anses
French agency for food, environmental and occupational health & safety

Strategies for a flawless determination of prohibited drugs in contaminated foods: a case study of deliberate fish exposure to multi-dyes

Estelle DUBREIL - Eric VERDON

Anses (France)

AOAC INTERNATIONAL

AOAC

Educate Network Collaborate

ANNUAL MEETING & EXPOSITION

133rd Annual Meeting | September 6-12, 2019 | Denver, Colorado

EURL

European Union Reference Laboratory for Antimicrobial Residues and dye in Food

Content

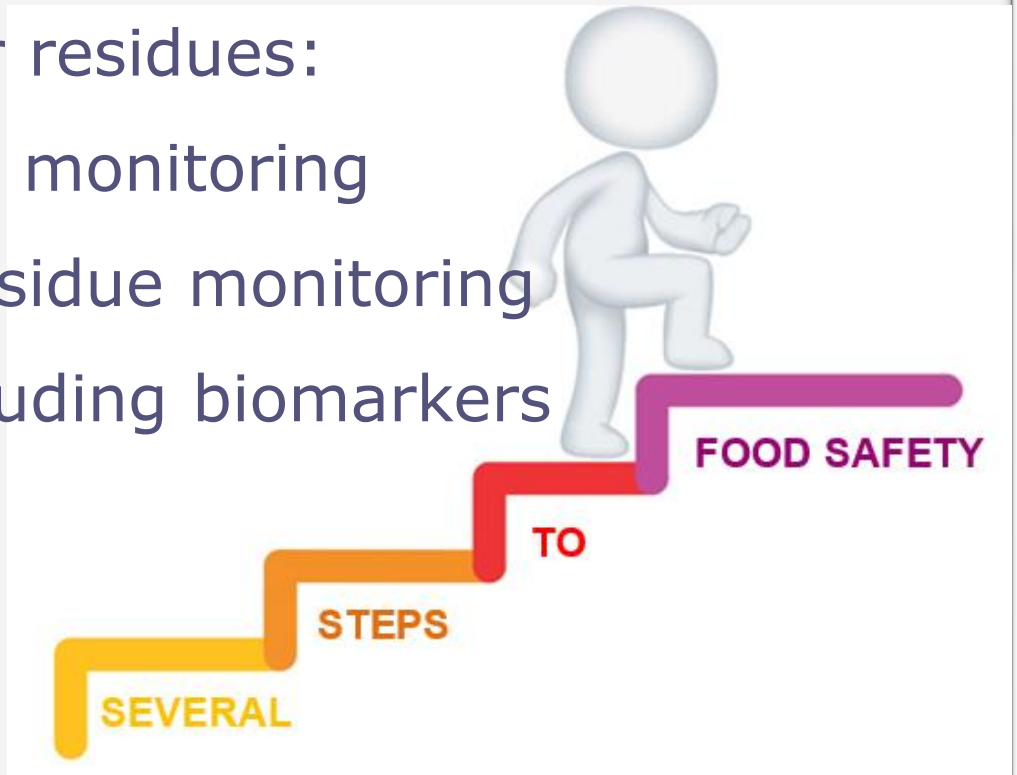
-Use of prohibited vet drugs

-Regulation

-Overview of existing analytical strategies

-Considerations to monitor residues:

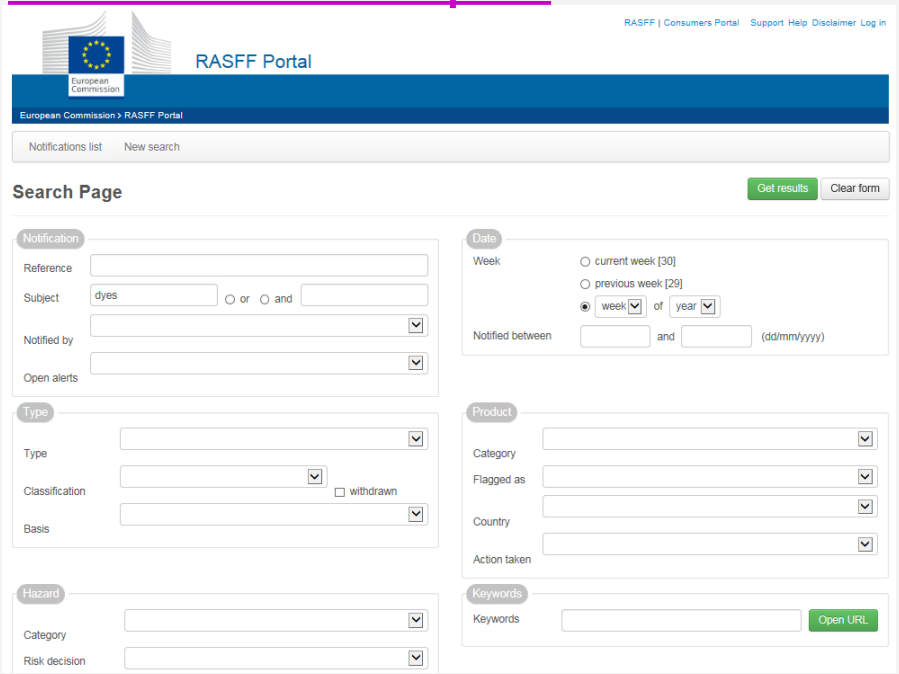
- Restrictive residue monitoring
- Extensive multi-residue monitoring
- Multi-residues including biomarkers /metabolites



Use of prohibited vet drugs

E.Dubreil - E.Verdon

Alerts in Europe:



Ronidazole : 13 alerts (since 2005)
AMOZ: 111 alerts (since 2002)
Chloramphenicol: 520 (since 2001)
Malachite green: 150 (since 2002)
Crystal violet: 15 (since 2002)
Victoria pure blue bo: 1 (in 2010)

Use of prohibited vet drugs

E.Dubreil - E.Verdon

Dyes: Toxicity and harmfull effects for consumers

efsa European Food Safety Authority

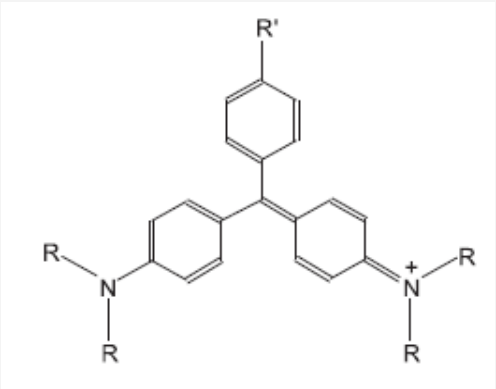
classified 20 dyes (2017) :

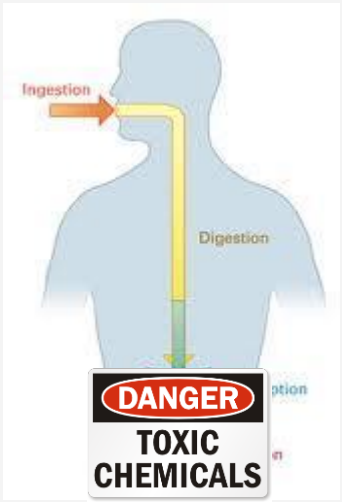
Substance belongs to **Group I.**
TSV is **0.0025** µg/kg bw per day

Residues

Food poisoning

Genotoxic
Mutagenic





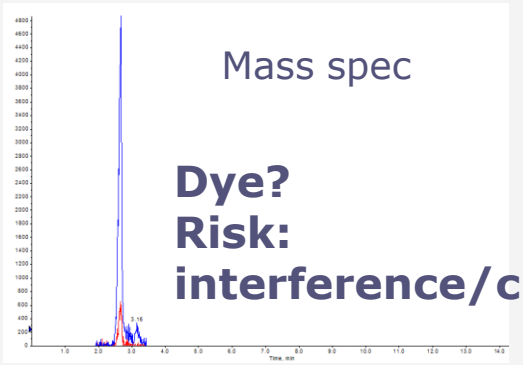
Malachite green- Crystal violet
Brilliant green- Victoria blue - Pararosaniline- Other dyes …

Regulation for dyes

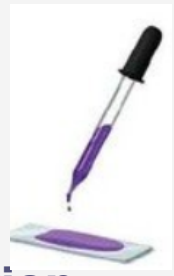
CODEX ALIMENTARIUS **WHO/FAO**

Policy of "zero tolerance" adopted in most countries
JECFA ⇨ ADI (acceptable daily intake)

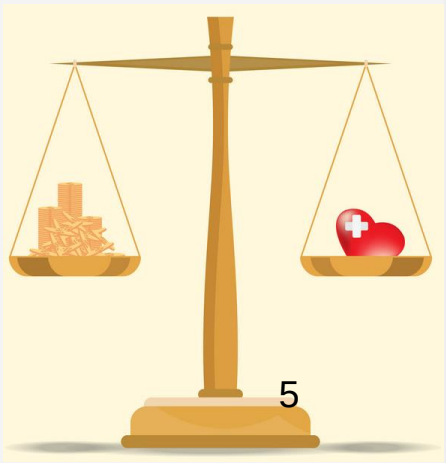
Analytical Methods: what is required for prohibited substances?
Identification points (in EU) to ensure reliability of analyte control



Dye?
Risk:
interference/cross contamination



Is it enough?



- Avoid false negatives: Safety for Health!
- But also false positives:
  Important consequences for farmers!

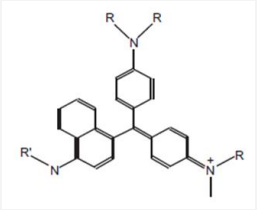
Overview of analytical strategies

E.Dubreil - E.Verdon

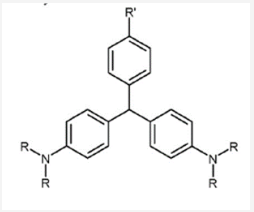
40 years

Multi-class methods

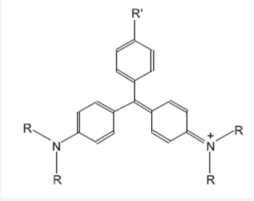
10 residues (multi-families of dyes)+ some metabolites



3 residues + metabolites



1 residue (+ leuco form)



1st option: Restrictive monitoring

Malachite green
Crystal violet
(Brilliant green)
+
Leuco
metabolites

in

by

Based on known **fraudulent treatment or occurrence**
Often Post-alerts: "curative"
Suited for National Residues control plans:
routine methods

Considerations for the monitoring

1st option: Restrictive monitoring

example

VETERINARY DRUG RESIDUES

Determination of Residues of Three Triphenylmethane Dyes and Their Metabolites (Malachite Green, Leuco Malachite Green, Crystal Violet, Leuco Crystal Violet, and Brilliant Green) in Aquaculture Products by LC/MS/MS: First Action 2012.25

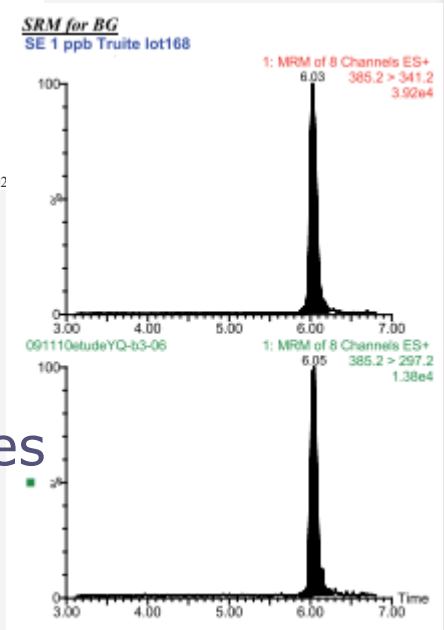
DOMINIQUE HURTAUD-PESSEL, PIERRICK COUÉDOR, and ERIC VERDON[1]
ANSES, French Agency for Food, Environmental and Occupational Health Safety, Fougères Laboratory, Veterinary Drug Residues Unit, EU-RL for Antibiotic and Dye Residues, La Haute Marche, Javené, BP 90203, 35302 Fougères, France
DAWN DOWELL
AOAC INTERNATIONAL, 481 North Frederick Ave, Suite 500, Gaithersburg, MD 20877-2417

+

RESIDUES AND TRACE ELEMENTS

Dye Residue Analysis in Raw and Processed Aquaculture Products: Matrix Extension of AOAC INTERNATIONAL *Official Method*[SM] 2012.25

WENDY C. ANDERSEN
U.S. Food and Drug Administration, Animal Drugs Research Center, Denver Federal Center, Bldg 20, Denver, CO 80225
CHRISTINE R. CASEY, TARA J. NICKEL, and SUSAN L. YOUNG
U.S. Food and Drug Administration, ORA Denver Laboratory, Denver, CO 80225
SHERRI B. TURNIPSEED
U.S. Food and Drug Administration, Animal Drugs Research Center, PO Box 25087, Denver Federal Center, Denver, CO 802



Very reliable and sensitive (range 0.5 - 2 µg/kg)
Often methods with extensive sample purification
Intensive validation: inter-species, inter-laboratories

Issues

- only three dyes monitored
- not all metabolites available
- some false results due to cross contamination is possible

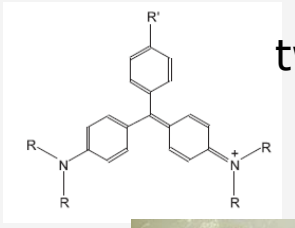
Considerations for the monitoring

2nd option: Extensive multi-families monitoring

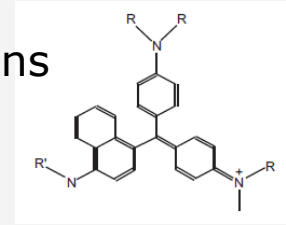
Triarylmethanes
Triarylnaphtylmethanes
Phenothiazines
Phenoxazines
Xanthènes

in

by



twins



Based on known **similar properties**

and/or therapeutic effects

(Alderman, Journal of Fish Diseases, 1982)

Not necessarily proofs of fraudulent use
More costly methods: not yet applied in routine

Parasitic fungi

Considerations for the monitoring

2nd option:Extensive multi-families monitoring

examples

ANALYTICA CHIMICA ACTA 625 (2008) 188–194

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/aca

ELSEVIER

Multiresidue determination of triarylmethane and phenothiazine dyes in fish tissues by LC–MS/MS

Jonathan A. Tarbin, Danny Chan, George Stubbings, Matthew Sharman*

Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK

Journal of Chromatography B, 953–954 (2014) 92–101

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

ELSEVIER

Multi-dye residue analysis of triarylmethane, xanthene, phenothiazine and phenoxazine dyes in fish tissues by ultra-performance liquid chromatography–tandem mass spectrometry

Tim Reyns[a,*], Claude Belpaire[b], Caroline Geeraerts[c], Joris Van Loco[a]

[a] *Scientific Institute of Public Health, Food, Medicines and Consumer Safety, Chemical Residues and Contaminants, Juliette Wytsmanstraat 14, 1050 Brussel, Belgium*

[b] *Research Institute for Nature and Forest, Duboislaan 14, 1560 Groenendaal-Hoeilaart, Belgium*

eraardsbergen, Belgium

Journal of Chromatographic Science 2012;50:591–597
doi:10.1093/chromsci/bms054 Advance Access publication April 26, 2012

Article

Simultaneous Determination of Malachite Green, Crystal Violet, Methylene blue and the Metabolite Residues in Aquatic Products by Ultra-Performance Liquid Chromatography with Electrospray Ionization Tandem Mass Spectrometry

Ying-Jiang Xu[1,2], Xiu-Hui Tian[1,2], Xiu-Zhen Zhang[1,2], Xiang-Hong Gong[1,2], Hui-Hui Liu[1,2], Huan-Jun Zhang[1,2], Hui Huang[3] and Li-Min Zhang[1,2*]

[1]Marine Fisheries Research Institute of Shandong Province, Yantai 264006, People's Republic of China, [2]Shandong Province Key Laboratory of Restoration for Marine Ecology, Yantai 264006, People's Republic of China, and [3]College of Food Science and Technology, Shanghai Ocean University, Shanghai 200000, People's Republic of China

Reliable and sensitive, sometimes a compromise between molecules with ± extensive purification

Validation intra-lab in line with the regulatory requirements

Considerations for the monitoring

2nd option: Extensive multi-families monitoring

example

Oxidation step to convert unknown leuco forms

Food Chemistry 294 (2019) 355–367

Contents lists available at ScienceDirect

Food Chemistry

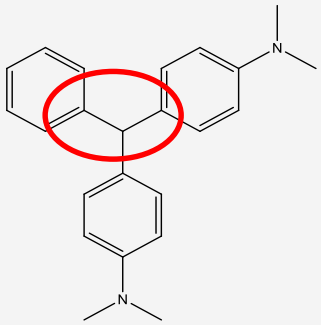
journal homepage: www.elsevier.com/locate/foodchem

Review

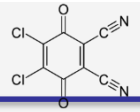
Dye residues in aquaculture products: Targeted and metabolomics mass spectrometric approaches to track their abuse

E. Dubreil[a,*], S. Mompelat[a], V. Kromer[a], Y. Guitton[b], M. Danion[c], T. Morin[c], D. Hurtaud-Pessel[a], E. Verdon[d]

[a] ANSES Fougeres Laboratory, Unit of Analysis of Residues and Contaminants, CS 40608-Javene, F-35306 Fougeres, France
[b] LABERCA, Oniris, INRA, Université Bretagne-Loire, 44307 Nantes, France
[c] ANSES Ploufragan-Plouzane Laboratory, Unit of Viral Pathology in Fish, F-22440 Ploufragan-Plouzane, France
[d] ANSES Fougeres Laboratory, European Union Reference Laboratory for Antibiotic and Dye Residue in Food, CS 40608-Javene, F-35306 Fougeres, France

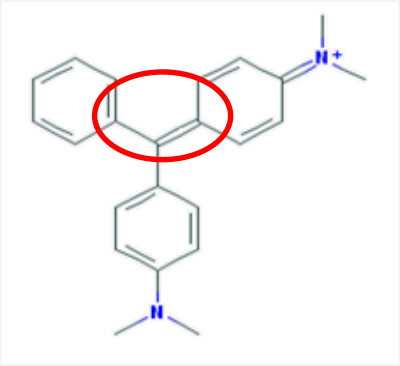


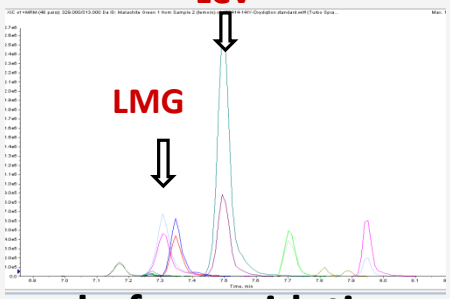
Chemical oxidation



DDQ

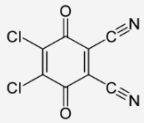
Enzymatic reduction



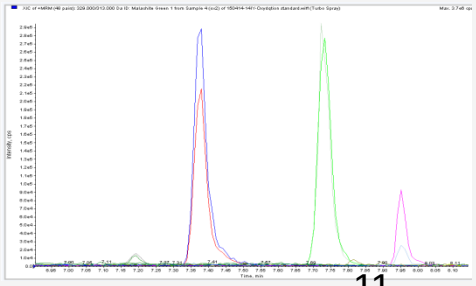


before oxidation

Extract of spiked sample

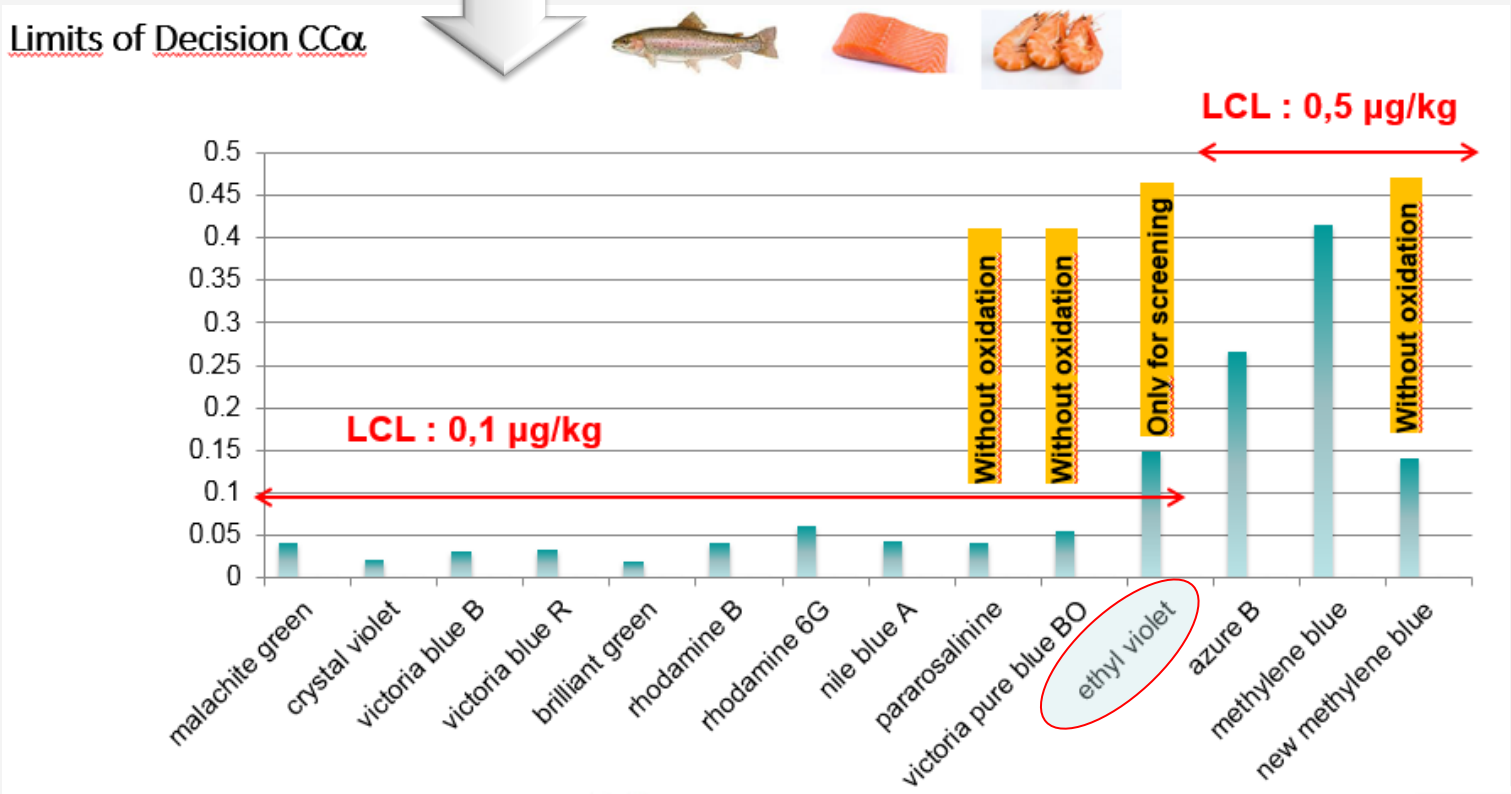


After addition of DDQ



after oxidation

2nd option: Extensive multi-families monitoring



Issues

- Oxidation solves the non-availability of standards of metabolites powders but no more proof of treatment
- Some false results due to cross contamination/ interference still possible

Considerations for the monitoring

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anses

3rd option: Multi-class of veterinary drugs monitoring

Multi-class of veterinary drugs (banned or not)

i n

b y

High resolution MS

Issues

. Expensive instruments
. Often for screening and not enough sensitive for confirmation of banned VD
. More suited for Applied Research (exposomics) and for risk assessment

Considerations for the monitoring

4th option: Multi-families monitoring including metabolites/biomarkers

Multi-families of Dyes

+

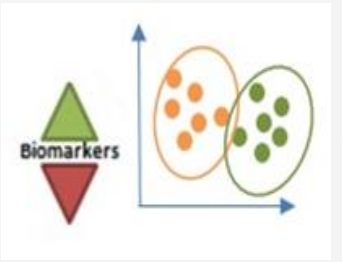
Relevant metabolites

+

Biomarkers of effects

in

by



Issues

. Based on endogenous/exogenous metabolism

. **Need to implement *in vitro/in vivo* experiments**

. Bring better proof of fraudulent use

. More suited for research (new practices) and for risk assessment

Considerations for the monitoring

4th option: Multi-families monitoring including metabolites/biomarkers

Chemosphere
Available online 10 August 2019, 124538
In Press, Journal Pre-proof

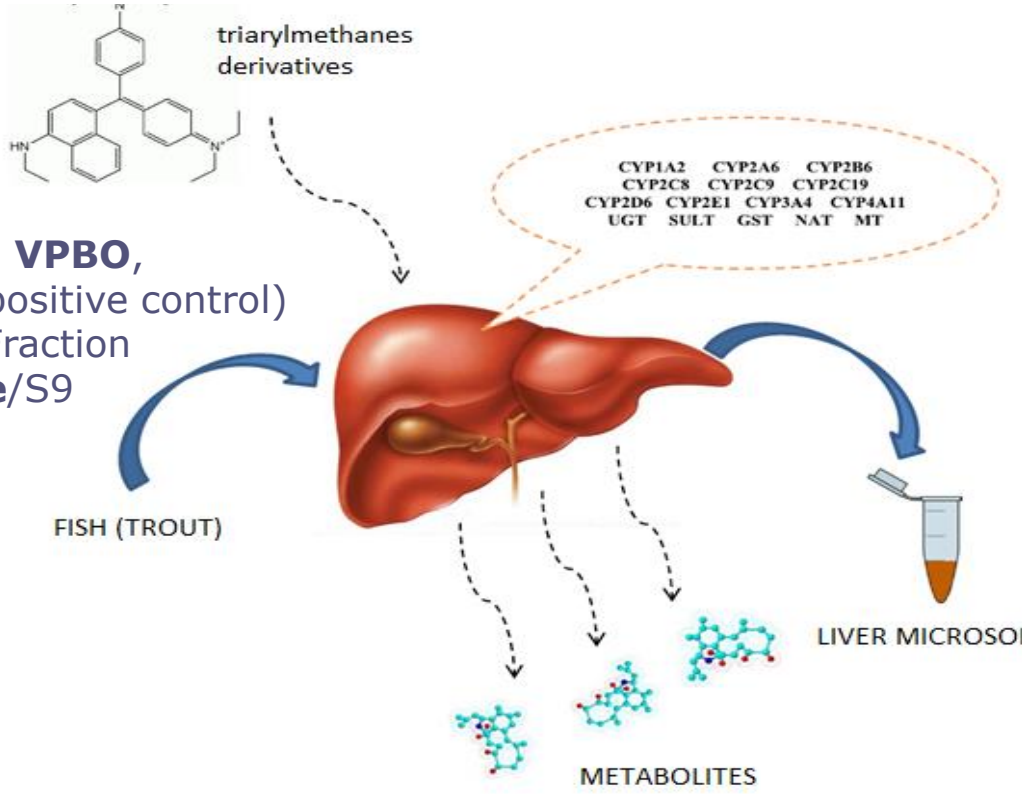
In vitro investigations of the metabolism of Victoria pure blue BO dye to identify main metabolites for food control in fish

Estelle Dubreil [a], Luc Sczubelek [a], Viktoriia Burkina [b], Vladimir Zlabek [b], Sidika Sakalli [b], Galia Zamaratskaia [c], Dominique Hurtaud-Pessel [a], Eric Verdon [a]

example

Step 1: *in Vitro* investigations for search of metabolites

LC-HRMS analysis



Substrate = **VPBO**,
or MG (for positive control)
Enzyme = Fraction **microsome**/S9

LTQ-Orbitrap Thermo

Metworks + Bibliography

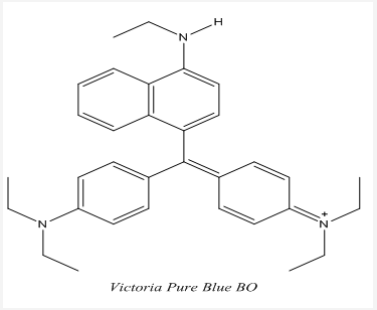
Considerations for the monitoring

4th option: Multi-families monitoring including metabolites/biomarkers

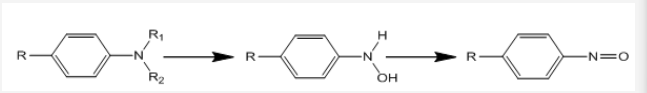
. Rapid to implement
. Incubations for each dye

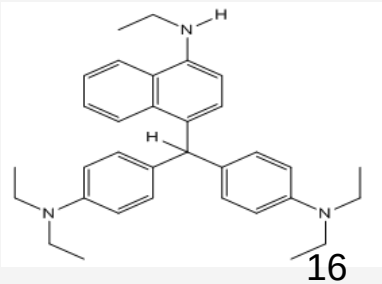
Step 1: *in Vitro* investigations for search of metabolites

Métabolite	Δm/z	Métabolisation proposé	Liver Micro	Liver S9 BNF
VPBO	0	-	67,0%	77,4%
Deéthylation (-28,03130)	-28,03130	N-deethylation	25 197 736	83 207 072
	-56,0626	Double Deethylation	34 875 279	47 038 661
	-84,0939	triple Deethylation	6 868 805	10 527 223
	-112,1252	quadruple Deethylation	642 658	1 156 568
	-140,1565	quintuple Deethylation	49 302	433 473
Demethylation (-14,01565)	-14,01565	N-demethylation*	538 242	1 556 792
	-42,04695	Deethyl + DM*	85 220	77 755
Hydroxylation (+15,99491)	15,99491	Oxidation de VPBO**	331 525	1 299 942
	-12,03639	Deethyl + O** de VPBO	357 525	1 248 496
	-40,06769	Double Deethylation + O** de VPBO	261 357	478 910
	-68,09899	triple Deethylation + O** de VPBO	88 259	205 989
	-96,13029	quadruple Deethylation + O** de VPBO	-	-
Hydroxylation + désaturation	13,97926	O** + DS*** de VPBO	NF	360 560
	-14,05204	Deethyl + O** + DS*** de VPBO	NF	327 640
	-42,08334	double Deethylation + O** + DS*** de VPBO	NF	393 826
	-70,11464	triple Deethylation + O** + DS*** de VPBO	99 634	237 218
Leuco-DE-VPBO	-26,01565	Deethyl-Leuco-VPBO	NF	544 773



$n\ (-CH_2 + H)$



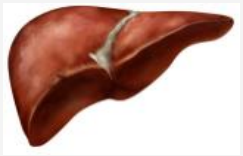


Considerations for the monitoring

4th option: Multi-families monitoring including metabolites/biomarkers

Trout exposed to VPBO in water bath, 1 day of treatment, 64 days of depuration

Step 2 optional: *in Vivo* investigations for metabolites



liver

muscle

skin

plasma

ACN extraction

+

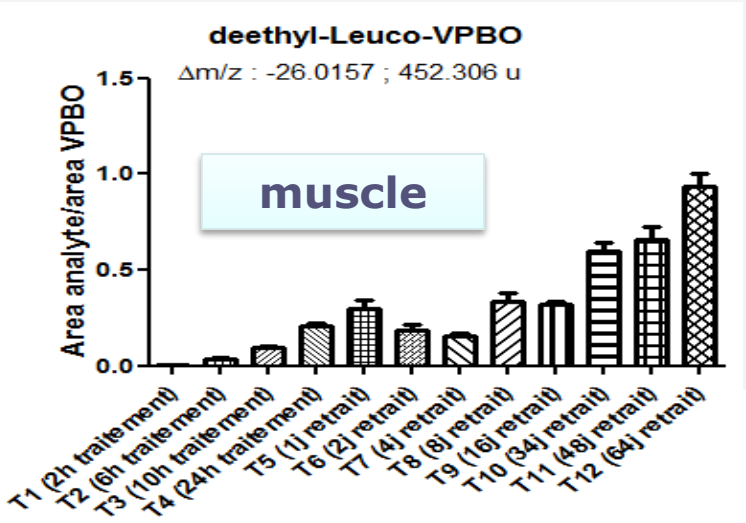
LC-HRMS analysis

LTQ-Orbitrap Thermo®

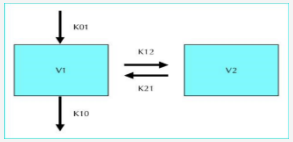
Considerations for the monitoring

E.Dubreil-E.Verdon

4th option: Multi-families monitoring including metabolites/biomarkers

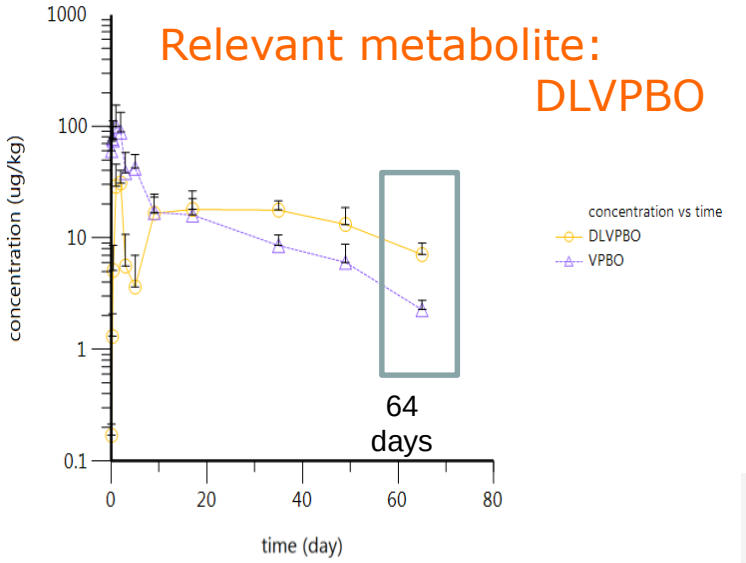


Step 2: *in Vivo* investigations for metabolites



⇒ **Modelisation 2 compartments: phoenix software**

Relevant metabolite: DLVPBO



	Muscle	
	VBPO	DLVBPO
Lambda_z (/days)	0.0352	0.03079
HL_Lambda_z (d)	19.69	22.51
AUC_{last} (µg d/l ou kg)	956.9 ± 56.7	945.1 ± 63.8
AUC_{INF} (µg h/l ou kg)	1010.2	1174.6
AUC_{Extrap} (%)	5.52	19.53
MRT_{last} (d)		
MRT_{INF} (d)	12.25	110.80
T_{max} (d)	1	1
C_{max} (µg/l ou kg)	99.9 ± 22.2	28.9 ± 6.9

Considerations for the monitoring

E.Dubreil - E.Verdon

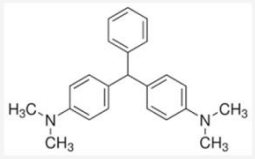
4th option: Multi-families monitoring including metabolites/biomarkers

Other possibility to metabolite investigation: Untargeted metabolomic study

Step 3: *in Vivo* investigations for biomarkers

Workflow

Animal experiment

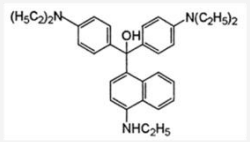


Malachite Green

×27 trouts

0,1 mg.l^{-1}

×9 ×9 ×9



Victoria pure blue BO

×27 trouts

0,05 mg.l^{-1}

×9 ×9 ×9

Control

×27 trouts

≠

×9 ×9 ×9

Sample preparation

-80° C

- ✓ ACN extraction
- ✓ Mix/centrifuge
- ✓ Evaporation to dryness
- ✓ Reconstitution in ACN/water
- ✓ Centrifugation and filtration

LC-ESI-HRMS

Full scan mode within m/z [100-1000] uma, ESI+
FWHM: 60000
Use of QC samples

Data processing

Alignment + filters, denoising, normalization for batch effects, filters for QC variation (< 30%)

Data analysis

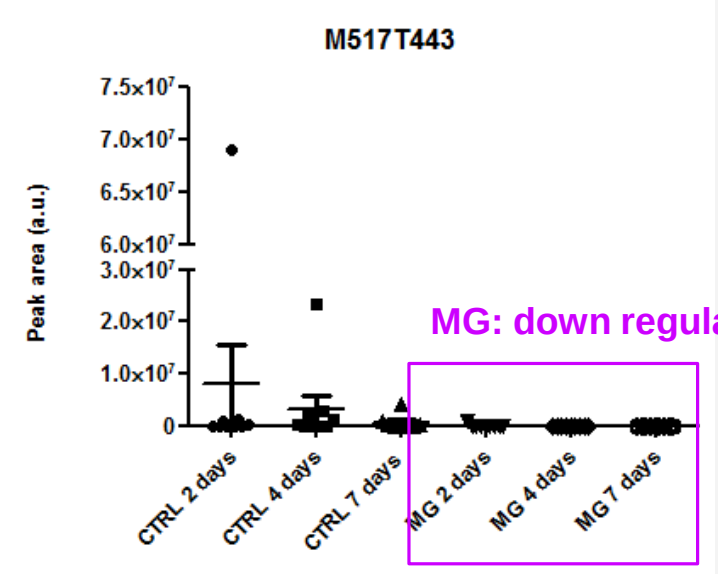
Workflow4metabolomics

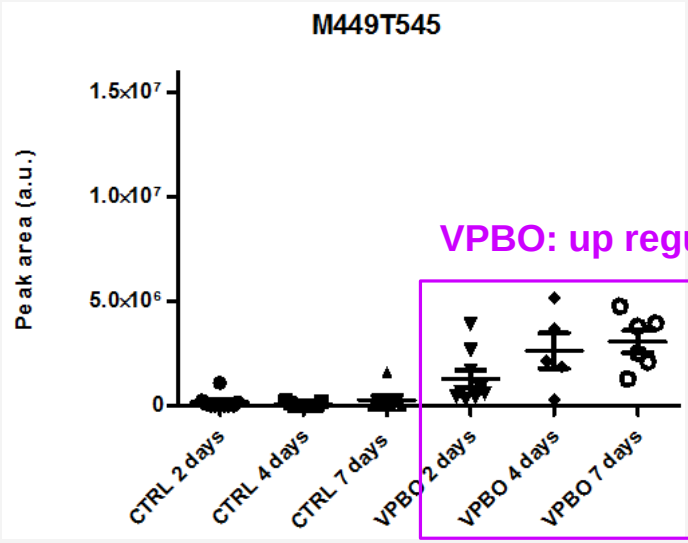
Multivariate statistical analysis
Candidate biomarkers validation

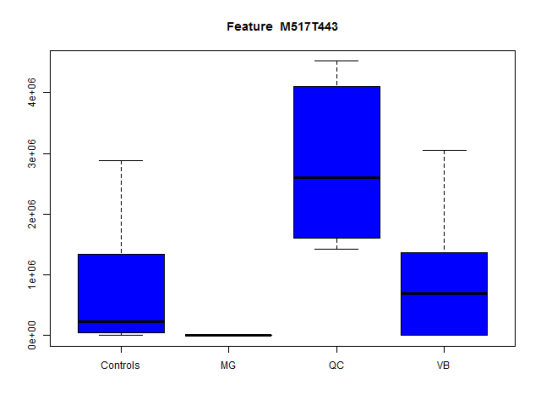
Considerations for the monitoring

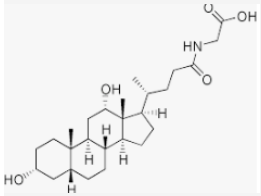
4th option: Multi-families monitoring including metabolites/biomarkers

Step 3: *in Vivo* investigations for biomarkers









Endogenous metabolites involved

Identification as bile acids

Considerations for the monitoring

4th option: Multi-families monitoring including metabolites/biomarkers

example

Dyes (15)
Malachite green (MG)
Crystal violet (CV)
Azur B (AZB)
Victoria blue B (VBB)
Victoria pure blue bo (VPBO)
Victoria blue R (VBR)
Methylene blue (MB)
New methylene blue (NMB)
Brilliant green (BG)
Rhodamine B (RHB)
Ethyl violet (EV)
Nile blue A (NBA)
Rhodamine B (RHB)
Rhodamine G (RHG)
Pararosalinine (PRRA)

Combination …

Metabolites (4)
Leuco MG (LMG)
Leuco CV (LCV)
-
-
Deethyl leuco VPBO (DLVPBO)
-
-
-
Leuco BG (LBG)
-
-
-
-
-

Endogenous biomarkers (3)
Taurodeoxycholic acid (TRDA)
Glycodeoxycholate (GCDA)
Deoxycholic acid (DCA)

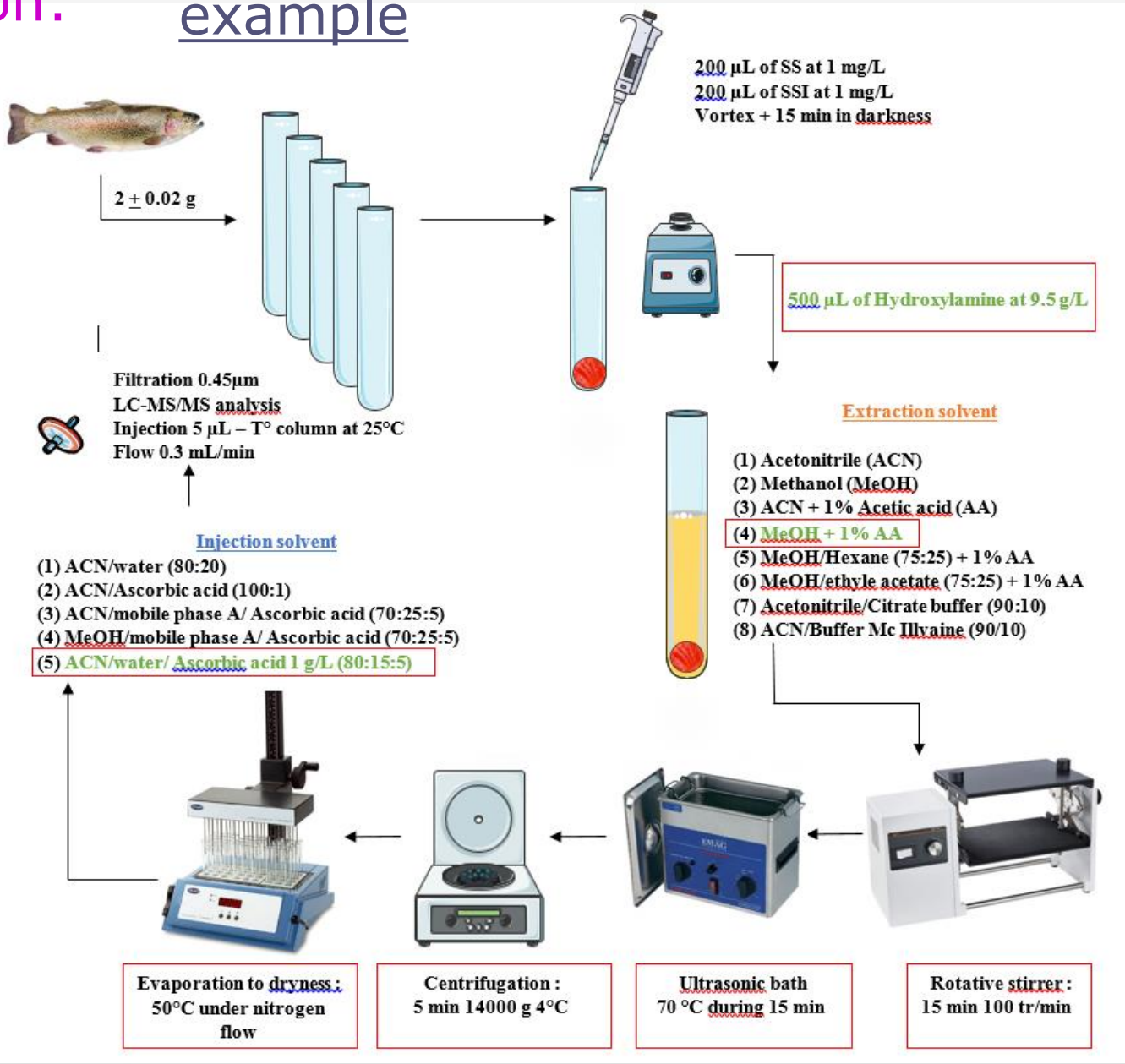
Internal standards (4)
MG-d5, LMG-d5
CV-d6, LCV-d6

… to be continued

→ To find transitions of DLVPBO, contaminated extract was injected in different modes (SIM, MS/MS et ≠ CE (V))

Considerations for the monitoring

4th option: example



Considerations for the monitoring

4th option: Multi-families monitoring including metabolites/biomarkers

Applicability: analysis of a positive sample from a routine lab

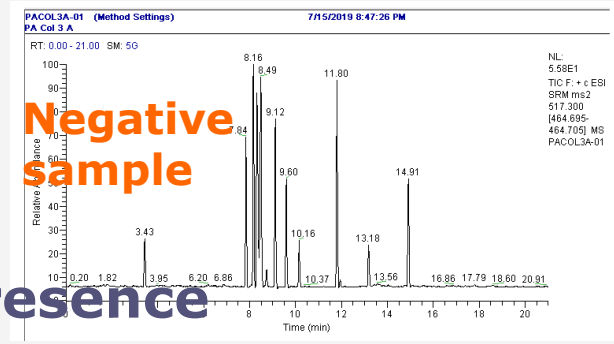
Catfish

Malachite green: **0,905 µg/kg**
Leuco Malachite green: **23,062 µg/kg**

Levels of bile acids:
DCA: 49 µg/kg
TRDA: 15 µg/kg

Up-regulation
MG confirmation of presence

Ex:TRDA



Conclusion

Considerations for the monitoring of residues of banned Vet Drugs:

Multi-res. incl. biomarkers /metabolites

Extensive multi-residue monitoring

Restrictive residue monitoring