CONffIDENCE: Contaminants in food and feed: Inexpensive detection for control of exposure

DEVELOPMENT OF A RAPID TEST FOR MALACHITE GREEN IN FISH: A COMPARATIVE STUDY BETWEEN ANTIBODY, APTAMER AND RECEPTOR MG-BINDERS

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I. INTRODUCTION

Malachite Green (MG) is a synthetic dye that is sometimes illegally used in aquaculture as antifungal, antimicrobial and antiparasitic agent. Due to its (mutagenic and carcinogenic) toxic effects on human health, this dye has been banned in animal product for human consumption in Europe. The European Commission has established a Minimum Required Performance Limit (MRPL) for the analysis of MG and its metabolite Leucomalachite Green (LMG) at 2 µg/kg-ppb (2004/25/EC).
In order to develop a rapid dipstick-based assay for the detection of MG/LMG in fish, we have characterized and compared 3 different types of anti-MG/LMG binding molecules including a polyclonal antibody, a RNA aptamer and a biological receptor.
The best reagent has been chosen and implemented to a lateral flow device format developed to detect MG/LMG at ppb level in fish tissue and feeds.

II. EVALUATION OF THE 3 BINDING MOLECULES FOR MG/LMG

LMG ANTIBODY

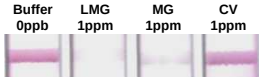
ELISA results show that LMG Ab has a good sensitivity for LMG and a significant cross-reactivity for MG (**a**). One optimal buffer was selected for an indirect competitive dipstick Ab-based assay development (**b**). This Ab is able to recognize excess of LMG and MG in buffer using competitive dipstick format (**c**). The Ab-based dipstick assay can easily detect 10 ng/ml (ppb) of LMG and 100 ng/ml (ppb) of MG in buffer.

a **ANTIBODY CHARACTERIZATION IN ELISA**

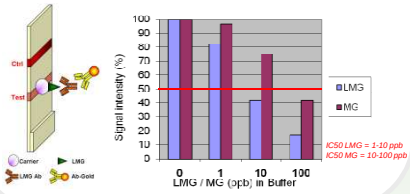
	IC_{50} (ng/ml)	CR (%)
Leucomalachite green	2.2	100
Malachite green	6.1	36
Brilliant green	485	0.5
Crystal violet	>1000	ND
Leucocrystal violet	>1000	ND

b **DIPSTICK BUFFER SELECTION**

c **DIPSTICK SELECTIVITY**



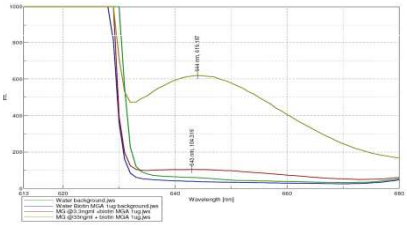
d **INDIRECT ANTIBODY-BASED DIPSTICK EVALUATION**



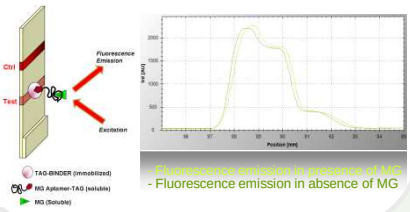
MG APTAMER

A sequence of MG RNA aptamer[1] was modified to have a biotine in 5' and an inversed dT in 3'. MG binding property of the RNA aptamer was first evaluated in solution using spectrofluorimetric detection (**a**) and then in dipstick format (**b**) using a fluorescent dipstick reader (ESE-quant, Ex590nm/Em635nm) to monitor the appearance of a fluorescence shift indicative of MG recognition[2].
Fluorescent signal significantly increases for solutions containing MG (2 higher curves) compared to control buffers (**a**). Unfortunately, no significant difference is observed in this direct-dipstick format (**b**). Further studies revealed that most of the fluorescent signal generated in this dipstick format was due to background emission from TAG-Binder present at the test line.

a **APTAMER CHARACTERIZATION IN FLUORIMETER**



b **DIRECT APTAMER-BASED DIPSTICK EVALUATION**



MG RECEPTOR

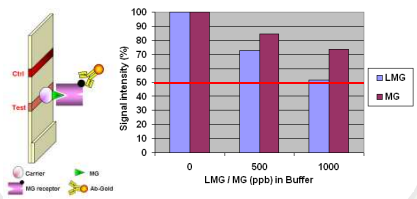
A mutant form of MG receptor was engineered to have a better solubility/stability in buffer and a tag for its purification/detection. The capability of this gold-labeled receptor to bind appropriate immobilized conjugate was evaluated in dipstick format. A specific signal appeared at the test line in presence but not in absence of this receptor meaning that this receptor is exploitable as binding reagent in dipstick development (**a**).
Preliminary evaluation of this competitive dipstick assay shows that excess of LMG in solution can significantly decrease the test line signal of the dipstick as expected. However this format did not allow the detection of MG/LMG concentration in buffer < 1 mg/kg (ppm) (**b**). Condition of this dipstick assay must be optimized for a better MG/LMG sensitivity of detection.

a **RECEPTOR CHARACTERIZATION IN DIPSTICK**

1:
- Immobilized conjugate
- Soluble receptor
- Soluble gold-antibody

2:
- Immobilized conjugate
- Soluble gold-antibody

b **INDIRECT RECEPTOR-BASED DIPSTICK EVALUATION**



III. ANTI-LMG DIPSTICK ASSAY DEVELOPMENT

Analyses of fish tissue samples and buffer by LMG Ab-based dipstick assay show an important "matrix interference" on the test line intensity of the dipstick for fish tissues compared with buffer alone. This effect makes the test line difficult to appreciate by visual observation. Although this issue must be improved by assay/buffer optimization, preliminary data obtained after instrumental measure (Readsensor) of the dipsticks following a SPE-based concentration step[2] of MG/LMG contaminants indicate that the performance of the Ab binding reagent should be sufficient for the detection of LMG at the EU MRPL. The detection of the parent form (MG) at MRPL may not be possible based on the Ab cross-reactivity profile. However, more than 80% of the total LMG/MG residue content in dosed fish tissue will be present as the metabolite form (LMG) thus, a sample containing LMG/MG at or above the MRPL (sum of LMG/MG) would be detected as non-compliant (positive result).

MG/LMG EXTRACTION[2]

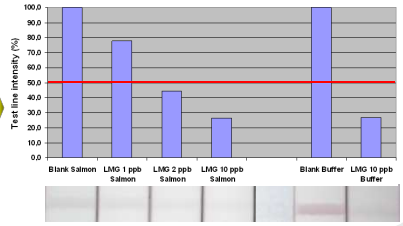
SAMPLE
8 gr. Fish tissue

EXTRACTION
MeCN – CH3COOH 0,5%

PURIFICATION
SPE (OASIS MCX LP 500mg/6cc)

DISSOLUTION
Dried extract dissolution in Buffer

MG/LMG DIPSTICK ASSAY



IV. CONCLUSION

In conclusion different binding molecules for MG/LMG were evaluated and an antibody was chosen for further development of a dipstick assay for the rapid detection of MG/LMG in fish tissue and feed. Despite some "matrix interference" from fish tissues, the dipstick assay should be compatible with MRPL detection of MG/LMG and is being optimized within EU Conffidence project.

[1]Flinders et al, Chembiochem, 2004, 3;5(1):62-72. [2]Stead et al, Anal.Chem, 2010, 82, 2652-2660.

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