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High accuracy determination of malachite green and leucomalachite green in salmon tissue by exact matching isotope dilution mass spectrometry

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ABSTRACT

A high accuracy method for the quantification of malachite green (MG) and leucomalachite green (LMG) in salmon is described. Analytical challenges including the effects of analyte instability and matrix suppression were minimised by the use of exact matching isotope dilution mass spectrometry. The developed method included overnight extraction in acidified acetonitrile/ammonium acetate buffer and analysis by LC–MS/MS utilising isotopic internal standards. This method was used to determine the level of MG and LMG in a sample of salmon used in an international inter-comparison organised by the Comité Consultatif pour la Quantité de Matière (CCQM). The sum of MG and LMG was found to be $9.32 \pm 0.98\,\mathrm{ng\,g^{-1}}$ at the 95% confidence interval (relative expanded uncertainty 10.5% ($k = 2$)). This encompassed the mean and median of the CCQM inter-comparison.

1. Introduction

Malachite green (MG), a parasiticide and antifungal agent, has been used in the fish farm industry to prevent fin rot and proliferative kidney disease in trout and salmon [1]. MG, a multi-organ toxin to mammals [2], is metabolised to leucomalachite green (LMG); this metabolite has a wide range of toxicological effects including potential carcinogenesis [3]. The commercial use of MG has been banned in the European Union since 2002 with the minimum required performance limit (MRPL) for laboratories carrying out surveillance for these compounds being $2\,\mu\mathrm{g\,kg^{-1}}$ for the sum of MG and LMG [4] reported as "total MG". Regulators request results to be reported as total MG as a result of the conversion of MG to LMG, the more abundant nature of LMG and uncertainties in metabolism rates. The structures of MG and LMG are shown in Fig. 1.

The typical method for the analysis of MG and LMG [5–10] involves extraction by vortex mixing or shaking in acetonitrile/buffer mixtures; the inclusion of anti-reductants and radical scavengers has been common practice [11]. Solid phase extraction (SPE) and/or liquid–liquid extraction with dichloromethane has been performed as a sample pre-treatment before analysis. Liquid chromatography-tandem mass spectrometry, either with or without post-column oxidation of LMG to MG, has been the most common instrumental technique used. Isotopic internal standards are available and have been used to overcome problems such as matrix suppression during electrospray ionization. Reported decision limits (CCα) and detection capabilities (CCβ) for laboratories employing analysis by LC–MS/MS range from 0.02 to $0.3\,\mu\mathrm{g\,kg^{-1}}$ [5–6,11] for MG and LMG, which is well below the MRPL level for total MG of $2\,\mu\mathrm{g\,kg^{-1}}$.

Although the ability to detect MG and LMG at regulated levels has been dramatically improved by the use of LC–MS/MS and SPE clean-up procedures, the analysis of MG in fish tissue remains a challenge, primarily due to issues surrounding extraction and analyte stability. To the best of the author's knowledge, scientific literature to date does not provide any information on published methods' ability to efficiently extract incurred MG from fish tissue. This paper describes the development of a high accuracy method, with SI-traceability including a full uncertainty budget, for the quantification of total MG in fish tissue, at part per billion levels, by exact matching isotope dilution mass spectrometry (IDMS). Full optimisation of the extraction conditions are described, as well as an assessment of the conversions occurring during sample preparation. Unlike the majority of published methods, the developed method employs a longer 16 h extraction time and minimal sample clean-up. Application of this method to accurately measure total MG in salmon in an international inter-laboratory comparison organised by the Comité Consultatif pour la Quantité de Matière (CCQM) has been described.

2. Materials and methods

2.1. Sample details

Samples were received from the Central Science Laboratory (CSL, York, UK). Each unit consisted of approximately 30 g of

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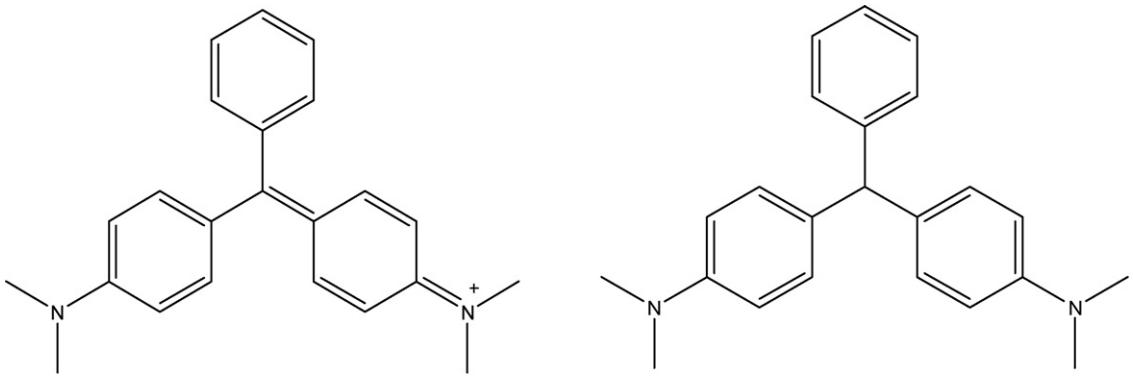

Fig. 1. Structure of malachite green cation (left) and leucomalachite green (right).

homogenised salmon muscle. The material had been prepared by blending MG incurred salmon with "blank" salmon. These samples were stored in a freezer at −20 °C until required for analysis. Blank fish tissue used to prepare matrix matched calibration blends was obtained from Cefas Weymouth Laboratory and had been prepared from salmon reared in a MG-free environment.

2.2. Standard materials and preparation

MG oxalate (purity 94.2 ± 1.4%) and LMG (purity 98.8 ± 0.8%) certified reference materials were obtained from LGC Standards (Teddington, UK). d_5-MG picrate was obtained from Witega laboratories (Berlin, Germany). $^{13}C_6$-LMG was obtained from Cambridge Isotope Laboratories (via LGC Standards, Teddington, UK).

MG solutions were prepared in 1% (v/v) glacial acetic acid (analytical reagent grade, Fisher, Loughborough, UK) in acetonitrile (HPLC special grade, LGC Standards, Teddington, UK). LMG solutions were prepared in acetonitrile (HPLC special grade, LGC Standards, Teddington, UK). This is in accordance with a stability study conducted in-house [12]. All standard working solutions were prepared gravimetrically.

2.3. Preparation of sample blends

Sample blends were prepared gravimetrically by the addition of d_5-MG and $^{13}C_6$-LMG isotopic internal standards to frozen sample (2 g) in extraction solvent (20 g). The extraction solvent consisted of 80:20 (v/v) acidified acetonitrile (1% acetic acid):ammonium acetate buffer (50 mM, pH 4.5) and was added pre-mixed to the frozen sample, before any additions of standard or internal standard. The concentrations of the internal standard solutions used to prepare the blends were such that the ratio of natural/labelled analyte measured on the mass spectrometer was close to unity (±5%). Gravimetric addition of internal standard solutions was carried out immediately after the addition of extraction solvent to the frozen fish in the following order: d_5-MG and $^{13}C_6$-LMG (0.8 g each).

2.4. Preparation of calibration blends

Calibration blends were prepared gravimetrically by the addition of standard solutions of MG and LMG and their isotopic internal standards to frozen blank fish matrix (2 g) in the same extraction solvent (20 g). The concentration of the standard solutions used to prepare the blends was such that the final sample and calibration blend concentrations were equimolar with respect to MG and LMG. In order to obtain these exact matching blends, several iterations were required, including an initial assessment to determine the approximate level of MG and LMG in the sample. The amount of internal standard added to the calibration blends was exactly the same as that which was added to the sample blends. Gravimetric addition of standard and internal standard solutions was carried out immediately after the addition of extraction solvent to the frozen fish in the following order: MG, d_5-MG, LMG and $^{13}C_6$-LMG (0.8 g each).

2.5. Extraction and clean-up procedure

Each sample and calibration blend was mechanically agitated, using a magnetic stirrer bar, in the extraction solvent. In order to assess the extraction efficiency during method development, 200 μL aliquots were removed at defined time intervals, added to 120 μL ammonium acetate buffer (50 mM, pH 4.5) and centrifuged for 10 min at 3000 rpm before analysis by LC–MS/MS. In order to carry out full IDMS experiments, agitation was carried out for 16 h on a 15-place magnetic stirrer plate. An aliquot (10 mL) of the extractant was centrifuged at 3000 rpm for 15 min. The supernatant was then evaporated to dryness at 60 °C under a stream of nitrogen. The dried extract was reconstituted in 1 mL 50:50 (v/v) acetonitrile:ammonium acetate buffer (50 mM, pH 4.5) and vortex mixed for 20 s. After the addition of isopropanol (40 μL), the extract was centrifuged for a further 2 min before analysis.

2.6. Instrumental analysis

All analyses were carried out using a 4000 Q TRAP® (Applied Biosystems, Warrington, UK) mass spectrometer coupled with an 1100 Liquid Chromatography (Agilent, West Lothian, UK) instrument. MG and LMG were separated using a Symmetry C_{18} 3.5 μm column, 75 mm × 4.6 mm (Waters, Watford, UK) at 45 °C. The mobile phase consisted of solvent (*A*) acetonitrile/0.1% formic acid (v/v) and solvent (*B*) ammonium acetate buffer (50 mM, pH 4.5). The compounds were separated using the following gradient at a flow rate of 1 mL min^{-1}. Initial conditions were 46:54 *A*:*B* and this was held for 4 min and increased linearly to 80:20 *A*:*B* by 4.5 min; this was held constant for a further 6 min after which the column was flushed for 5 min with 100% acetonitrile. The mobile phase was adjusted back to initial conditions over 1 min, and the column allowed to re-equilibrate for 10 min.

The Q TRAP was operated in electrospray positive ion mode with a capillary voltage of 2000 V. The source was heated to 600 °C and curtain gas, gas 1 and gas 2 were set at 15, 60 and 60 (arbitrary units), respectively. A declustering potential of 75 V was used. Selected reaction monitoring was performed and the following transitions were monitored and used for quantification, with a dwell time of 100 ms: MG (*m*/*z* 329.2 > 313.2), d_5-MG (*m*/*z* 334.2 > 318.2), LMG (*m*/*z* 331.2 > 239.1) and $^{13}C_6$-LMG (*m*/*z* 337.2 > 239.1). Confirmatory

transitions were only monitored during method development, in order to maximise the number of data points under each peak for optimum ratio measurement precision.

2.7. Measurement procedure

Three sample blends and three calibration blends were prepared for each unit to be analysed. Each sample blend was injected five times with every sample injection being bracketed by its corresponding calibration blend. The mass fractions of MG and LMG in each of the sample extracts were calculated using the shortened version of the double IDMS equation [13] (Eq. (1)):

$$W_x = W_z \frac{m_z}{m_{yc}} \frac{m_y}{m_x} \frac{R'_B}{R'_{Bc}} \quad (1)$$

where W_x is the mass fraction of MG (LMG) in the sample ($ng\,g^{-1}$), W_z the mass fraction of the natural MG (LMG) solution used to prepare the calibration blend ($ng\,g^{-1}$), m_z the mass of the natural MG (LMG) solution added to the calibration blend, m_x the mass of the sample used, m_{yc} the mass of the labelled MG (LMG) solution added to the calibration blend, m_y the mass of the labelled MG (LMG) solution added to the sample blend, R'_B the measured ratio (peak area MG/peak area d_5-MG or peak area LMG/peak area $^{13}C_6$-LMG) of the sample blend and R'_{Bc} is the average measured ratio (peak area MG/peak area d_5-MG or peak area LMG/peak area $^{13}C_6$-LMG) of the calibration blend injected before and after the sample.

The mass fraction of total MG in the sample is defined as the sum of the overall mean mass fractions of MG and LMG (Eq. (2)):

$$W_{Total} = \overline{W_{MG}} + \overline{W_{LMG}} \quad (2)$$

2.8. Calculation of uncertainty

The uncertainty of each individual measurement was calculated by combining the uncertainties associated with the concentrations of the natural standard solutions, the precision of the instrument and the weighing by the following equation:

$$u_c = w_x \sqrt{\left(\frac{u_{Wz}}{w_z}\right)^2 + \left(\frac{u_{PR}}{p_R}\right)^2 + \left(\frac{um_x}{m_x}\right)^2 + \left(\frac{um_y}{m_y}\right)^2 + \left(\frac{um_z}{m_z}\right)^2 + \left(\frac{um_{yc}}{m_{yc}}\right)^2} \quad (3)$$

where u_{Wz} is the standard uncertainty associated with the mass fraction of the calibration solution, u_{PR} the standard deviation of the ratios of R'_B/R'_{Bc} ($n = 5$), P_R the mean of R'_B/R'_{Bc} ($n = 5$), u_{mx} the uncertainty associated with the mass of sample used, u_{my} the uncertainty associated with the mass of labelled MG (LMG) added to the sample, u_{mz} the uncertainty associated with the mass of MG (LMG) added to the calibration blend and u_{myc} is the uncertainty associated with the mass of labelled MG (LMG) added to the calibration blend.

The combined final uncertainty for each analyte in the homogenised material was calculated by combining the average measurement uncertainty with the blend-to-blend variation (Eq. (4)).

$$u = \sqrt{b_{var}^2 + (\bar{u}_c)^2} \quad (4)$$

where b_{var} is the standard deviation of individual sample mass fractions.

The combined uncertainty for total MG was calculated by combining the final uncertainties for MG and LMG (Eq. (5)).

$$u_{Total} = \sqrt{u_{MG}^2 + u_{LMG}^2} \quad (5)$$

Uncertainties for MG, LMG and total MG were expanded using a coverage factor of $k = 2$ to give a 95% confidence interval.

3. Results and discussion

3.1. Introduction

The use of exact matching IDMS has been described by Henrion [14] and guidelines for its use have been published by the Royal Society of Chemistry [15]. The ratio measurements of the sample and calibration blends are ideally indistinguishable by the mass spectrometer (i.e. the ratio of ratios approaches unity) [16] as they have been prepared at the same concentrations of natural and labelled analyte. Being a time-consuming and iterative process, this is not a suitable method for a high-throughput laboratory. When highly accurate and precise determinations are required on a material, for example when assigning a value to a certified reference material, exact matching IDMS has proved invaluable. Full assessment and calculation of individual components of the overall uncertainty are carried out in order to provide traceability to SI units, in-line with the ISO Guide to Expression of Uncertainty in Measurement (GUM) [17].

The principle of using exact matching IDMS to quantify MG and LMG in fish is dependent upon having traceable standards and upon the exhaustive extraction of the analytes from the fish. It is also crucial to achieve equilibration between the natural and isotopically labelled forms of the analyte, particularly with respect to the conversions which MG and LMG may undergo. Sample and calibration blends are prepared and taken though identical extraction and sample preparation procedures before analysis by LC–MS/MS.

MG/LMG calibration blends exhibit different chemical behaviour when in matrix or solvent; this may be due to the presence of stabilising compounds in the matrix. In order to match the sample blends, the calibration blends were prepared in matrix (blank salmon) and taken through the entire procedure. Each sample blend was injected five times, with its corresponding calibration blend injected before and after (bracketing). The double IDMS equation was then used to calculate the mass fraction of MG and LMG in the original sample.

3.2. Extraction

When using IDMS for the traceable measurement of the analytes in a solid matrix, an exhaustive extraction with minimum compound breakdown, and full equilibration of the analyte and internal standard, is required. Several methods were investigated for the exhaustive extraction of MG from fish, including vortex mixing, Soxhlet extraction and accelerated solvent extraction (ASE). None of these methods were considered suitable due to incomplete extraction of MG (vortex mixing and ASE), severe degradation and/or inter-conversion (Soxhlet extraction and ASE) and carry-over (ASE). Mechanical agitation was also investigated and considered to be the most promising extraction technique, the principle being that the fish sample would be agitated in extraction media for a time period sufficient to attain equilibrium between the natural and isotopically labelled analytes.

In order to determine the time required to achieve equilibration, a sample was placed in a bottle with the extraction solvent and then spiked with the isotopically labelled analogues. The sample and the extraction solvent were then stirred together for several hours with a magnetic stirrer bar. Aliquots of the extraction solvent were removed at regular intervals and analysed by LC–MS/MS.

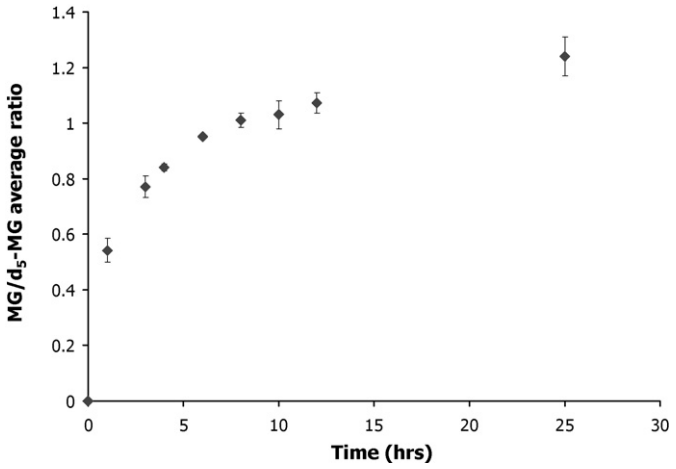

Fig. 2. Equilibration profile for MG and its isotopic internal standard d_5-MG during the extraction of MG from an incurred salmon sample by mechanical agitation in acidified acetonitrile/acetate buffer (50 mM, pH 4.5). Error bars reflect standard deviation of four replicate samples each of which were injected twice on the LC–MS/MS.

The ratios of MG/d_5-MG and LMG/$^{13}C_6$-LMG were plotted against time and the data was assessed to indicate when equilibration was achieved.

Equilibration of the analytes is deemed to be achieved when a stable ratio of the natural to the isotopically labelled analyte is obtained. The data displayed in Fig. 2 indicated that equilibration between the natural and labelled MG had not occurred by 12 h due to MG being continuously extracted from the fish. LMG, on the other hand, is extracted immediately and equilibration between natural and labelled forms was established after 1 h of mechanical agitation (Fig. 3). This implies that LMG and MG have different chemical behaviour in the fish tissue. One possible explanation is that LMG is dissolved in the fish oils and is therefore easily extracted by organic solvent, whilst MG is more strongly bound to the fish tissue. Attempts to improve the rate of extraction for MG by adjusting the organic: aqueous proportions, changing the pH and by sonication were unsuccessful.

Full IDMS experiments were then carried out on duplicate samples which had been extracted for different amounts of time, in order to more precisely define the optimum extraction length for MG. Time points were chosen that encompassed the last data point

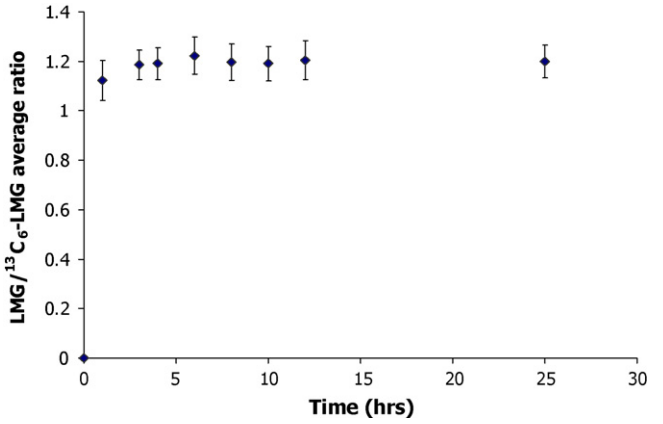

Fig. 3. Equilibration profile for LMG and its isotopic internal standard $^{13}C_6$-LMG during the extraction of LMG from an incurred salmon sample by mechanical agitation in acidified acetonitrile/acetate buffer (50 mM, pH 4.5). Error bars reflect standard deviation of four replicate samples each of which were injected twice on the LC–MS/MS.

Table 1
SRM transitions of selected MG and LMG degradation products

Compound	Transition (m/z)	Collision energy (eV)	Dwell time (ms)
MG carbinol (natural)	347.3 > 255.2	40	100
MG carbinol (labelled)	352.3 > 255.2	40	100
MG monode (natural)	315.2 > 299.2	36	100
MG monode (labelled)	320.2 > 304.2	36	100
LMG monode (natural)	317.2 > 225.2	36	100
LMG monode (labelled)	323.2 > 225.2	36	100

from the equilibration study at 25 h. No significant differences in MG concentrations were observed for extraction times of 16, 30 and 40 h (MG mass fractions of 2.5 ± 0.2, 2.6 ± 0.3 and 2.5 ± 0.1 μg kg^{-1}, respectively) and the conclusion was that an extraction time of 16 h was required.

3.3. Stability

The stability of MG and LMG in matrix during extraction and sample preparation was assessed. The use of acidified acetonitrile in the extraction media dramatically reduced any conversion of MG to its carbinol form, with no detrimental effect on LMG, which was stable under these extraction conditions. Both MG and LMG can undergo demethylation, typically producing the mono-demethylated (monode) and di-demethylated forms. This is catalysed by light and therefore the extraction and sample preparation were carried out in the absence of light wherever possible. During initial method development the carbinol form of MG and monode forms of MG and LMG were monitored using appropriate SRM transitions (Table 1) and were found to be low to negligible (data not shown).

Analyte inter-conversion was assessed with the use of separate MG/d_5-MG and LMG/$^{13}C_6$-LMG matrix standards at concentrations equivalent to that of the sample, which were taken through the entire extraction and sample preparation procedure. Comparison of pre- and post-extraction matrix standards was performed to ensure any conversions identified were the result of the extraction and/or sample preparation and not, for example, due to an impurity in the standard solutions. Conversion of LMG to MG was found to be negligible (approximately 0.1%), whereas conversion of MG to LMG was found to be significant; up to 15% MG can be converted to LMG during the evaporation step. The use of isotopic internal standards and exact matched sample and calibration blends will account for any such conversion, provided full equilibration has been achieved. It is important to note that the calibration blends in this case were required to be matrix matched as the conversion rate of the analytes in solvent was dramatically different (data not shown).

3.4. Chromatography

Ion suppression or matrix suppression is known to occur during the electrospray process. In order to improve the repeatability of analyte peak areas, elution of analytes is preferred to be away from areas of matrix suppression. In order to assess the separation of the analytes with respect to signal suppression, a matrix suppression profile for the analytes was obtained. This involved the infusion of a strong solution of MG and LMG at 1 μg g^{-1}, into the mobile phase whilst injecting and analysing a blank matrix extract spiked with d_5-MG and $^{13}C_6$-LMG (10 ng g^{-1}). Mobile phase conditions were as described in Section 2.6.

The matrix suppression profile for blank salmon tissue (with peaks for d_5-MG and $^{13}C_6$-LMG superimposed) is shown in Fig. 4. Signal suppression is identified by a negative peak in the

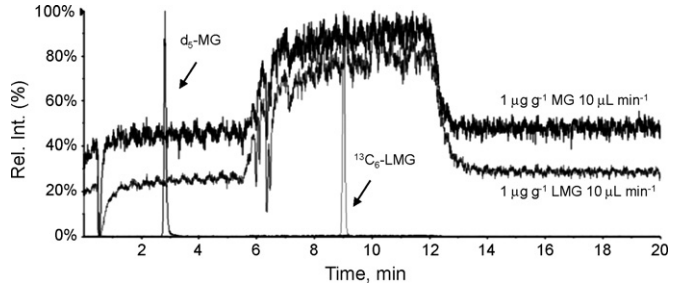

Fig. 4. Suppression of MG (top trace) and LMG (bottom trace) signals by matrix (salmon tissue extract). Peaks for isotopic internal standards d_5-MG and $^{13}C_6$-LMG are superimposed.

chromatogram. MG exists as a cation in solution and therefore shows very little signal suppression due to matrix under these conditions. LMG is more heavily suppressed, particularly during the first 2 min and during the first few minutes after the organic proportion is increased to 80%.

Chromatographic separation of MG and LMG in a sample extract was achieved. A high flow rate and elevated temperature ensured sharp chromatographic peaks with a corresponding improvement of peak area precision. A signal to noise ratio (peak to peak) of >800 and >2000 for levels of MG and LMG at 2 and 7 $\mu g\,kg^{-1}$, respectively, indicates that this method would be applicable to detect levels of total MG at the MRPL level of 2 $\mu g\,kg^{-1}$ (Fig. 5).

3.5. Homogeneity of material

Assessment of homogeneity of the sample material was conducted, sampling ten random units in duplicate and quantifying using the exact matching IDMS method described. Relative standard deviation of values from the mean for MG, LMG and total MG were 13%, 5% and 6%, respectively. The spread of data obtained for total MG in the sample material is shown in Fig. 6.

3.6. Determined concentration of total MG

Two units were sampled three times each and the final iteration results for the levels of MG, LMG and total MG in the material are

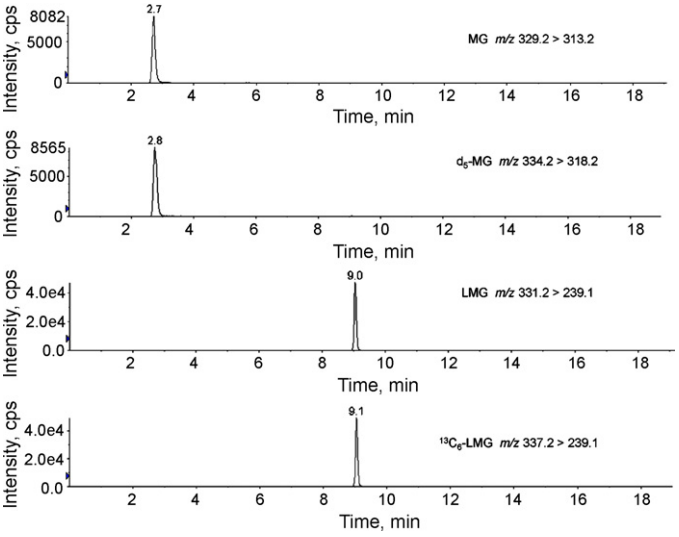

Fig. 5. SRM chromatograms showing separation of MG, d_5-MG, LMG and $^{13}C_6$-LMG in a typical sample extract (corresponding to 2 $\mu g\,kg^{-1}$ MG and 7 $\mu g\,kg^{-1}$ LMG in fish).

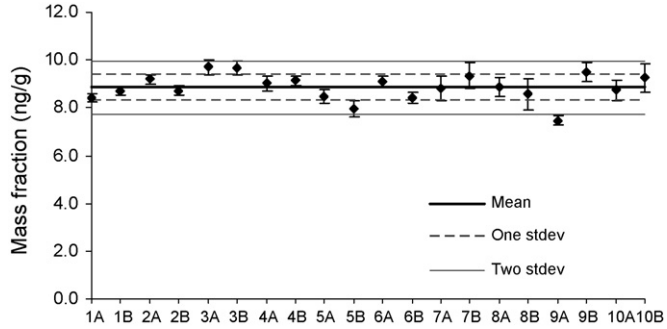

Fig. 6. Homogeneity results for total MG in sample material (10 duplicate units, sample size 2 g) determined by exact matching IDMS. Error bars represent expanded ($k = 2$) measurement uncertainty.

Table 2
Summary of results for the determination of malachite green in fish tissue by exact matching IDMS

Analyte	Mean ($ng\,g^{-1}$)	Uncertainty ($ng\,g^{-1}$)	Expanded uncertainty ($ng\,g^{-1}$, $k = 2$)
MG	2.52	0.18	0.36
LMG	6.80	0.43	0.86
Total MG	9.32	0.49	0.98

summarised in Table 2. Quality control samples were extracted and analysed with each batch of sample and calibration blends in order to monitor possible conversions and contamination. These included reagent and matrix blanks, MG/d_5-MG matrix standard, LMG/$^{13}C_6$-LMG matrix standard, MG/LMG matrix standard and d_5-MG/$^{13}C_6$-LMG matrix standard.

The material was found to contain $2.52 \pm 0.36\,ng\,g^{-1}$ MG, $6.80 \pm 0.86\,ng\,g^{-1}$ LMG and $9.32 \pm 0.98\,ng\,g^{-1}$ total MG. The uncertainties associated with these numbers are expanded (coverage factor $k = 2$) to give a 95% confidence interval; relative uncertainty values are 14.3%, 12.6% and 10.5% for MG, LMG and total MG, respectively. The major source of uncertainty (~95%) was the blend-to-blend variation; which includes any possible material inhomogeneity.

The results obtained by LGC for total MG in the sample material, in the context of results obtained by other laboratories participating in the inter-comparison, are shown in Fig. 7. Each laboratory used its preferred methodology for the extraction, clean-up and

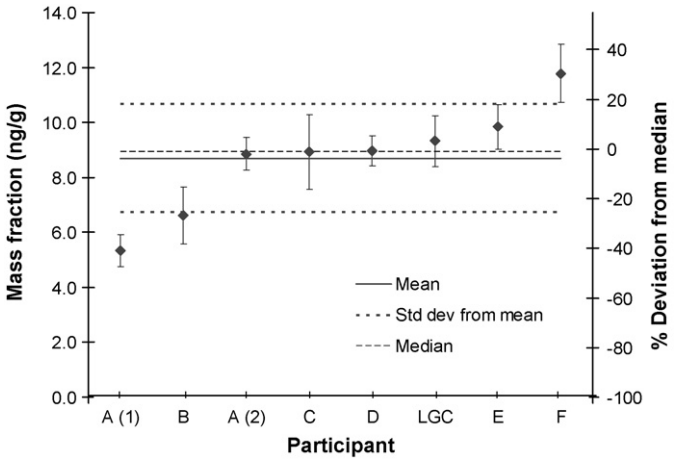

Fig. 7. Total MG in sample as determined by laboratories participating in pilot CCQM inter-comparison for the determination of total MG in fish. Error bars represent the estimated expanded uncertainty (95% confidence interval).

analysis. Participating laboratories were either national measurement institutes or designated national measurement institutes and three continents were represented. Participant A reported separate results for each of the two units analysed due to large inter-unit differences for LMG; this was attributed to suspected inhomogeneity of the material. The mean and median values for total MG obtained in this study were 8.71 and 8.95 ng g^{-1}, respectively.

4. Conclusions

The determination of MG in fish tissue is a complex analysis involving the extraction of trace levels of potentially unstable analytes from a solid matrix. A high accuracy method has been developed for the analysis of part per billion levels of total MG in salmon. Analytical challenges such as analyte instability and inter-conversions can be overcome and matrix effects minimised by the use of exact matching IDMS with matrix matched calibration blends. An assessment of the extraction method showed that whilst the total extraction and equilibration of LMG was achieved in under an hour, further MG could still be extracted up to 16 h. This clearly highlights the difference in chemical behaviour of the two analytes in the fish matrix and the necessity for a lengthy extraction time. Future work could concentrate on improving the rate of release of MG from fish tissue, for example by the use of enzymatic and/or microwave digestion. Using the described method, a value for total MG of 9.32 ± 0.98 ng g^{-1}, at the 95% confidence interval, was assigned to salmon tissue (relative uncertainty of 10.5%). This value and its associated uncertainty encompassed the mean and median of a blind inter-laboratory comparison.

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