

DEVELOPMENT OF A COMPETITIVE ELISA FOR THE DETECTION OF MALACHITE GREEN AND LEUCOMALACHITE GREEN

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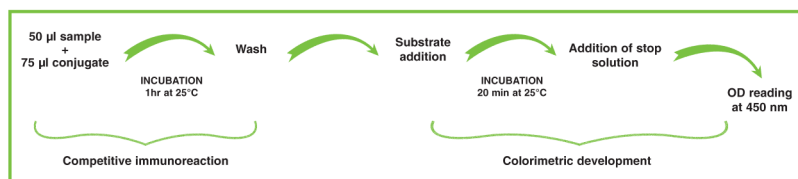
Introduction

The use of malachite green (MG) to treat infections in farmed edible fish and shellfish is banned in several countries because of public health risk. Following absorption, MG is rapidly reduced to leucomalachite green (LMG), the primary metabolite, which is the prevalent residue in incurred tissues because of its slower elimination rate. The European minimum required performance limit (MRPL), a quality parameter for residue laboratories, is set at 2 ppb for the sum of MG and LMG in meat of aquaculture products⁽¹⁾. Several methods have been reported for the determination of MG and LMG in a variety of matrices, the majority of these methods are based in liquid chromatography with ultra-violet, or electrochemical detection, and may include subsequent confirmation with mass spectrometry^(2,3,4). The chemical conversion of the LMG to MG is usually required for visible absorbance detection. The use of a competitive enzyme-linked immunosorbent assay (ELISA) to directly detect and quantify LMG and MG without the need of conversion to MG offers a rapid alternative. At the same time, the analysis capacity is increased as several samples can be tested at the same time with the corresponding implications in the cost-effectiveness of the assay. To our knowledge, we report the first ELISA applicable to the direct detection and quantification of the parent compound MG and its breakdown metabolite LMG. The development of the immunoassay is described, the suitability of the immunoreagents generated was assessed by MALDI-TOF mass spectrometry and the initial performance evaluation of the immunoassay is shown. A 96 well microtiter plate was precoated with the specific antibodies to both LMG and MG. Competition between free MG/LMG, present in the calibrator/sample, and HRP-labelled conjugate for antibody binding sites was measured by reading the absorbance at 450 nm, which was inversely proportional to the concentration of analyte. The assay has the capacity to analyse 40 samples in 90 min and could be used for screening LMG and MG in various sample matrices to monitor legislative compliance

Methodology

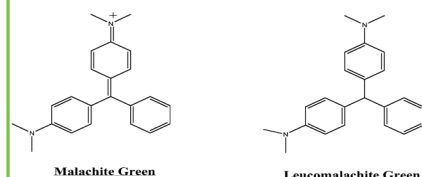
Generation of the hapten, immunogen, specific antibodies and horseradish peroxidase conjugate have been described in the European patent application 05077175.7.

The competitive ELISA was performed as follows: a 96 well microtiter plate was precoated with the specific antibodies to LMG and MG. Free LMG/MG, present in the calibrator/sample, and HRP-labelled conjugate compete for antibody binding sites. Measurement was carried out by reading the absorbance at 450 nm, which was inversely proportional to the concentration of analyte. The immunoassay steps are outlined in the flow chart.

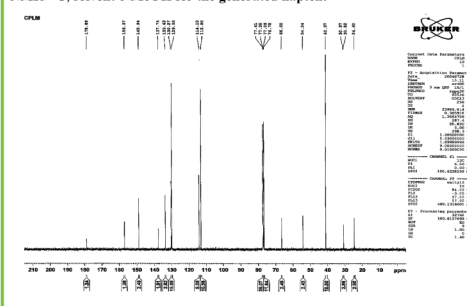


Results

Structural formulae of malachite green and leucomalachite green

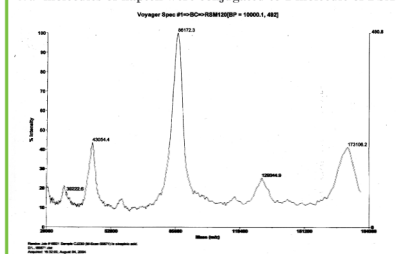


NMR ¹³C, solvent δ-MeOH for the generated hapten:



Immunogen generated evaluated using matrix assisted UV laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS).

47.9 molecules of hapten were conjugated to 1 molecule of BSA

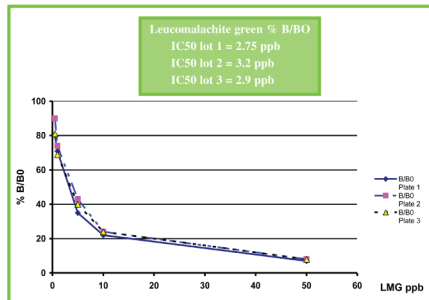


Initial evaluation parameters of the competitive ELISA are described:

Specificity of the competitive ELISA for leucomalachite green and malachite green.

Analyte	%Cross-reactivity
Leucomalachite green (LMG)	100
Malachite green (MG)	20

Calibration curve for leucomalachite green in the competitive ELISA.



Intra-assay precision

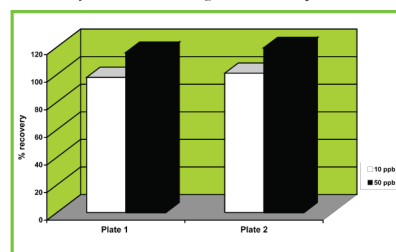
LMG 0.5 ppb		LMG 1 ppb		LMG 5 ppb		LMG 50 ppb	
Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
n=12	n=12	n=12	n=12	n=12	n=12	n=12	n=12
% CV	% CV	% CV	% CV	% CV	% CV	% CV	% CV
2.8	3.1	3.2	6.0	2.9	4.8	1.7	3.9

Inter-assay precision

17 separate runs in duplicate

LMG 0.5 ppb	LMG 1 ppb	LMG 5 ppb	LMG 50 ppb
n=17	n=17	n=17	n=17
% CV	% CV	% CV	% CV
2.8	7.8	4.7	2.5

% Recovery for leucomalachite green in the competitive ELISA



Conclusion

To our knowledge, this is the first ELISA applied to the direct detection of LMG and MG. The antibody used in the development of the ELISA was designed to facilitate detection of the LMG metabolite, to avoid the need for any chemical conversion step to convert LMG to MG. The components used for the development of this immunoassay, including hapten, immunogen, polyclonal antibodies, calibrators and HRP conjugate were successfully generated. Initial evaluation parameters in the competitive ELISA show % cross-reactivity of 100% for LMG and c.a. 20% for MG. IC50 for LMG was <3.5 ppb. Initial intra-assay and inter-assay precision values expressed as % CV were <10% and the % recovery values for LMG levels of 10 ppb and 50 ppb were between 70 and 120%. Further evaluation studies with spiked and incurred matrices are in progress.

This ELISA represents a simple and applicable alternative for the rapid screening of LMG and MG as it detects directly the two compounds without chemical conversion. In addition, the ELISA format allows the analysis of sets of samples at one time, which is also advantageous in monitoring legislative compliance.

References

- (1) European Commission decision No. 2004/25/EC of 22 December 2003 amending Decision 2002/657/EC. *Official Journal of the European Union*, L 6/38.
- (2) Mitrowska, K., and Posnyniak, A. (2004). Determination of Malachite Green and Its Metabolite, Leucomalachite Green, in Fish Muscle by Liquid Chromatography. *Bull. Vet. Inst. Pulawy*. **48**: 173-176.
- (3) Halme, K., Lindfors, E., and Peltonen K. (2004). Determination of Malachite Green Residues in Rainbow Trout Muscle with Liquid Chromatography and Liquid Chromatography Coupled with Tandem Mass Spectrometry. *Food Addit. Contam.* **21**(7) :641-648.
- (4) Turnipseed, S.B., Andersen, W.C., and Roybal, J.E. (2004). Determination and Confirmation of Leucomalachite Green in Salmon Using No-Discharge Atmospheric Pressure Chemical Ionisation LC-MS. *Laboratory Information Bulletin* 4333, **20**(11).

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