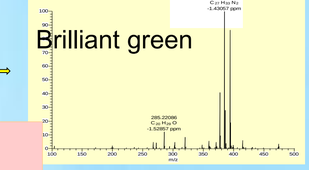
-Ions are sorted by lowest p-value – the lowest indicating the most **statistically significant differences** between blank control and treated fishes.
- Ions grouped by retention time

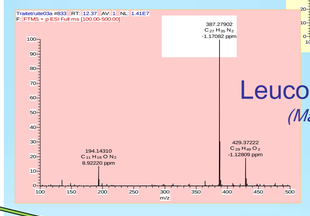
FrameID	pValue	Average Time	Average m/z	MZStart	MZStop	TimeStart	TimeStop	Ratio
15	8.914E-11	12.7368	387.279	387.269	387.289	11.4868	13.9868	423.471
499	7.63212E-9	12.7368	194.645	194.635	194.655	11.4868	13.9868	23.4527
166	1.11536E-8	12.7963	194.143	194.133	194.153	11.5463	14.0463	116.095
88	1.52514E-8	12.7963	388.282	388.272	388.292	11.5463	14.0463	251.448
57	2.54684E-7	8.88219	359.248	359.238	359.258	7.63218	10.1322	51.261
479	6.93342E-7	6.44377	384.31	384.3	384.32	5.19377	7.69377	10.9317
175	9.25873E-7	6.96329	386.267	386.257	386.277	5.71329	8.21329	7.2466
477	1.75513E-5	6.39916	428.337	428.327	428.347	5.14916	7.64916	12.5942
37	2.26705E-5	6.99279	385.264	385.254	385.274	5.74279	8.24279	3.81795
204	4.90222E-5	8.88219	360.251	360.241	360.261	7.63218	10.1322	8.32198
587	6.01214E-5	12.7963	389.285	389.275	389.295	11.5463	14.0463	255153
641	6.28737E-5	6.39916	441.332	441.322	441.342	5.14916	7.64916	6.74141
97	8.79363E-5	12.0088	140.002	139.992	140.012	10.7588	13.2588	0.0827744
312	0.000146443	12.7219	181.028	181.018	181.038	11.4719	13.9719	0.109765
221	0.000147813	6.42902	402.253	402.243	402.263	5.17902	7.67902	2.4405
113	0.000162488	6.44377	356.279	356.269	356.289	5.19377	7.69377	10.3162

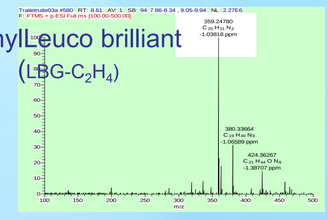
Identification of peaks of interest => Potential metabolites

- Extracted ion chromatograms of differentially expressed ions









QUANTITATION OF INCURRED MUSCLES OF TROUTS USING LC/MSMS in MRM mode

Animal	Treatment brilliant green		Brilliant Green (MRM 385 > 341)	Leuco Brilliant green (MRM 387 > 343)
	Bath at 100 ng/ml	Bath in clear water before slaughtering		
Trout 1	-	-	-	-
Trout 2	1 hour	0	7.9 µg/kg	7.8 µg/kg
Trout 3	1 hour	1 hour	27.4 µg/kg	18.2 µg/kg
Trout 4	1 hour	2 hours	25.4 µg/kg	14.9 µg/kg

Conclusions

Metabolites identification of brilliant green in trouts was performed using LC-HRMS : Like others triphenylmethane compounds, Brilliant green is metabolized in its reduced form, the leuco brilliant green. Using an untargeted approach, another metabolite, the desethyl leuco brilliant green was identified.
Further experiments shoud be carried out to measure the depletion of these metabolites and establish the target marker residue of a treatment of brillant green and control the misuse of this compound.