1. INTRODUCTION

This method describes the analysis of leucomalachitegreen (LMG) in samples of salmon. After extraction of the samples, clean-up and concentration on aromatic sulfonic acid extraction columns, the purified extracts are washed, dried and injected on the GC-MS. The method can be used for both screening and quantification. The MRPL for samples of salmon is 2 µg/kg based on the sum of both MG and LMG. A semi-quantification validation experiment on this MRPL level is performed and described. The validation experiment was performed when the internal standard LMG-d5 was not yet available.

The method was validated according to the criteria laid down in Commission Decision 2002/657/EC [6.1].

2. MATERIALS

Reference to a company and/or product is for purposes of identification and information only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environment (RIVM) to the exclusion of others which might also be suitable.

2.1. Chemicals and reagents

All chemicals, including standards and solutions, are of defined quality. Pure chemicals are of "Pro Analyse" quality or better. Standards of MG and LMG are obtained from the department of food of animal origin (V&V Utrecht).
The internal standard of LMG-d5 was obtained from Daniel R. Doerge (National Center for Toxicological Research, Jefferson, AR 72079 USA).

2.1.1. Ammonium acetate anhydrous
2.1.2. Acetic acid
2.1.3. Acetonitril
2.1.4. Ammonium hydroxide 25%
2.1.5. Ascorbic acid
2.1.6. Citric acid monohydrate
2.1.7. Dichloromethane
2.1.8. Sodium acetate anhydrous
2.1.9. N,N-Dimethylformamide (DMF)
2.1.10. Methanol
2.1.11. Sodium sulphate anhydrous
2.1.12. di-Sodium hydrogen phosphate dihydrate
2.1.13. N,N,N',N',-Tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD)
2.1.14. para-Toluenesulfonic acid (p-TSA)
2.1.15. Phosphate buffer 0,2 M. Dissolve 36,6 g of di-sodium hydrogen phosphate dihydrate in 1000 ml water.
2.1.16. Leuco crystal violet.
2.1.17. N,N,N',N',-Tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) (1 mg/ml ethanol).
2.1.18. TSA solution. Dissolve 9,5 gram para-Toluenesulfonic acid in 50 ml of water.

2.1.19. Ascorbic acid solution 1.0 mg/ml. Dissolve 0.10 g ascorbic acid in 100 ml methanol.
2.1.20. Acetate buffer 0,112 M ; pH 4.0. Dissolve 2.45 g sodium acetate in 800 ml of water, add 4.92 g acetic acid, mix and add water to a volume of 1000 ml.
2.1.21. McIlvaine buffer pH 3.0. Mix 81.1 ml citric acid 0.10 M with 18.9 ml phosphate buffer and 100 µl TSA solution and 50 µl TMDP solution.
2.1.22. McIlvaine buffer pH 6.0. Mix 37.5 ml citric acid 0.10 M with 62.5 ml phosphate buffer.
2.1.23. Citric acid 0.10 M. Dissolve 10.5 g citric acid mono hydrate in 500 ml water.
2.1.24. SPE conditioning solvent. Mix 80 ml acetonitril with 20 ml dichloromethane.
2.1.25. SPE-eluent. Mix 2.5 ml ammonium hydroxide, 2.5 ml ascorbic acid and 45 ml methanol. Prepare just before use!
2.1.26. Internal standard solution leucomalachitegreen-d5 1 mg/ml methanol.

Stock solutions of LMG and LMG-d5 containing 0.1 mg/ml were prepared in methanol and stored at -20°C. Working solutions were prepared by sequential 10-fold dilutions of the 0.1 mg/ml solutions to a single serie of appropriate standard solutions. These solutions were stored in the dark at approximately 4°C (range 1-10°C) for a maximum period of 12 months.

2.2. Apparatus

Standard laboratory glassware and equipment is used, with the addition of:

2.2.1. Aromatic sulfonic acid column (3 ml, 500 mg, Baker).
2.2.2. Vacuum manifold (SPE-12G, Baker).
2.2.3. Adaptors 20 ml to place on top of the SPE-columns.
2.2.4. GC-MS equipment:

Gas chromatograph (Hewlett Packard, type 6890).
GC-column, fused silica CpSil24CB 25 m x 0,25 mm ID, film thickness 0.12 µm (Varian art.5814).
Automatic injector (7673A, H.P.).
Mass selective detector (Electron Impact) (5973N, Agilent).

The following conditions are used during GC-MS analysis:
Injectionport: splitless 260°C.
Temp.program oven: 80°C (1 min.); rate 25°C/min. to 340°C.
Constant flow 1.1 ml/min helium.
Temperature transferline: 280°C.
Solventdelay of MS: 10 min.
ions for screening and confirmation: m/z 165-210-253-330-335.

3. ANALYTICAL PROCEDURE

Samples are stored in the dark at -20°C.

3.1. Extraction

3.1.1. Weigh 2 gram of mixed salmon in a polypropylene tube of 50 ml and add 4 ng of the internal standard LMG-d5 (2 µg/kg).
3.1.2. Add 2 ml McIlvaine buffer pH 3.0, vortex for 1 min.
3.1.3. Add 12 ml acetonitrile.
3.1.4. Shake for 10 min. on a rotating apparatus and 10 min. in an ultrasonic water bath.
3.1.5. Cool the centrifuge to 15°C for 10 min. prior to use.
3.1.6. Centrifuge at 15°C for 10 min at 3400 g.
3.1.7. Decant the solution into a second 50 ml polypropylene tube.
3.1.8. Add 2 ml McIlvaine buffer pH 6.0.
3.1.9. Add 12 ml acetonitrile.
3.1.10. Shake for 10 min on a rotating apparatus and 10 min. in an ultrasonic water bath.
3.1.11. Centrifuge at 15°C for 10 min at 3400 g.
3.1.12. Decant the solution into the second 50 ml polypropylene tube.
3.1.13. Add 6 ml dichloromethane to the second tube and vortex mix.
3.1.14. Centrifuge at 15°C for 10 min at 3400 g.
3.1.15. The top layer is collected and cleaned-up and concentrated using SPE.

3.2. SPE-clean-up and concentration.

3.2.1. Place the SPE column on a vacuum manifold.
3.2.2. Condition column: 3 ml condition solvent: Prevent the column(s) from running dry.
3.2.3. Attach the reservoir(s) on top of the column(s).
3.2.4. Transfer top layer of the sample with a pipette onto the reservoir. Allow the column(s) to run dry.
3.2.5. Wash the column(s) with 1.5 ml methanol.
3.2.6. Dry the column(s) for 10 min: vacuum, prepare SPE-eluent.
3.2.7. Elute the LMG with 3 ml SPE-eluent in a 10 ml tube.
3.2.8. Evaporate the eluate at 50°C to dryness in a stream of nitrogen.

3.3. Further clean-up.

3.3.1. Dissolve the extract in 2.5 ml of water and 0,075 ml of acetic buffer 0,112 M, pH 4.0 and 0,425 ml of acetonitril by placing for 1 min. in an ultrasonic water bath
3.3.2. Add 2.5 ml of dichloromethane and extract by placing on a vortex.
3.3.3. Centrifuge for 10 min. at 2000 g. at 15°C.
3.3.4. Remove the top layer by suction.
3.3.5. Add some anhydrous sodium sulphate and shake.
3.3.6. Transfer the dichloromethane through a funnel filled with glass wool and anhydrous sodium sulphate into a clean tube.
3.3.7. Wash the first tube with dichloromethane and transfer to the funnel.
3.3.8. Evaporate till a volume < 1 ml and transfer into a 2 ml vial and evaporate till dry.
3.3.9. Evaporate the solvent and dissolve the extract in 0.05 ml of methanol.
3.3.10 Transfer the solvent into injection-vails and inject 0.002 ml splitless on the GC-MS.

4. INTERPRETATION AND CALCULATION.

Quantitative results are obtained by construction of a calibration curve. The peak area of the selected ion of LMG: m/z 330 and the peak area of the selected ion of the internal standard of LMG-d5: m/z 335 are calculated and the ratio is the response variable. A calibration curve is constructed by the ratio between the response variable versus the concentration of the standards. Unknown samples are calculated by interpolation.

For identification according to the EC-criteria (Sanco 2002/657/EC [6.1]) it is mandatory that at least 4 ions are monitored. Each ion monitored (response) should fulfil the criterion that the maximum exceeds the average noise + 3 S.D. If this criterion is fulfilled the 3 different ratios are calculated. The same ratios are calculated for the standard analyte, preferably at the corresponding concentration. For positive identification the responses obtained for the unknown sample should be:

Relative intensity (% of base peak)	relative range of the response for EI
> 50%	± 10%
> 20% <50%	± 10%
> 10%-20%	± 20%
≤ 10%	± 50%

5. VALIDATION.

The results of the in-house validation study are described. It should be noted that this experiment is performed when LMG-d5 was not yet availabe.
Validation is performed according to ARO SOP 475 using Resval 1.2 [6.3].

5.1 Calibration information

	Calibration Line (y = ax + b)		
	Slope (a)	Y-intercept (b)	Correlation (r^2)
Exp. 1	0.496	-0.230	0.9834
Exp. 2	0.582	0.039	0.9973
Exp. 3	0.632	-0.215	0.9883
Exp. 4	0.600	-0.032	0.9985

	Samples (y = ax + b)				
	Slope (a)	Y-intercept (b)	Correlation (r^2)	Stdev.Y-intercept	n
Exp. 1	0.266	-0.037	0.9829	0.0248	21
Exp. 2	0.326	-0.067	0.9326	0.0620	21
Exp. 3	0.359	-0.181	0.9600	0.0518	21

5.2 Accuracy Means, STANDARD deviations and Covariations

Performance level	Exp.1 Accuracy	Exp 2 Accuracy	Exp 3 Accuracy	Exp 1 Stdev.	Exp 2 Stdev.	Exp 3 Stdev.
1	91.8%	44.0%	72.2%	10.3%	5.6%	10.1%
2	66.4%	49.5%	53.1%	4.1%	2.7%	9.9%
3	71.3%	42.4%	55.5%	4.1%	16.9%	10.2%

Performance level	Exp.1 C.V.	Exp 2 C.V.	Exp 3 C.V.
1	11.2%	12.7%	14.0%
2	6.2%	5.4%	18.6%
3	5.8%	39.7%	18.4%

5.2 Critical levels according to EC/2002/657

	CC_α ng/g	CC_β ng/g
Exp. 1	0.22	0.37
Exp 2	0.44	0.76
Exp 3	0.34	0.57
Average	0.33	0.57

Decision limit CC_α means the limit at and above which it can be concluded with an error probability of α (=1%) that a sample is not compliant.
Detection capability CC_β means the smallest content of the substance that may be detected, identified in a sample with an error probability of β (95%).

5.3 Results from ISO 5725-2:1994

Performance level	Exp.1 C.V.	Exp 2 C.V.	Exp 3 C.V.
1	11.2%	12.7%	14.0%
2	6.2%	5.4%	18.6%
3	5.8%	39.7%	18.4%

Results are given as s^2 and are used in 5.5 for the determination of the reproducibility

5.4 Accuracy Means & Standard deviations experiment 4

Performance level	Accuracy	Accuracy	Accuracy
2	29.7%	7.8%	26.2%

The results of this experiment are used in 5.5 for the determination of the matrix effect

5.5 Measurement uncertainty

Description	S^2
Reproducibility Matrix effects Other uncertainties	0.045
Total S^2	0.045
Measurement Uncertainty (U)	0.43

The uncertainty is determined at level 1.
By using the result of 5.5 measured concentrations should be report by value x ± U (95%)

6. REFERENCES.

6.1. Commission Decision 2002/657/EC

6.2. Determination of residues of malachite green in aquatic animals. Aldert A. Bergwerff, Peter Scherpenisse, J. Chrom. B,788 (2003) 351-359.

6.3. SOP ARO 475A Method Validation using ResVal.