CCQM-K85

Malachite Green in fish tissue

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Introduction

Malachite green (MG) is a triphenylmethane dye which has powerful antifungal properties. It has been historically used to prevent fin rot and proliferative kidney disease in trout and salmon[1]. MG persists in the environment and has been found in the human food chain. MG, a multi-organ toxin to mammals, is metabolised to leucomalachite green (LMG), which itself has a wide range of toxicological effects including carcinogenesis[2]. This has led to the total ban of its use in aquaculture on many continents. Whilst the detectable presence of any MG can constitute an offence, many enforcement bodies carrying out surveillance for these compounds are working to a minimum required performance limit (MRPL) for testing, typically 2 µg kg^{-1}, measured as the sum of MG and LMG[3].

In 2005 the organic analysis working group (OAWG) reviewed its activities in the foods area. LGC were requested to review the area of trace contaminants in fish. This resulted in the proposal that the analysis of MG and its metabolite LMG would serve as an appropriate indicator to assess the capabilities of NMIs for the analysis of trace (µg kg^{-1}) contaminants in fish. The determination of malachite green in salmon tissue is a complex analysis involving the extraction of trace levels of potentially unstable analytes from a solid matrix. A pilot study was co-ordinated by LGC (CCQM-P88) in 2007 and the results were presented to the OAWG in October 2007 and detailed in the final pilot study report. This pilot study indicated a laboratories' ability to measure µg kg^{-1} levels of medium to high polarity residues in fish tissue and demonstrated a high level of analytical capability in extraction from a solid matrix and the ability to measure inter-converting and unstable analytes. A follow-on key comparison (CCQM-K85) was agreed at the OAWG in April 2009, however the pilot study indicated that the material used in the initial comparison did not exhibit the required homogeneity and therefore a new production method was investigated prior to commencing a key comparison. Therefore, a bespoke material was prepared by LGC for use in CCQM-K85.

Description of the measurand

Malachite green is normally administered as a solution preparation and is available in solid form as the oxalate or hydrochloride salt (Figure 1 shows the cation form). The parent compound is quickly metabolised to its major metabolite leucomalachite green (Figure 2) and this is normally the most abundant form found in samples. The conversion of LMG to MG and vice versa, the more abundant nature of LMG and uncertainties in metabolism rates, have resulted in many regulators requesting that results be reported as "total malachite green". This is the sum of MG and LMG in the sample (the sum of the separate mass fractions of MG and LMG). For this study this approach was also adhered to. Therefore, the measureand was the total mass fraction of leucomalachite green (4,4'-Benzylidenebis(N,N-dimethylaniline)) and malachite green (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline)) expressed as 'total malachite green'.

Participants were requested to submit results for the individual measurands of MG (as the free cation shown in Figure 1) and LMG to enable better assessment of the approaches taken by individual laboratories.

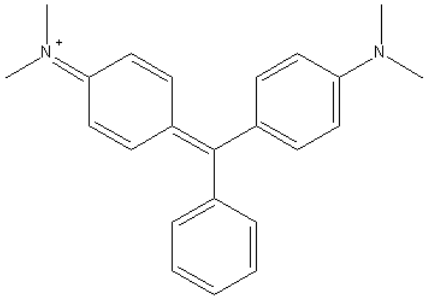

Figure 1: Malachite green

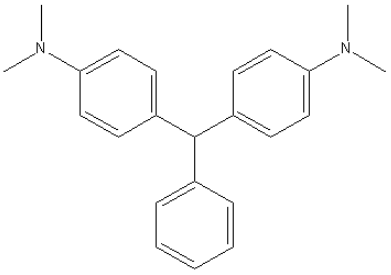

Figure 2: Leucomalachite green

Preparation of samples

The sample distributed for CCQM-K85 was sourced by a third party, and not by LGC. It was prepared by blending the muscle tissue of a salmon with elevated levels of naturally incurred malachite green, with that of 'blank' salmon muscle tissue. This material was then "cryomilled" at LGC by grinding 25g portions of salmon tissue under liquid nitrogen. The individual 25g sub samples were transferred under liquid nitrogen to a pooled sample which was stirred under liquid nitrogen for one hour. This pooled sample was then transferred to plastic sample pots which had been purged and filled with nitrogen gas. Between 5 and 6 g of the homogenised material was placed in individual plastic pots and stored below -70 °C in the dark until distributed.
The final levels of malachite green and leucomalachite green in the homogenised sample were expected to be in the following range: MG 0.5 – 5 µg/kg; LMG 3 – 10 µg/kg.

Homogeneity testing

Twelve vials were selected from the seventy vials produced. These vials were analysed in triplicate using a precise method (LC-IDMS/MS). The homogeneity study was run in three batches over three days, forming a balanced nested design with units nested within days. Results are displayed in Figures 3 and 4.

Homogeneity assessment

Between-run, within-unit and between-unit variance components are listed in Table 1. The outlying point in the MG data was omitted (outliers inflate within-unit effects and lead to underestimation of between-unit effects). The analysis used restricted maximum likelihood estimation for both sets to allow for the small imbalance caused by the missing value for MG and to avoid negative variance estimates. The between-run effects differ as expected from visual inspection; the run effect for MG was small, while that for LMG was the largest contribution to the variance (a likelihood ratio test shows it to be very strongly significant, with $p<<0.001$). After controlling for run effect, however, both analytes show essentially negligible inhomogeneity, shown by the very small between-unit standard deviation. . The combined relative standard deviation of the thirty six results for MG and LMG was less than 3 %, which was the requested level of maximum heterogeneity by the OAWG. With appropriate treatment of between-day effects and removal of one outlying observation for MG, the between unit relative standard deviation was determined as 1×10^{-4} % and 3.6×10^{-5} % for MG

and LMG respectively, demonstrating that between-unit inhomogeneity was negligible. The sample size used for assessing the homogeneity was 2 g.
Note that the presence of one within-unit outlier might indicate occasional within-unit inhomogeneity in the form of rare local 'hot spots'.

Table 1: Variance components

Source of variance	Malachite green			Leucomalachite green		
	Variance	StdDev	DF*	Variance	StdDev	DF*
Run	7.00×10^{-5}	8.37×10^{-3}	2	1.11×10^{-1}	3.33×10^{-1}	2
Unit	7.95×10^{-12}	2.82×10^{-6}	9	5.64×10^{-12}	2.37×10^{-6}	9
Residual	5.30×10^{-3}	7.28×10^{-2}	29	8.98×10^{-3}	9.48×10^{-2}	30

*DF=degrees of freedom based on classical ANOVA table, included for information only.

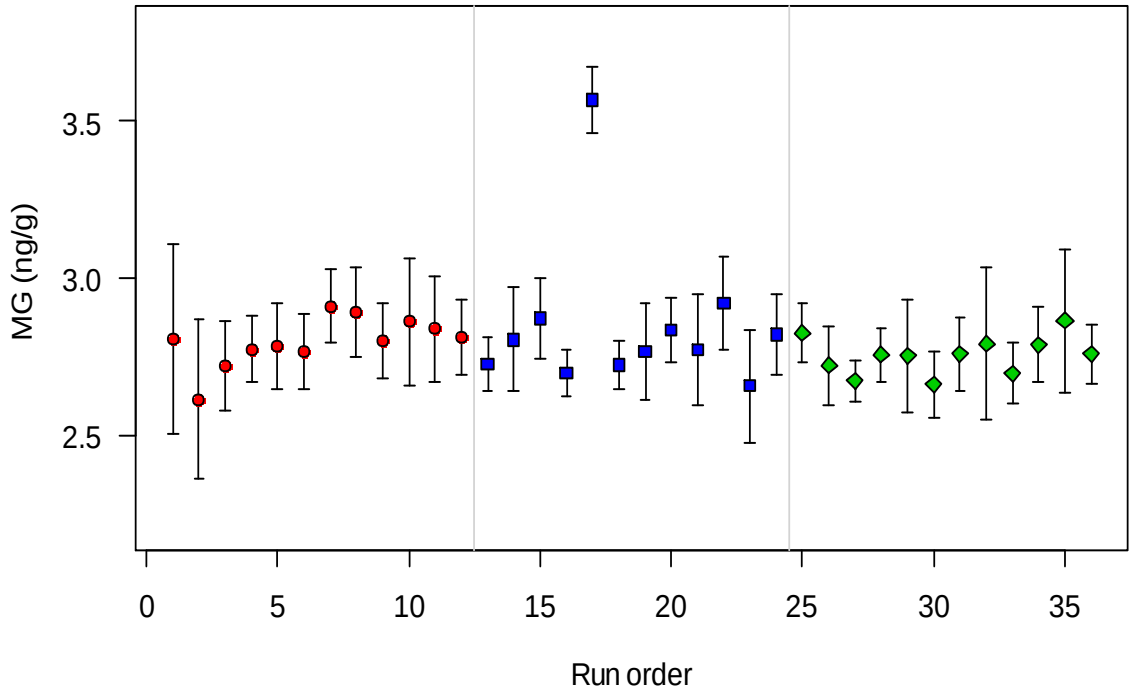

Figure 3. MG homogeneity in material CCQM-K85 as determined by exact matching IDMS performed on 12 samples in triplicate (2g sample size). The different colors indicate analysis performed on different days. Error bars represent the standard deviation.

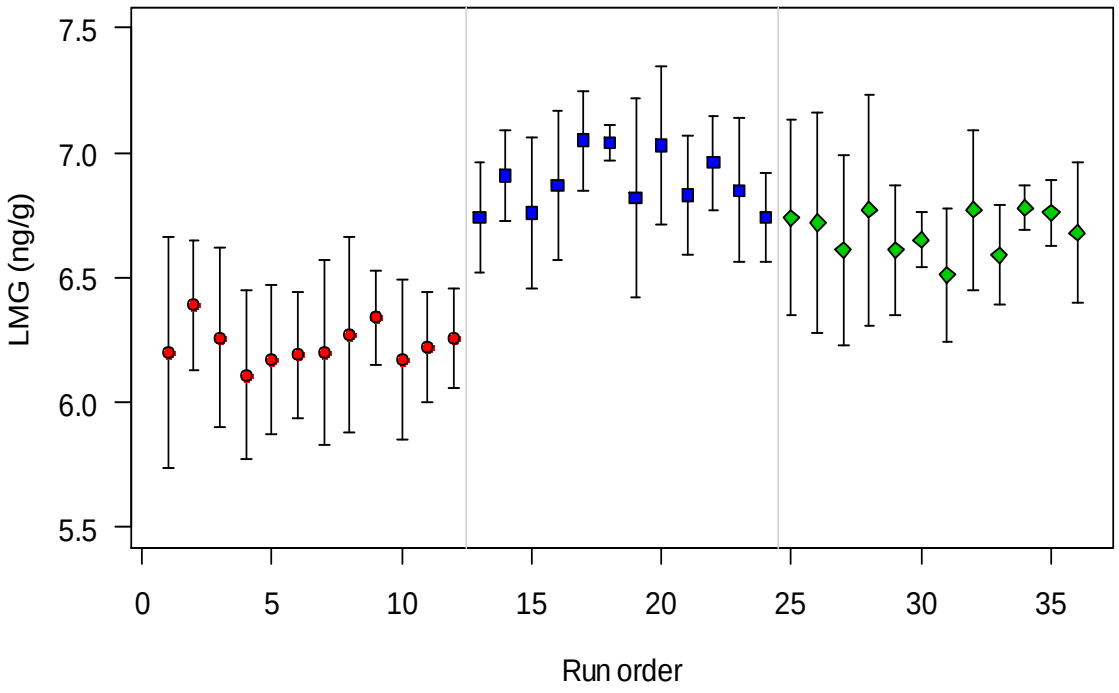

Figure 4. LMG homogeneity in material CCQM-K85 as determined by exact matching IDMS performed on 12 samples in triplicate (2g sample size). The different colors indicate analysis performed on different days. Error bars represent the standard deviation.

Stability

As this material was specifically prepared for this study, advanced long and short term stability studies were not performed. Instead two sample subsets, containing 3 vials of material each, were separated from the main batch of materials. On shipment of the samples one subset containing three vials was placed in a shipment container and sealed at the same time as the samples shipped to participants. On confirmation of receipt of all samples the samples were returned to the storage at < -70 °C. After completion of the study two sub samples from each of the six vials were analysed. No significant differences between the determined mass fractions for MG and LMG were found.

Participant instructions

Each participant received three pots of the K85 material. They were requested to store the material in the dark below -70 °C and advised that subsamples should be taken whilst the material was still frozen as freeze-thaw cycles were known to alter the MG/LMG ratio. As the material was cryomilled the material should have consisted of a "free moving" powder-like particulate while at temperatures below -70°C. Participants were instructed to stir or agitate the material, if needed, to realise this.
Participants were requested to analyse two separate subsamples from each of two pots. The measurand was the mass fraction of "total malachite green" and therefore individual laboratories were requested to assess their methods for any likely conversion between the different forms during extraction and analysis. The individual mass fractions of the malachite green and leucomalachite green were requested, if

measured, to aid in the overall assessment of participants' results and the outcome of the study.
The minimum recommended sample size for each determination was 2 g.

Reporting instructions
Two replicate measurements for total malachite green were requesed for each of two pots of the CCQM-K85 material received. A single estimate for MG, LMG and total malachite green based on four replicate measuremetns was requested. A data reporting sheet was emailed directly to participants on requesting samples and was used for the submission of results (see appendix 1).
It was requested that all results returned should include,

- The mass fraction of total malachite green in the salmon tissue as µg/kg
- A full uncertainty budget
- The source and details of all primary standards used
- The source and details of any labelled materials used
- An outline of the methodology, a full measurement equation and a breakdown of the uncertainty estimation submitted

Comparison timetable

Report on assessment of study material:	OAWG meeting November 2009
Deadline for signup to study:	30th July 2010
Distribution of sample materials:	week of 2nd August May 2010
Deadline for submission of results:	29th October 2010
Draft A Results:	March 2011

Due to reported difficulties in receipt of samples by some laboratories the deadline for submission of results was extended to mid December 2010. All results were reported to the coordinating lab by 10th December 2010.

Participating laboratories

The participating laboratories are listed in Table 2; all laboratories submitted results.

Table 2. Participating laboratories

Institute	Acronym	Country
The Federal Office of Consumer Protection and Food Safety	BVL	Germany
Government Laboratory of Hong Kong	GLHK	Special administrative region of Hong Kong
Korea Research Institute of Standards and Science	KRISS	Korea
LGC*	LGC	UK
National Institute of Metrology (China)	NIM	China
National Institute of Metrology (Thailand)	NIMT	Thailand

* Coordinating laboratory

Analytical methods used

Participants were requested to use their preferred methodology for the analysis of malachite green. These may have include a variety of extraction methods including, but not limited to, maceration, sonication, ultra turrax, liquid and Soxhlet extraction as a variety of these were used in preceding pilot study. Clean up methods including, but not limited to, filtration, SPE and solvent exchange were used by the participants.LC/MS and LC-MS/MS with isotope dilution were the only methods used in the pilot study. Therefore, only results submitted using these approaches would be used in the calculation of the KCRV.

Most participants attempted to achieve exhaustive extraction with the length of extraction time varying from a few minutes to several hours. In all cases the extraction technique involved some form of agitation in solvent under room temperature/pressure conditions. This is noteworthy as the traditional routine methods of achieving exhaustive extraction from a solid matrix used Soxhlet extraction, or (more recently) accelerated solvent extraction (ASE). The use of ambient temperature solvent extraction is no doubt due to the problems associated with MG and LMG stability under harsher conditions, particularly with respect to their inter-conversion. Acetonitrile or acetonitrile/buffer was the common solvent used for extraction with some participants adding radical scavengers or compounds to prevent reduction of MG to LMG (N,N,N’,N’-tetramethyl-1,4-phenylenediamine dihydrochloride, hydroxylamine). One participant used dichloromethane as the solvent. A summary of each participant’s extraction conditions is shown in Table 3.

Table 3. Summary of analysis conditions and analytical procedures used by each participating laboratory

A) BVL

Participant	**BVL**
Approximate mass of the sample used for replicate analysis:	2g
Method of sub sampling:	Taped the pot gently on a hard surface and stirred the sample briefly with a spatula, before we weighed a sub-sample.
Extraction method:	Liquid/liquid (1. 2 mL McIlvaine buffer (pH 3), 100 µL 1M p-TSA und 50 µL methanolic TMPD solution, 12 mL acetonitrile; 2. 2 ml McIlvaine Puffer (pH 6), 12 mL Acetonitrile)
Solvent:	Acetonitrile and McIlvaine buffer
Post extraction clean-up:	SPE (Bakerbond SPE, aromatic sulfonic acid, 500mg/3mL), Eluent: 25% ammonium hydroxide, 2,5 mL methanolic ascorbic acid (1 mg/mL) and 45 mL of methanol
Analytical method: (e.g. LC/MS)	LC-MS/MS
Separation details: (column dimensions etc)	Symmetry C18: 150 mm x 3,9 mm ID, 5 µm, Waters (30°C, 0,6 mL/min)
Method of quantification: (e.g. IDMS, internal standard)	IDMS; d5-Leucomalachite green (d5-LMG), d5-Malachite green (d5-MG)
Solvent used for the preparation of Standards:	Acetonitrile
Type of calibration (single point, bracketing, curve)	Calibration curve with 7 calibration points, internal standard calibration curve with recovery correction
Method for determining "Total malachite Green" (e.g. MG+LMG)	MG + LMG

Table 3. Summary of analysis conditions and analytical procedures used by each participating laboratory
B) GLHK

Participant	**GLHK**
Approximate mass of the sample used for replicate analysis:	2g
Method of sub sampling:	sub sampling in dry air bag with relative humidity <10%
Extraction method:	Vortexing (overnight for >16hr + 2hr)
Solvent:	Acetonitrile/0.1M Ammonium acetate(pH 4.5)/1M p-TSA/hydroxylamine
Post extraction clean-up:	DCM extraction and solid phase extraction clean-up with Alltech neutral alumina (500mg, 3mL) and Waters Oasis MCX (500mg, 6mL)
Analytical method: (e.g. LC/MS)	LC/MS/MS with 2 MRM transitions
Separation details: (column dimensions etc)	Waters XSelect Phenylhexyl 4.6x150mm, 5u preceded by Securityguard guard cartridge of same stationary phase, Mobile phase: 50mM pH 4.5 ammonium acetate buffer / 0.1% formic acid in acetonitrile
Method of quantification: (e.g. IDMS, internal standard)	IDMS
Solvent used for the preparation of Standards:	Malachite green stock solution (1% acetic acid in acetonitrile); Leucomalachite green stock solution (acetonitrile); Working standard solutions for both analytes (1:1 v/v acetonitrile:0.1M pH 4.5 ammonium acetate buffer with 1000ppm hydroxylamine)
Type of calibration (single point, bracketing, curve)	Matrix match calibration curve
Method for determining "Total malachite Green" (e.g. MG+LMG)	MG+LMG

Table 3. Summary of analysis conditions and analytical procedures used by each participating laboratory
C) KRISS

Participant	**KRISS**
Approximate mass of the sample used for replicate analysis:	2g
Method of sub sampling:	Take 2 g from the top of the sample
Extraction method:	Liquid-liquid extraction
Solvent:	Dichlormethane
Post extraction clean-up:	Alumina and propylsulfonic acid SPE, then elute with mobile phase A
Analytical method: (e.g. LC/MS)	LC/MS
Separation details: (column dimensions etc)	Prodigy ODS-3 (5 micron, 4.6 x 250 mm), Mobile phase A: 50 mM ammonium acetate and acetonitrile (50:50) , mobile phase B: acetonitrile
Method of quantification: (e.g. IDMS, internal standard)	ID-MS
Solvent used for the preparation of Standards:	MG: Acetonitrile with 1% acetic acid, LMG: acetonitrile with ascorbic acid (10 ug/mL)
Type of calibration (single point, bracketing, curve)	Not available for ID-MS
Method for determining "Total malachite Green" (e.g. MG+LMG)	Concentration of MG and LMG were measured respectively and add up those concentration

Table 3. Summary of analysis conditions and analytical procedures used by each participating laboratory
D)LGC

Participant	**LGC**
Approximate mass of the sample used for replicate analysis:	2g
Method of sub sampling:	take aliquot after stirring contents of vial with spatula pre-chilled in dry ice
Extraction method:	stirring in extraction solvent at room temperature for ca 38 hours
Solvent:	acetonitrile+1% glacial acetic acid/50mM ammonium acetate pH 4.5 (80/20,v/v)
Post extraction clean-up:	centrifugation, concentration and solvent change
Analytical method: (e.g. LC/MS)	LC-MS/MS
Separation details: (column dimensions etc)	separation on Halo C18 2.1 x 100mm 2.7um; gradient elution with acetonitrile containing 0.1%formic acid/50mM ammonium acetate pH 4.5
Method of quantification: (e.g. IDMS, internal standard)	exact matching IDMS (EM-IDMS)
Solvent used for the preparation of Standards:	MG: Acetonitrile +1% acetic acid, LMG: Acetonitrile
Type of calibration (single point, bracketing, curve)	single point, exact matched
Method for determining "Total malachite Green" (e.g. MG+LMG)	sum of MG and LMG

Table 3. Summary of analysis conditions and analytical procedures used by each participating laboratory
E) NIM China

Participant	**NIM**
Approximate mass of the sample used for replicate analysis:	2g
Method of sub sampling:	The sample was taken with a dry pre-chilled spatula
Extraction method:	Liquid extraction using a shaker for 15 hours.
Solvent:	15mL acetonitrile, 2mL 0.1moL/L ammonium acetate, 1mL hydroxylamine, 0.05mL1mol/Lp-TSA
Post extraction clean-up:	The extractant was centrifuged at 3500rpm for 10min.The supernatant was extracted by 20mL dichloromethane for two times (each time 10mL). The layer with dichloromethane was evaporated into about 2mL and cleaned up by basic alumina SPE column. The sample solution was concentrated into 1mL by N_2 and was added 1mL ammonium acetate buffer
Analytical method: (e.g. LC/MS)	LC-MS/MS
Separation details: (column dimensions etc)	ZORBAX Eclipse Plus C18, 2.1*100mm, 3.5Micron
Method of quantification: (e.g. IDMS, internal standard)	IDMS
Solvent used for the preparation of Standards:	
Type of calibration (single point, bracketing, curve)	single point (exacting matching)
Method for determining "Total malachite Green" (e.g. MG+LMG)	by adding individual value of MG & LMG

Table 3. Summary of analysis conditions and analytical procedures used by each participating laboratory
F) NIMT

Participant	**NIMT**
Approximate mass of the sample used for replicate analysis:	2g
Method of sub sampling:	Subsamples were carried out whilst the material still frozen. The sample pot was stirred and tapped before doing subsampling.
Extraction method:	Fish tissue (2g) was weighed into a 40 mL amber glass vial. Liquid-liquid extraction was carried out by adding 3 mL of ammonium acetate buffer (50 mM, pH 4.5 at RT), 100 □L of 1 M *p*-TSA and 50 □L of 1 mg/mL TMPD, followed by 10 mL of acetonitrile into the sample vial before gravimetrically adding the internal standard solutions (D_5-MG and D_6-LMG). The sample tubes were vortexed and shaken vigorously using a mechanical shaker for 16 hours. The sample tubes were then centrifuged at 4200 rpm for 10 minutes. The top layer was collected into a clean 50 mL polypropylene tube. Solid phase extraction (SPE) was then carried out using VertiPak SXC SPE cartridges (3 mL, 500 mg).
Solvent:	Acetonitrile was used for liquid-liquid extraction.
Post extraction clean-up:	The SCX SPE column was conditioned with 2 mL of methanol and 2 mL of acetonitrile:ammonium acetate buffer pH 4.5 at RT (90:10 v/v) before loading sample. The sample solutions obtained from the liquid-liquid extraction (15 mL) were loaded onto the cartridges. The wash solvent of 30% methanol (2 mL) was applied, followed by 2 mL of a second wash solvent of hexane. The cartridges were dried by forcing air through each cartridge. Eluting solvent (6 mL of 2 % (v/v) NH_4OH in methanol and 3 mL of acetonitrile) was added to elute the analytes from the cartridges. The eluants were carefully evaporated to dryness under a stream of nitrogen at 45 °C and reconstituted in 1 mL of 60:40% (v/v) acetonitrile in ammonium acetate buffer (50 mM, pH 4.5 at

Analytical method: (e.g. LC/MS)	RT) by vigorous vortex-mixing. The reconstituted samples were transferred to 1.5 mL micro centrifuge tubes. The samples were centrifuged for 5 minutes at 9000 rpm and transferred to sample vials for LC-MS/MS analysis. The Shimadzu 20A series liquid chromatography coupled with API 4000, AB Sciex Instruments, MS/MS system was used for analysis.
Separation details: (column dimensions etc)	HPLC analyses were performed on Clipeus C18 chromatography column (100 x 3.0 mm i.d., 5 mm packing, Higgins Analytical, Inc.) The column temperature was maintained at 40 □C. The mobile phase was composed of solvent A (50 mM ammonium acetate buffer pH 4.5 at RT) and solvent B (acetonitrile). The gradient program was: 0-9 min 25% B; 9-12 min 95% B; 12-12.5 min 25% B (constant flow rate of 0.6 ml/min).The data were acquired in the positive multiple reaction monitoring (MRM) mode.
Method of quantification: (e.g. IDMS, internal standard)	An exact-matching double IDMS was employed.
Solvent used for the preparation of Standards:	Acetonitrile was used to prepare LMG and D_6-LMG. The solution of 1% (v/v) acetic acid in acetonitrile was used to prepare MG and D_5-MG solutions.
Type of calibration (single point, bracketing, curve)	An exact-matching IDMS using one point calibration for bracketing was employed. The matrix-matched calibration blend was used.
Method for determining "Total malachite Green" (e.g. MG+LMG)	Quantification of MG was performed by measuring the integrated peak areas at m/z 329.2>313.1 (primary ion), m/z 329.2 > 208.1 (secondary ion) and m/z 334.2>318.1 (primary ion), m/z 334.2 > 213.10(secondary ion) for the D_5-MG. Quantification of LMG was carried out by measuring the integrated peak areas at m/z 331.2>239.1 (primary ion), m/z 331.2>316.0 (secondary ion) and m/z 337.2>240.2 (primary ion), m/z 337.2>322.1 (secondary ion) for the D_6-LMG.

All participants used certified pure materials; be they sourced from another NMI with CMCs for the provision of pure CRMs or from alternative suppliers with a full characterization being performed in house. Many participants identified the advantage of making up MG standards in acidified acetonitrile for stability, whereas LMG was stable in acetonitrile. There was an even split of those who chose to use exact matching IDMS and those who used IDMS in conjunction with a calibration curve; isotopic diluents used were deuterated MG (d_5-MG) and either deuterated LMG (d_5-LMG) or $^{13}C_6$-LMG. Details of pure materials and internal standards used by the participating laboratories are shown in Table 4.

Table 4. Details of pure materials and internal standards used by participants

Institute	Pure materials	Internal Standards
BVL	Malachite green (MG) oxalate (LGC 1706, purity 94.2 ±1.4%) Leucomalachite green (LMG) (ERM, ACA 303a, purity 98.8 ± 0.8%)	d5-Malachite green picrate (>99%) d5-Leucomalachite green (>99%) were obtained from Witega laboratories (Berlin, Germany).
GLHK	Malachite green oxalate (LGC1706, LGC, purity 94.2%); Leucomalachite green (LGC, purity 98.8%)	Malachite green d5 picrate (Witega, 99% purity); Leucomalachite green ^{13}C6 (Cambridge Isotope Laboratories)
KRISS	MG from LGC LMG form Dr. Ehrenstorfer and purity assessments of MG and LMG In-house value assignment by KRISS	Not reported
LGC	Malachite Green oxalate, LGC 1706, purity 94.2%, (67% as MG), Leucomalachite Green ERM AC303a purity 98.8%,	Malachite Green-d5 Picrate, purity>99%, from Witega Laboratorien Berlin Phenyl-^{13}C6-Leucomalachite Green, purity >=98%, from Cambridge Isotope Laboratories,
NIM China	Malachite green: 98.3% ± 1% from NIM China Leucomalachite green: 99.2% ± 0.6% from NIM China	D_5-Malachite green 98% is from WITE Laboratorien Berlin $^{13}C_6$- Leucomalachite green 100µg/mL is from Cambridge Isotope Laboratories
NIMT	MG standard LGC1706, purity 94.2±1.4% LMG standard ERM-AC303a, purity 98.8±0.8%	Malachite green d5 picrate (Chemical purity 99.5%, Isotopic purity >98%) and Leucomalachite green d6 (Isotopic purity 98%) were purchased from Dr. Ehrenstorfer.

The majority of participants used some form of SPE clean up, and several had additional liquid-liquid extraction clean-up steps. In all cases analysis was carried out using electrospray ionisation LC-MS or LC-MS/MS. Separation was typically

achieved using a C_{18} stationary phase with acetonitrile/buffer (acetate or formate) mobile phase.

Approaches to uncertainty

A variety of different approaches were used to estimate the uncertainty. BVL estimated the uncertainty based on combining the standard uncertainties attributed to the weight of sample, preparation of the standards and historical repeatability, reproducibility, matrix and method effect data gained from running this method in their laboratory over a long period of time. Other participants based their uncertainty estimates on the measurement equation used for the calculation of the mass fraction of the individual measurands of MG and LMG. Most included additional components in their uncertainty budgets to address potential inter-conversion and possible interference. Common components were, the uncertainty resulting from the preparation of the calibration solution (which includes the uncertainty of the purity of the standard), uncertainties in weighings, and the overall method precision. LGC and NIMT included a separate component for the instrumental precision of ratio measurements. GLHK and NIMT included a recovery component in their uncertainty budget. These additional components were all Type A uncertainties and estimated though repeat experimentation. The major contribution to the overall uncertainty in all cases was the observed dispersion of results (which would include possible inhomogeneity).

Expanded uncertainties were calculated using a coverage factor of k = 2 or in the case of one participant, calculated using the 95% two tailed critical value of Student's t using degrees of freedom calculated from the Welch-Satterthwaite equation. The combined uncertainty for total MG was calculated by the majority of participants using the following equation:

$$u_{Total} = \sqrt{u_{MG}^{2} + u_{LMG}^{2}}$$

A summary of the approaches used for the estimation of measurement uncertainty by the individual labs is provided in Appendix A.

Results and discussion

The results from the participating laboratories for MG, LMG and total MG are summarised in Table 6 to 8 respectively. The reported results for MG and LMG show appreciable evidence of inconsistency, which is not attributed to any one laboratory. The LMG results also shows evidence of inconsistency therefore the results for these were considered subject to general overdispersion. However, the agreement between laboratories for MG was a considerable improvement, with a CV of 10%, in comparison to the pilot study CCQM-P88 (CV 52%). The dispersion of the LMG results was also reduced with a CV of 6% in comparison to CCQM-P88 (CV 21%). The absolute values for these two measurands were not considered for the calculation of KCRVs or DoEs. The reason for this is simply a chemical one, in that the reporting of malachite green for regulatory purposes are the combined sum of LMG and MG. This is due to the fact that LMG and MG are rapidly converted from one form to the other. There is some evidence to support this as the agreement of the interlaboratory results is better for the sum “total malachite green” than it is for the individual

species. There is also evidence of negative correlation between reported MG and LMG for individual participant results. That is, that those returning low answers for MG returned a high answer for LMG and vice versa. Since the agreed measureand in the Key comparison study was total malachite green, DoEs and UDoE have only been calculated for the sum total of LMG and MG referred to as "total malachite green".

Table 6. Participants' reported results for MG

Lab id	CCQM-K85 Malachite Green Mean (ng/g)	u	relative u u%	K	U_{95}
BVL	2.26	0.36	15.9	2.00	0.72
KRISS	2.28	0.05	2.3	2.78	0.14
NIMT	2.52	0.11	4.4	2.03	0.23
GLHK	2.65	0.21	7.8	2.00	0.41
NIM	2.79	0.095	3.4	2.00	0.19
LGC	2.91	0.08	2.7	2.00	0.16

Table 7. Participants' reported results for LMG

Lab id	CCQM-K85 Lucomalachite Green Mean (ng/g)	u	relative u %	K	U95
BVL	6.76	0.69	10.2	2.00	1.37
GLHK	6.83	0.15	2.2	2.00	0.31
LGC	6.86	0.10	1.5	2.00	0.20
NIM	7.04	0.12	1.7	2.00	0.24
NIMT	7.25	0.28	3.8	2.02	0.56
KRISS	7.98	0.34	4.3	2.45	0.84

Table 8. Participants' reported results for "total" Malachite Green

Lab id	CCQM-K85 "Total" Malachite Green Mean (ng/g)	u	relative u %	K	U95
BVL	9.02	1.70	18.9	2.00	3.40
GLHK	9.47	0.26	2.7	2.00	0.51
NIMT	9.77	0.30	3.1	2.00	0.60
LGC	9.77	0.12	1.2	2.00	0.24
NIM	9.83	0.27	2.7	2.02	0.53
KRISS	10.26	0.35	3.4	2.45	0.85

The results are represented graphically in figures 6, 7 and 8.

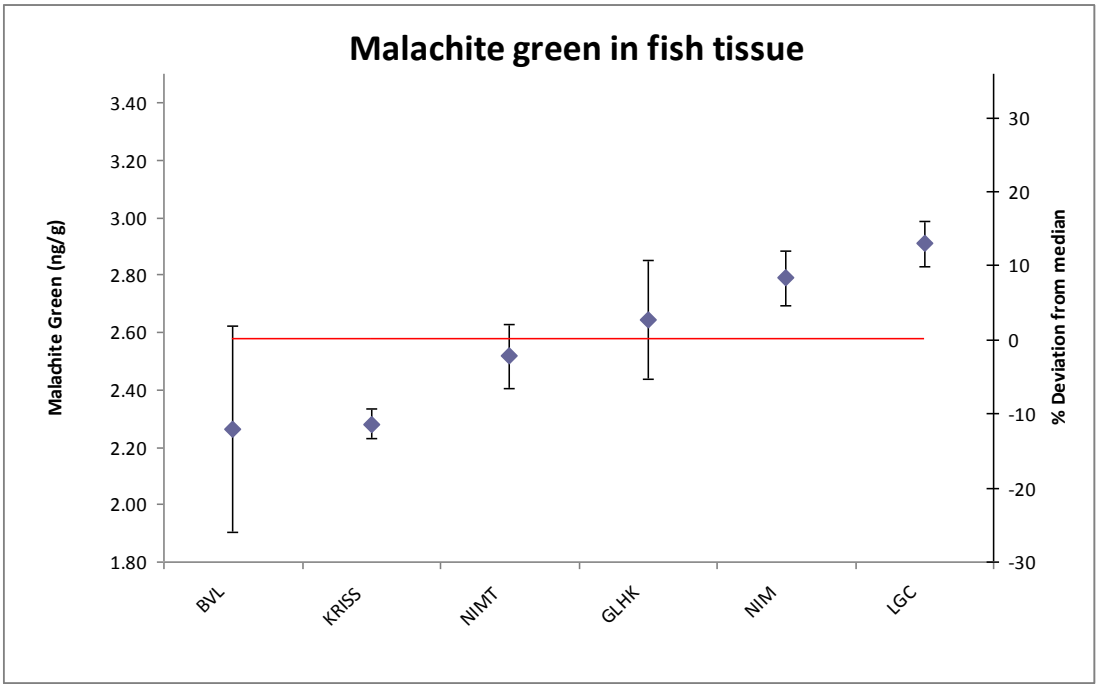

Figure 6. Participants' reported results for MG in CCQM-K85 material with their reported standard uncertainties. The solid red line is the median value of all participants (See table 4)

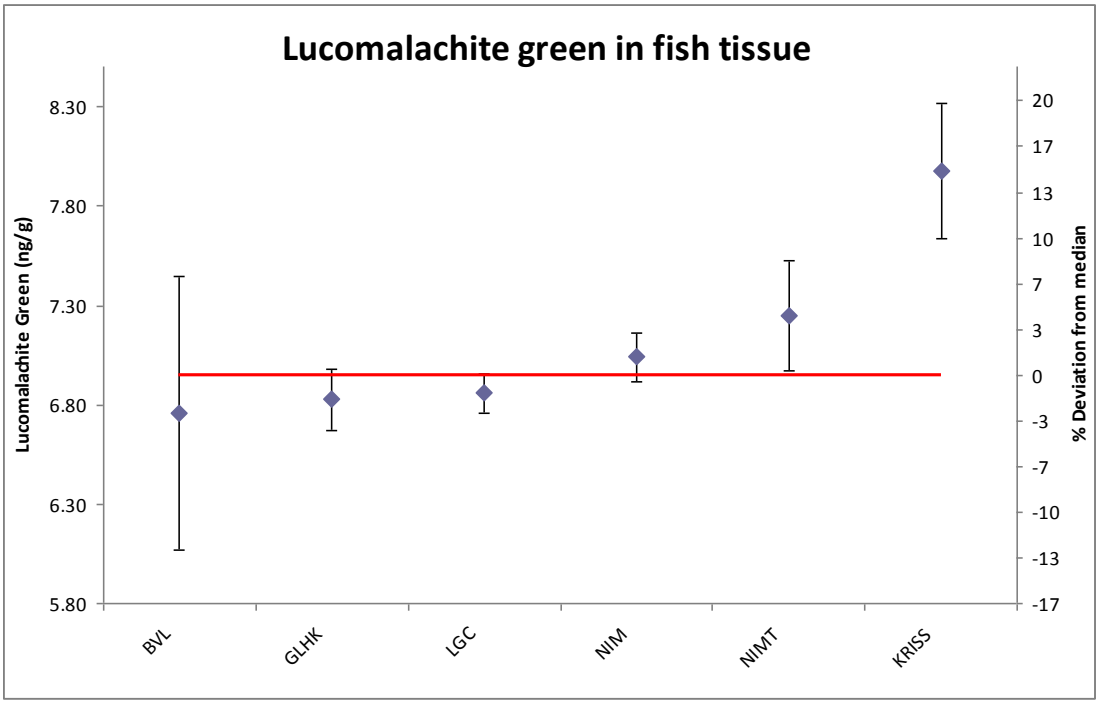

Figure 7. Participants' reported results for LMG in CCQM-K85 material with their reported standard uncertainties. The solid red line is the median value of all participants. (See table 4)

Assigning of the KCRV

The KCRV was calculated using all participants results. A number of different estimators were assessed for use as the KCRV and these are summarised in Table 9.

The suggestion for the use of the DerSimonian-Laird estimator was based on this being the simplest approach for dealing with general overdispersion. This may have been appropriate for the treatment of the individual MG and LMG results but the "Total MG" did not exhibit general overdispersion. This, combined with the fact that the DerSimonian-Laird approach converging to the classical weighted mean, with uncertainty close to the classical Graybill-Deal estimate was felt likely to be unrealistically low, resulted in the working group deciding that the median and its uncertainty estimate was the most appropriate approach for assigning the KCRV and its uncertainty. Therefore a KCRV of 9.77 ng/g ± 0.35 ng/g was agreed.

Table 9. Comparison of different estimators for us in the calculation of the KCRV for "total" Malachite Green

Arithmetic Mean	9.69 ng/g
Standard deviation	0.41 ng/g
No. of data used (N)	6
Standard uncertainty (=s.d./(n)$^{0.5}$)	0.17 ng/g
DerSimonian-Laird	9.77 ng/g
No. data used	6
Standard uncertatiny	0.09 ng/g
Median	9.77 ng/g
MADe	0.27 ng/g
No of data points(N)	6
Standard uncertainty	0.14 ng/g

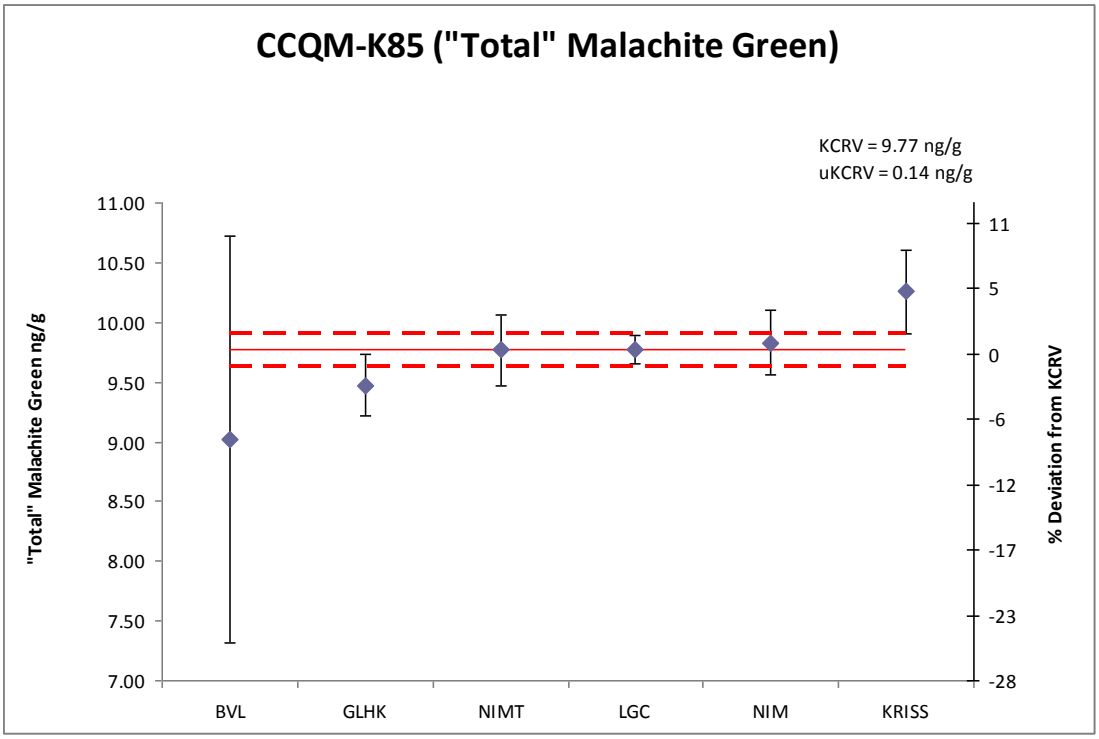

Figure 8. Participants' reported results for "total malachite green" in CCQM-K85 material with their reported standard uncertainties (see table 4). The KCRV is represented by the solid red line whilst the uncertainty associated with the KCRV "uKCRV" expressed as the standard uncertainty is represented by the broken red lines.

Calculation of Degree of Equivalence

The degrees of equivalence (DoE) and relative degrees of equivalence were calculated from the key comparison reference value using the following standard approaches:

$$DoE = \left(x_i - x_{ref}\right)$$

Where x_i is the individual participants results and x_{ref} is the comparison reference value.
The uncertainty associated with the DoE for each participant was estimated as:

$$uDoE = \sqrt{\left(u_{x_i}\right)^2 + \left(u_{x_{ref}}\right)^2}$$

Whilst the expanded uncertainty of DoE was calculated for 95% coverage by using a coverage factor of 2.

$$UDoE = k \cdot uDoE$$

The calculated Doe and UDoE for each Key participant are shown in Table 9 with graphical representation of the results shown in Figures 9 and 10.

Table 10: Degree of equivalence and relative degree of equivalence for key participants in CCQM-K85

Laboratory	Total malachite green (ng/g)		Total malachite green	
	Di	***U(d)***	**Di (%)**	***U%***
BVL	-0.75	3.42	-7.7	35.0
GLHK	-0.29	0.58	-3.0	5.9
KRISS	0.49	0.75	5.0	7.7
LGC	0.00	0.36	0.0	3.7
NIM	0.06	0.60	0.6	6.1
NIMT	0.00	0.66	0.0	6.7

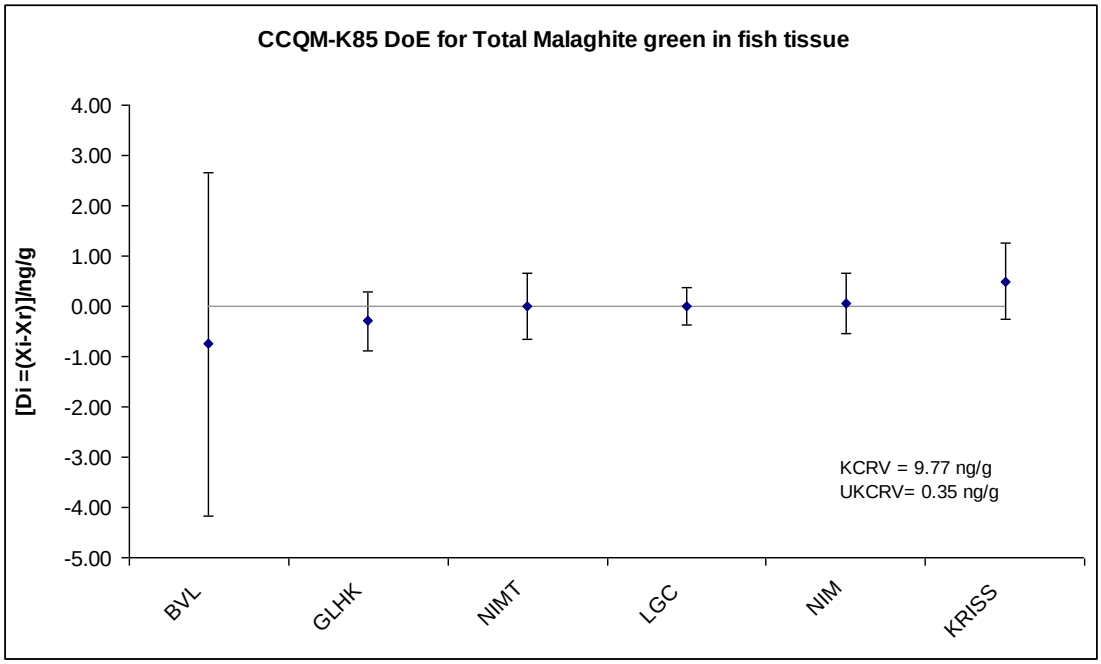

Figure 9. Participants' calculated DoE for "total malachite green" in CCQM-K85 material with their associated expanded uncertainties (See table 9).

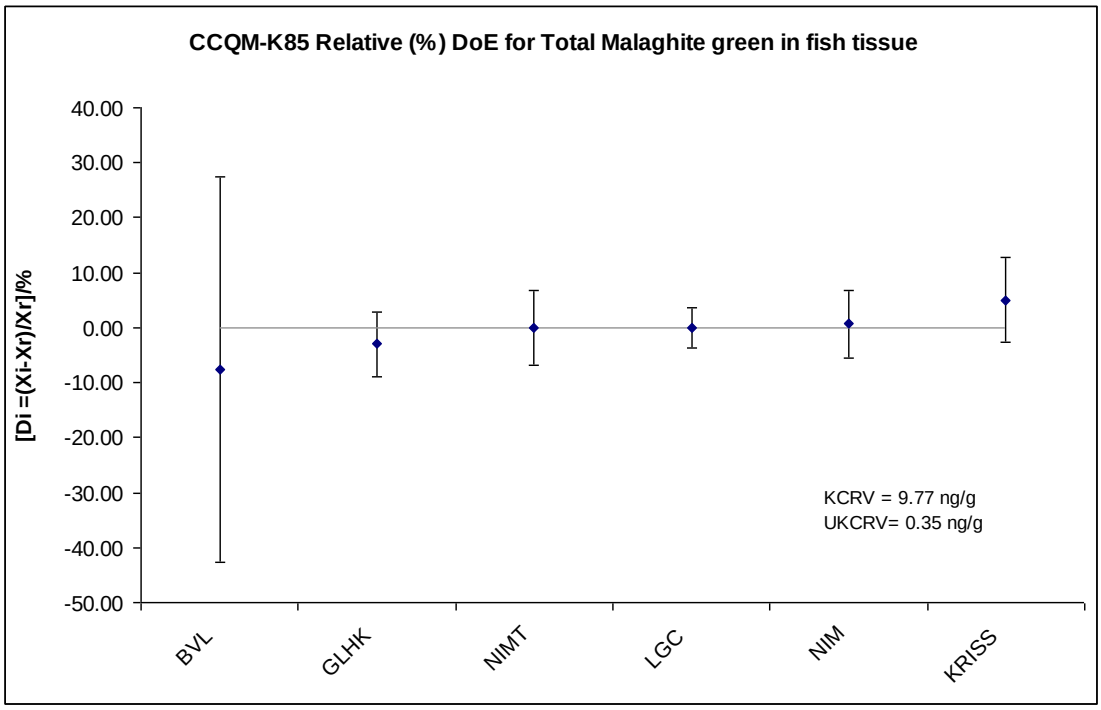

Figure 10. Participants' calculated relateive % DoE for "total malachite green" in CCQM-K85 material with their associated expanded uncertainties (See table 9).

Use of study in support of CMCs

The determination of malachite green in salmon tissue is a complex analysis involving the extraction of trace levels of potentially unstable analytes from a solid matrix. Successful participation is indicative of a laboratory's ability to measure low levels (1-100 µg/kg) of medium to high polarity residues in muscle tissue. It also demonstrates a high level of analytical capability in extraction of labile compounds from solid matrices.

Conclusions

The ability of participating laboratories to measure total malachite green in fish has been demonstrated through participation in this key comparison. All of the participants successfully determined the mass fraction of "total malachite green". The key comparison involved extraction, clean-up and separation of two labile compounds requiring precautionary steps be taken to reduce degradation of the compounds.

The relative standard deviation of all participants results for MG and LMG were 10% and 6% respectively suggesting that further work may be required by some laboratories to reduce inter-conversion in the extraction and analysis of the individual forms. The relative standard deviation of participant's results for "total malachite green" was less than 5%. This is considered excellent especially when the complexity of the matrix and the sub 10 ng/g levels of individual compounds measured.

Acknowledgements

The coordinators wish to thank all the participating laboratories and Mrs Gill Holcombe (LGC) for organizing the distribution of the samples and collation of results.

References

1. Clifton-Hadley, R.S., Alderman, D.J. The effects of malachite green on proliferative kidney disease. Journal of Fish Diseases. 10 (1987) 101-107.
2. Srivastava, S., Sinha, R., Roy, D. Toxicological effects of malachite green. Aquatic Toxicology. 66 (2004) 319-329.
3. Byrne, D., European Commission. Settling of minimum required performance limits (MRPL's) for certain residues in food of animal origin. 2002/657/EC, L 6-38-L 6/39. 22-12-2003.
4. CCQM Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence

Appendix A:

Summary of approaches taken to uncertainty estimation used by each participating laboratory

A) BVL

Malachite green							
contributions to measurement uncertainty:							
	u		**target**			**u(x)/X [%]**	
u calibration solution:	0.00398	ug/g	0.12987	ug/g		3.067	9.406114998
u sample weight:	0.01977	mg	2000	mg		0.0010	9.77132E-07
in-house reproducibility method:	0.33384	ng/g	2.14	ng/g		15.600	243.36
(incl. repeatability, lab/method condition, time effect, matrix effect)							
					k=	2	
					u=	**15.9** %	
					U=	**31.8** %	

Leucomalachite green							
contributions to measurement uncertainty:							
	u		**target**			**u(x)/X [%]**	
u calibration solution:	0.00153	ug/g	0.12987	ug/g		1.175	1.380923238
u sample weight:	0.01977	mg	2000	mg		0.00099	9.77132E-07
in-house reproducibility method:	0.74235	ng/g	7.35	ng/g		10.100	102.01
(incl. repeatability, lab/method condition, time effect, matrix effect)							
					k=	2	
					u=	**10.2** %	
					U=	**20.3** %	

Summary of approaches taken to uncertainty estimation used by each participating laboratory

B) GLHK

Malachite green

Parameter	Value	Unit	Standard Uncertainty, u(x)	Relative Standard Uncertainty, u(x)/x	Source of data
(1) Stock standard solutions					
Purity of standard	94.2	%	0.57	0.006041	Certificate of reference material LGC1706 with assessment purity of 94.2% and uncertainty of 1.4% (k=2.46)
Mass of neat standard	0.05000	g	0.0000515	0.001030	In-house calibration indicated an half load expanded uncertainty of $1.03x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Mass of solvent used to dissolve the neat standard	180	g	0.000127	0.00000071	In-house calibration indicated an full load expanded uncertainty of $2.54x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Mass of standard solution taken for serial dilution x 4	1.5	g	0.000508	0.00034	In-house calibration indicated an full load expanded uncertainty of $2.54x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Mass of water used for serial dilution x 4	40	g	0.000508	0.0000127	In-house calibration indicated an full load expanded uncertainty of $2.54x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Calibration Blend, native standard and internal standard	1.5	g	0.000254	0.0001693	In-house calibration indicated an full load expanded uncertainty of $2.54x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
			u(S)	**0.006140**	
(2) Sample blend					
Mass of sample	1.5	g	0.00005	0.00003	In-house calibration indicated an half load expanded uncertainty of $1.03x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Mass of internal standard solution added x 2	1.5	g	0.000103	0.00007	In-house calibration indicated an half load expanded uncertainty of $1.03x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
			u(SB)	**0.0000768**	
(3) Method precision					
Run-to-run variability	1		0.07676	0.07676	From a pool of results
			u(P)	**0.07676**	
(4) Spike Recovery					
Recovery	1		0.01259	0.01259	From spike recovery data (n=4)
			u(R)	**0.012594**	

Combined standard uncertainty, u(MG), ng/g = Mass fraction of MG x $\sqrt{u(S)^2+u(SB)^2+u(P)^2+u(R)^2}$

= 0.206

Expanded uncertainty, U(MG), ng/g = u(MG) x k (where k = coverage factor of 2)

= 0.413

. Summary of approaches taken to uncertainty estimation used by each participating laboratory

B) GLHK (cont.d)

Leucomalachite green

Parameter	Value	Unit	Standard Uncertainty, u(x)	Relative Standard Uncertainty, u(x)/x	Source of data
(1) Stock standard solutions					
Purity of standard	98.8	%	0.3200000	0.003239	In-house validation of reference material by assessing chromatographic purity by HPLC and water content by Karl Fisher titration
Mass of neat standard	0.02000	g	0.0000515	0.002575	In-house calibration indicated an half load expanded uncertainty of $1.03x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Mass of solvent used to dissolve the neat standard	90	g	0.000127	0.00000141	In-house calibration indicated an full load expanded uncertainty of $2.54x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Mass of standard solution taken for serial dilution x 4	1.5	g	0.000508	0.00034	In-house calibration indicated an full load expanded uncertainty of $2.54x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Mass of solvent used for serial dilution x 4	40	g	0.000508	0.0000127	In-house calibration indicated an full load expanded uncertainty of $2.54x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Calibration Blend, native standard and internal standard	1.5	g	0.000254	0.0001693	In-house calibration indicated an full load expanded uncertainty of $2.54x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
			u(S)	**0.004155**	
(2) Sample blend					
Mass of sample	2.0	g	0.00005	0.00003	In-house calibration indicated an half load expanded uncertainty of $1.03x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
Mass of internal standard solution added x 2	1.5	g	0.000103	0.00007	In-house calibration indicated an half load expanded uncertainty of $1.03x10^{-4}$ g with a coverage factor of 2. (QA/BAL/E142)
			u(SB)	**0.0000733**	
(3) Method precision					
Run-to-run variability	1		0.01847	0.01847	From a pool of results
			u(P)	**0.01847**	
(4) Spike Recovery					
Recovery	1		0.01198	0.01198	From spike recovery data (n=4)
			u(R)	**0.011984**	

Combined standard uncertainty, u(LMG), ng/g = Mass fraction of LMG x $\sqrt{u(S)^2+u(SB)^2+u(P)^2+u(R)^2}$

= 0.153

Expanded uncertainty, U(LMG), ng/g = u(LMG) x k (where k = coverage factor of 2)

= 0.306

"Total Malachite Green"

Combined standard uncertainty, u(MG+LMG), ng/g = $\sqrt{u(MG)^2+u(LMG)^2}$

= $\sqrt{(0.206)^2 + (0.153)^2}$

= 0.257

Expanded uncertainty, U(MG+LMG), ng/g = u(MG+LMG) x k (where k = coverage factor of 2)

= 0.514

Summary of approaches taken to uncertainty estimation used by each participating laboratory

C) KRISS

KRISS Uncertainty Estimation

The total standard uncertainty of sum of MG and LMG was combined with the standard uncertainty of MG and the standard uncertainty of LMG.

Each standard uncertainty of MG and LMG was obtained by combining the systematic uncertainty and random uncertainty as shown below equation.

$$u_{total.} = \sqrt{u_{\text{systematic}}^2 + u_{\text{random}}^2}$$

The systematic uncertainty included the uncertainty of calibration standard solutions (u_{cal}), the uncertainty of isotope ratio standard ($u_{isotope}$) and the uncertainty of multiple measurements of isotope ratio standard for sample measurements (u_{multi}). Each component was combined.

$$u_{systematic} = \sqrt{u_{\text{cal}}^2 + u^2{}_{isotope} + u_{\text{multi}}^2}$$

The uncertainty of calibration standard solutions (u_{cal}) was obtained by combining the uncertainty of preparation of standard solutions and the uncertainty of purities for pure materials that are obtained by purity assessment in our laboratory. The uncertainty of isotope ratio standard ($u_{isotope}$) was obtained from the uncertainty of preparation for mixing pure materials and their isotopes.

The random uncertainty was calculated from the standard deviation of multiple measurement results (two subsamplings from two bottles).

. Summary of approaches taken to uncertainty estimation used by each participating laboratory

D) LGC

Calculation of uncertainty

The amount of MG and LMG in each of the sample extracts was calculated using the double IDMS equation:

$$W_x = W_z \cdot \frac{m_z}{m_{yc}} \cdot \frac{m_y}{m_x} \cdot \frac{R'_B}{R'_{BC}}$$

Where:

W_x = the mass fraction of MG (or LMG) in sample

W_z = the mass fraction of the natural MG (or LMG) used to prepare the calibration blend

m_z = mass of the natural MG (or LMG) solution added to the calibration blend.

M_x = mass of the sample used.

M_{yc} = mass of the labelled MG (or LMG) solution added to the calibration blend.

M_y = mass of the labelled MG (or LMG) solution added to the sample blend.

R'_B = measured ratio of the sample blend.*

R'_{BC} = average measured ratio of the calibration blend injected before and after the sample.*

* The measured ratios were as follows:

MG (peak area MG/peak area d5-MG)

LMG (peak area LMG/peak area 13C-LMG)

The mass fraction of total MG and LMG in sample is defined in Equation 2.

$$W_{Total} = \overline{W_{MG}} + \overline{W_{LMG}}$$

The uncertainty of each individual measurement was calculated using the following equation:

$$u_c = w_x \sqrt{\left(\frac{u_{Wz}}{w_z}\right)^2 + \left(\frac{u_{P_{R'B}}}{p_{R'B}}\right)^2 + \left(\frac{u_{P_{R'BC}}}{p_{R'BC}}\right)^2 + \left(\frac{um_x}{m_x}\right)^2 + \left(\frac{um_y}{m_y}\right)^2 + \left(\frac{um_z}{m_z}\right)^2 + \left(\frac{um_{yc}}{m_{yc}}\right)^2}$$

Where

u_{Wz} = the standard uncertainty associated with the mass fraction of the calibration solution.
W_z = the mass fraction of the calibration solution.
Um_x = the uncertainty associated with the mass of sample used.
M_x = the mass of sample used.
Um_y = the uncertainty associated with the mass of labelled MG/LMG added to the sample.
M_y = the mass of labelled MG/LMG added to the sample.
Um_z = the uncertainty associated with the mass of MG/LMG added to the calibration blend.
M_z = the mass of MG/LMG added to the calibration blend.
Um_{yc} = the uncertainty associated with the mass of labelled MG/LMG added to the calibration blend.
M_{yc} = the mass of labelled MG/LMG added to the calibration blend.
$U_{PR'B}$ = the standard deviation of ratio R'_B (n=5)
$U_{PR'BC}$ = the standard deviation of ratio R'_{Bc} (n=5)
$P_{R'B}$ = the mean of R'_B(n=5)
$P_{R'BC}$ = the mean of R'_{Bc} (n=5)

The combined final uncertainty for each analyte was calculated using:

$$u = \sqrt{b_{var}^2 + \bar{u}_{ci}^2}$$

Where

b_{var} = the standard deviation of individual sample mass fractions

$\bar{u}_{ci}$ = average of the individual sample uncertainties u_{ci}

The combined final uncertainty for the total MG in the sample was calculated by combining the final uncertainties of MG and LMG.

$$u_{Total} = \sqrt{u_{MG}^2 + u_{LMG}^2}$$

The final uncertainty for MG, LMG and total MG and LMG was expanded using a factor of k=2 (95 % confidence).

$$U = 2u$$

. Summary of approaches taken to uncertainty estimation used by each participating laboratory

E) NIM China

Mean result and uncertainty of malachite green, leucomalachite green and total malachite green

Mean Result and uncetainty	**Malachite green**	**leucomalachite green**	**Total malachite green**
Mean result (µg/kg)	2.79	7.04	9.83
Uncertainty from method (µg/kg)	0.080	0.087	0.216
Uncertainty from calibration solution (µg/kg)	0.050	0.082	0.155
Combined Standard Uncertainty (µg/kg)	0.095	0.120	0.266
Coverage factor, (k)	2	2	2
Expanded Uncertainty to give 95% Confidence Level (µg/kg)	0.19	0.24	0.53

. Summary of approaches taken to uncertainty estimation used by each participating laboratory

NIM China (Cont.d) :

$$C_{sample} = \frac{R_{SM} \times C_{calib} \times f_{purity} \times M_{spike(sample)}}{R_{CM} \times M_{sample} \times C_{spike(calib)}}$$

R_{SM} Area ratio of target compound and labeled standard in sample solution
R_{CM} Area ratio of target compound and labeled standard in calibration solution
C_{calib} Mass faction of calibration solution
Mspike(sample) Mass of labeled standard to added into sample
Msample Sample mass
Cspike(calib) Mass fraction of labeled standard to add into calibration solution
fpurity Purity of Calibrant

Full uncertainty Budget for value assign process

Parameter		Description	Type A/B	Related paramenter
Um (Method uncertainty)	U_1	Random uncertainty in the process of certification	A	SD of C_{sample}
	U_2	Extraction efficiency	B	R_{SM}
	U_3	From weighing out of the sample	A+B	M_{sample}
	U_4	From blank	A+B	C_{calib}
U_C (calibration solution uncertainty)	U_1	From purity of calibrant	A+B	C_{sample}
	U_2	From weighing of calibration solution and internal standard solution	A+B	C_{calib}

Summary of approaches taken to uncertainty estimation used by each participating laboratory

F) NMIT

Expanded measurement equation:

$$w_x = F_P . F_E . F_I \; . w_{z,c} . \frac{m_y . m_{z,c}}{m_x . m_{y,c}} . \frac{R'_B}{R'_{B,C}}$$

$w_{Z,C}$	mass fraction of analyte in the calibration solution used to prepare the calibration blend
m_Y	mass of spike solution added to sample blend
$m_{Y,C}$	mass of spike solution added to calibration blend
m_X	mass of sample added to sample blend
$m_{Z,C}$	mass of standard solution added to calibration blend
R'_B $R'_{B,C}$	observed isotope amount ratios in the sample blend and the calibration blend, respectively
F_I	interference effect
F_P	method precision factor

$$\frac{u(w_x)}{w_x} = \sqrt{\left(\frac{u(w_{Z,C})}{w_{Z,C}}\right)^2 + \left(\frac{u(m_Y)}{m_Y}\right)^2 + \left(\frac{u(m_{Y,C})}{m_{Y,C}}\right)^2 + \left(\frac{u(m_X)}{m_X}\right)^2 + \left(\frac{u(m_{Z,C})}{m_{Z,C}}\right)^2 + \left(\frac{u(R_B')}{R_B'}\right)^2 + \left(\frac{u(R_{B,C}')}{R_{B,C}'}\right)^2 + \left(\frac{u(F_P)}{F_P}\right)^2 + \left(\frac{u(F_I)}{F_I}\right)^2 + \left(\frac{u(F_E)}{F_E}\right)^2}$$

$u(w_{z,c})$	is the standard uncertainty of the mass fraction of analyte in the calibration solution used to prepare the calibration blend.
$u(m_Y)$ $u(m_{Y,C})$ $u(m_X)$ $u(m_{Z,C})$	are standard uncertainties of the masses. These values were estimated from the bias and precision effects of the balance.
$u(F_I)$	is the standard uncertainty of interference factor. This value was estimated from potential bias between primary ion pair and secondary ion pair.
$u(F_P)$	is the standard uncertainty of the precision factor which is estimated from standard deviation of the mean of the multiple IDMS results.
$u(F_E)$	is the standard uncertainty of the extraction efficiency factor which is estimated from the liquid-liquid extraction and SPE clean-up.
$u(R_B')$, $u(R_{BC}')$	are the standard uncertainties due to the precision in measuring the isotope amount ratios of the analyte and the internal standard in the sample and calibration blends.

$$u(w_{totalMG}) = \sqrt{u(w_{MG})^2 + u(w_{LMG})^2}$$

$$\nu_{eff} = \frac{u_c^4(y)}{\sum_{i=1}^{N} \frac{\left(c_i^2 \cdot u^2(x_i)\right)^2}{\nu_i}}$$

The total effective degrees of freedom was used to calculate the appropriate k factors to expand the combined standard uncertainties to a 95% confidence interval.

Table 5. Summary of approaches taken to uncertainty estimation used by each participating laboratory
F) NMIT (Cont.d)

Uncertainty budget for MG			
Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
Parameter (unit)			
F_P (1)	1.0000	0.02700	2.700%
m_{zc} (g)	0.39560	0.000053	0.0134%
m_y (g)	0.29409	0.000053	0.0180%
m_{yc} (g)	0.29390	0.000053	0.0180%
m_x (g)	2.00288	0.000053	0.0026%
w_{zc} (g)	19.0869	0.434219	2.2750%
R'b (1)	0.7285	0.014116	1.9377%
R'bc (1)	1.0863	0.014761	1.3589%
Additional Factors			
F_I (1)	1.000	0.0073	0.728%
F_E (1)	1.000	0.0100	1.000%

Uncertainty Analysis Result of MG		
w_{MG}=	2.52	ng/g
$u(w_{MG})$ =	0.111	ng/g
u(x)/x =	4.43%	
Veff(total) =	33.1	
k=	2.03	(@ 95% level)
U(x) =	0.227	
%U(x) =	9.01%	

Uncertainty budget for LMG			
Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
Parameter (unit)			
F_P (1)	1.0000	0.01117	1.117%
m_{zc} (g)	0.81405	0.000053	0.0065%
m_y (g)	0.71051	0.000053	0.0075%
m_{yc} (g)	0.70955	0.000053	0.0075%
m_x (g)	2.00288	0.000053	0.0026%
w_{zc} (g)	20.1334	0.550796	2.7357%
R'b (1)	1.0052	0.016898	1.6810%
R'bc (1)	1.1237	0.015722	1.3992%
Additional Factors			
F_I (1)	1.000	0.0032	0.323%
F_E (1)	1.000	0.0100	1.000%

Uncertainty Analysis Result of LMG		
w_{LMG}=	7.25	ng/g
$u(w_{LMG})$ =	0.277	ng/g
u(x)/x =	3.82%	
Veff(total) =	42.3	
k=	2.02	(@ 95% level)
U(x) =	0.559	
%U(x) =	7.72%	

Total MG = MG + LMG 9.77 ng/g

Standard uncertainty of total MG was calculated using the following equation.

$$u(w_{totalMG}) = \sqrt{u(w_{MG})^2 + u(w_{LMG})^2}$$

u_c 0.30 ng/g

The total effective degrees of freedom was used to calculate the appropriate k factors to expand the combined standard uncertainties to a 95% confidence interval.

$$\nu_{eff} = \frac{u_c^4(y)}{\sum_{i=1}^{N} \frac{\left(c_i^2 \cdot u^2(x_i)\right)^2}{\nu_i}}$$

k =2.00

Expanded uncertainty, U = 0.60 ng/g