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RESEARCH PAPER

Spiking and homogenization of biological matrices for production of reference materials using cryogenic processes

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Abstract
Biological reference materials (RMs) are essential for quality assurance, traceability of measurement results and for method validation. When addressing new measurement questions or emerging regulatory issues, rigorous large-scale CRM production may not be time efficient or economically practical using current production methods. By amending a relatively small matrix batch with a compound(s) of interest at the homogenization step, the National Institute of Standards and Technology (NIST) can create a custom material on an "as-needed" basis and circumvent the time delay inherent in large-batch production, thereby generating a fit-for-purpose, rapid-response RM. Here, Coho salmon (*Oncorhynchus kisutch)* was cryohomogenized and spiked with an aquaculture antibiotic and antibiotic metabolite. The resultant material was analyzed using liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS/MS) to determine the effectiveness of the amendment technique in a fresh-frozen matrix by assessing homogeneity and accuracy to the target concentration (e.g. mass fraction). Target mass fractions were achieved for both spike components, with RSDs below 5% in replicate measurements of each compound ($n = 8$). The stability of the spiked compounds was assessed one year post-production and mass fractions were stable, within 1–6% of the initial measurement results, indicating minimal change to the amended analyte concentrations over time. The results support this method as a promising new technique for custom, small-batch RM generation.

Keywords Reference materials · Biological samples · Bioanalytical methods

Introduction

Biological reference materials (RMs) have been used to aid in calibration of measurements for quality control, method development/validation and value assignment for decades, and are important in maintaining a coherent and robust measurement system worldwide. An RM is defined as a material sufficiently homogenous and stable concerning one or more specified properties, which have been established to be fit for its intended use in a measurement process [1]. A Certified Reference Material (CRM) is an RM characterized by a metrologically valid procedure for one or more specified properties, accompanied by an RM certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability [1]. CRMs are generally used for trueness checks, as they carry a certified value against which experimental results can be compared and, in many cases, numerous mass fractions of compounds are certified in a single material using a variety of analytical platforms. Though useful for method harmonization and value assignment, the process of generating a CRM can be expensive, time consuming, labor intensive and may not always be necessary. RMs can be used in a similar way, however value assignment is generally less rigorously conducted.

Ideally, one should choose a RM that is matrix-matched, includes the analyte of interest at a comparable concentration, and is prepared in the same manner as the experimental samples (e.g. frozen, lyophilized, room temperature, etc.). It is also important for practical considerations to be evaluated. Frozen materials are more costly to store and generally require dry ice for shipment, rendering these materials inaccessible to some customers due to strict shipping regulations. However, frozen materials remain stable for long periods of time, preventing degradation of the matrix and ensuring characteristics inherent to the material remain intact, while also limiting the loss or modification of sensitive (i.e. volatile or labile) analytes of

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interest [2]. In contrast, freeze dried materials do not require deep freezing, so they are easier to ship and maintenance at room temperature results in lower storage costs, though material composition and integrity of certain analytes can be affected during lyophilization [2]. The National Institute of Standards and Technology (NIST) and other National Metrology Institutes (NMIs) around the world generate and maintain a wide breadth of RMs, though it is clear not every analytical space is covered due to monetary and practical constraints. In addition, the availability of naturally-occurring source materials and the time required to obtain and process large batches may impede research efforts or be impractical. The best alternative is to generate a custom material for preforming trueness checks by assessing the recovery of spiked components in a homogenous matrix.

In-house custom generation of RMs has provided a work around for unavailable materials and the modification of blank matrices in a laboratory setting has become a means to meet specific RM needs in the analytical community. Multiple RMs have been generated by the addition of an analyte directly to a matrix [3] or by generating mixtures via blending of blank and incurred material to reach a desired concentration [4, 5]. These methods have been used in production schemas at room temperature with great success. However, there are no studies to date examining the utility of amending a frozen material during production, which would be useful when biological samples, such as tissues or food items are to be analyzed. At NIST, these RMs are typically created using cryohomogenization and stored frozen at −80 °C or below [6].

One potential use for custom frozen RMs is the authentication of food products presumed to be adulterated, contaminated or misidentified. The food safety industry identifies food fraud based on falsification of species, provenance and weight, the result of which can endanger human and environmental health and negatively impact the domestic economy [7]. In this case, obtaining authentic samples and generating a large batch RM may be a lengthy process. However, the generation of rapid-response custom materials would be particularly useful to more quickly answer authenticity questions, while ensuring integrity and confidence in the associated measurement. Stocks of these small-batches of high-interest materials would likely become exhausted relatively quickly (i.e. one to two years), rendering long-term stability testing unnecessary, while providing a temporary, high-quality standardization material until rigorous CRM production can be completed.

To this end, NIST tested the efficacy of generating a small-batch cryogenic material for proof of principle. Antibiotics/metabolites in fish was chosen as the test system for this technique. Crystal violet (CV) and malachite green are both antibiotic/antimicrobial compounds historically used in aquaculture. Although both have been banned in the United States as they are thought to have carcinogenic and mutagenic affects [8, 9], their use is still suspected due to low cost and widespread availability. Both parent compounds and their metabolites are detectable in fish tissues, with malachite green being predominantly converted to leucomalachite green (LMG). The intent of this study was to spike a neat cryogenic material (salmon homogenate) with a high-level antibiotic (CV) and low-level metabolite (LMG) at concentrations similar to those referenced in the literature [10, 11] in order to test the technique at biologically relevant levels. Here we provide a detailed method for the amendment of cryogenic material and assess the success of the technique.

Materials/methods

Reagents and materials

Spike components, crystal violet (CV) and leucomalachite green (LMG), and internal standards, d6-crystal violet (d6-CV) and d5-leucomalachite green (d5-LMG), were all purchased from Sigma Aldrich (St. Louis, MO, USA) and stored at room temperature until preparation.

Frozen Coho salmon (*Oncorhynchus kisutch)* were purchased directly from a commercial vendor and kept frozen until processing. Salmon were thawed, scaled, rinsed with fresh water and fileted. Edible meat (including skin) was cut from the carcass, chopped into small chunks, and placed on stainless steel trays for refreezing. The tissue pieces were placed in fluorinated ethylene propylene (FEP) bags and stored in liquid nitrogen (LN_2) vapor phase freezers (−140 °C to −190 °C).

Cryohomogenization and spiking

Salmon pieces (~0.5 kg) were poured into a large pre-cooled polytetrafluoroethylene (PTFE) disk mill and the tissue was cryohomogenized for 3 min using a rotary shaker [12]. A small amount of the resultant fresh-frozen powder was removed for screening and generating a matrix-matched calibration curve.

Custom spike solutions were prepared so that the final mass fractions of CV and LMG would be ~150 ng/g and ~3.5 ng/g, respectively. Neat CV and LMG were dissolved in a 50% acetonitrile solution and diluted to a final concentration of 29.73 μg/mL and 0.628 μg/mL, respectively. Approximately 2.5 g of the final spike solution was added to a clean plastic resealable bag and frozen upright at −80 °C. This pre-freezing step was included to limit isolated thawing of the material, which can occur when liquids are added to frozen homogenate, potentially compromising the integrity of the material and negatively impacting homogenization efficiency. The frozen spike was added to the milled homogenate in the large disk mill as a frozen block and homogenized three

times for 3 min each. Approximately 5 g of amended material was subsampled into eight clean 15 mL perfluoroalkoxy (PFA) jars, ensuring the material was thoroughly mixed between each sampling event. Jars were stored at LN_2 temperature until analysis and care was taken to protect the spike components from light at all times.

Tissue extraction

Extraction of ~2 g of spiked homogenate was performed to assess homogeneity according to AOAC published methods [11]. Stock solutions of each internal standard were prepared in acetonitrile at 40 μg/L and 100 μL of each internal standard solution was added by mass to the homogenate in a clean centrifuge tube. Samples were allowed to equilibrate for 15 min in the dark. Next, 500 μL of 9.5 g/L hydroxylamine hydrochloride solution was added and samples were allowed to incubate in the dark for 10 min. Each sample was extracted using 8 mL of acetonitrile and 1 g of anhydrous magnesium sulfate, vortexed for 1 min and allowed to mix on a rotary stirrer for 10 min. Samples were centrifuged at 2000 x g for 5 min at 4 °C and the supernatant was transferred to a clean tube. The solution was evaporated to dryness at 50 °C under a stream of N_2 and reconstituted in 800 μL of 1 g/L ascorbic acid in acetonitrile.

Instrumental methods

Extracts were analyzed by LC-MS/MS using a Vanquish UPLC system coupled to a Fusion Lumos Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer. Separation was achieved using an XDB-C18 column (1.0 mm id × 150 mm length, Santa Clara, CA, USA) at 40 °C. Mobile phase A (0.05 M ammonium formate, 0.25% formic acid) and mobile phase B (acetonitrile) were used with the following LC method at an injection volume of 10 μL and a flow rate of 150 μL/min: 40% B for 1 min, a ramp to 90% B over 18 min, hold at 90% B for 1 min, ramp down to 40% B and held for 6 min for a total of 25 min.

The liquid chromatography effluent was introduced to the mass spectrometer using a heated electrospray ionization (HESI) source operating at 3500 V. The sheath gas was set to 35 au, the auxiliary gas was set to 7 au, the ion transfer tube was held at 300 °C and the vaporizer was held at 275 °C. The MS1 was set at a resolution of 120,000, the RF lens was set at 50%, the AGC (automatic gain control) was set at 4.0×10^5 and the maximum injection time was 100 ms. The MS2 was set at a resolution of 30,000, the quad isolation was set to 2 Da, the normalized HCD collision energy was 50%, the AGC was set to 5.0×10^4 and the maximum injection time was 100 ms. Targeted ions for each compound of interest are shown in Table 1.

Quantification

A six-point matrix-matched calibration curve was generated using the sub-sampled homogenate (non-amended) by adding 2 g of spike solution at 0, 5, 10, 20, 50 and 100 ng/g, resulting in the addition of approximately 0, 0.5, 1, 2, 5 and 10 ng of CV and LMG. The calibration curve was extracted and analyzed with the experimental samples.

Data were blank corrected and quantification of CV and LMG in each of the samples was completed using Xcalibur software (Thermo Electron) using the matrix matched standard addition curves generated by integration of product ions from the respective compounds (Table 1).

Stability assessment

One year post-production, eight aliquots that had been stored in LN_2 vapor-phase freezers (−140 to −190 °C) were analyzed using the same extraction method in order to assess stability of the amended compounds. Extracts were analyzed by LC-MS/MS using a Vanquish UPLC system coupled to a Q Exactive Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) high-resolution accurate mass spectrometer. The separation was accomplished with an Agilent XDB-C18 column (Santa Clara, CA, USA) using the same mobile phases and temperature program as previously described. The liquid chromatography effluent was introduced to the mass spectrometer using a heated electrospray ionization (HESI) source operating at 3500 V. The sheath gas was set to 40 au, the auxiliary gas was set to 10 au, the ion transfer tube was held at 250 °C and the vaporizer was held at 300 °C. The MS2 was set at a resolution of 17,500, the quad isolation was set to 4 Da, the normalized HCD collision energy was 60%, the AGC was set to 2.0×10^5 and the maximum injection time was

Table 1 Target ions for aquaculture antibiotic and antibiotic metabolite identification

ID	Precursor Ion (m/z)	Product Ions (m/z)
CV	372.243	251.15, 328.19, 356.21, 372.24
d6-CV	378.280	257.19, 334.19, 362.25, 378.28
LMG	331.218	239.15, 272.14, 286.16, 301.17, 315.19
d6-LMG	337.255	239.15, 277.17, 291.19, 300.20, 320.22

Table 2 Result for quantitative assessment of CV and LMG after material amendment and approximately one year after amendment

Amendment			**Stability**		
Sample Name	[CV] ng/g	[LMG] ng/g	Sample Name	[CV] ng/g	[LMG] ng/g
1	111	1.21	1	114	1.18
2	127	1.24	2	116	1.15
3	117	1.34	3	111	1.17
4	115	1.26	4	118	1.13
5	118	1.20	5	123	1.14
6	114	1.27	6	118	1.11
7	121	1.24	7	118	1.20
8	110	1.23	8	128	1.29
Average/SD	116 ± 5	1.25 ± 0.04	**Average/SD**	118 ± 5	1.17 ± 0.06
% RSD	4.60	3.52	**% RSD**	4.55	4.73

100 ms. Integration of product ions from the compounds of interest was completed as previously described.

Results and discussion

The mass fraction of CV and LMG in replicate amended salmon samples was 110–127 ng/g (average 116 ng/g) and 1.20–1.34 ng/g (average 1.25), respectively (Table 2 'Amendment'). These values were within the range of the target spike mass fractions of CV (~150 ng/g) and LMG (~3.5 ng/g). Both values were slightly lower than expected, which may be the result of using an approximate mass for the starting homogenate in order to avoid thawing of the fine powder. The %RSD for CV and LMG was 4.6% and 3.5%, respectively, indicating that mass fractions of both analytes were homogenously distributed at the sample size analyzed.

Because the amended analytes are only mechanically and not biologically incorporated into the matrix, it is particularly important to monitor stability. The measured mass fractions of CV and LMG in replicate amended salmon samples were 111–128 ng/g (average 118 ng/g) and 1.11–1.29 ng/g (average 1.17 ng/g), respectively (Table 2 'Stability'). The % RSD of CV and LMG was 4.6% and 4.7%, respectively. Data generated during the initial custom amendment assessment and the stability assessment were compared using a common method [13] (Fig. 1). The results indicate that the cryogenic method used here to amend fish tissue with CV and LMG generated a homogenous product at both high-level and low-level spikes.

It is important to note that results may vary with different matrices, spike components and spike levels. Specifically, analytes that are particularly labile or mixtures that are difficult to homogenize may be more problematic than the example system employed here. As such, the success of this technique should be assessed for different matrices and spikes. The spike solvent used should also be carefully chosen so as not to drastically alter the material or negatively impact downstream analysis. Furthermore, it should be noted that exogenous compounds spiked into a matrix will not be biologically bound to molecules within the matrix itself and, as a result, may be extracted more easily than a naturally-incurred matrix. Though the material may not behave in the same way as experimental samples during analysis, the material would still be useful for harmonization of measurements across laboratories and analytical platforms, providing a sufficient alternative until an analogous CRM is completed, assuming full production is necessary.

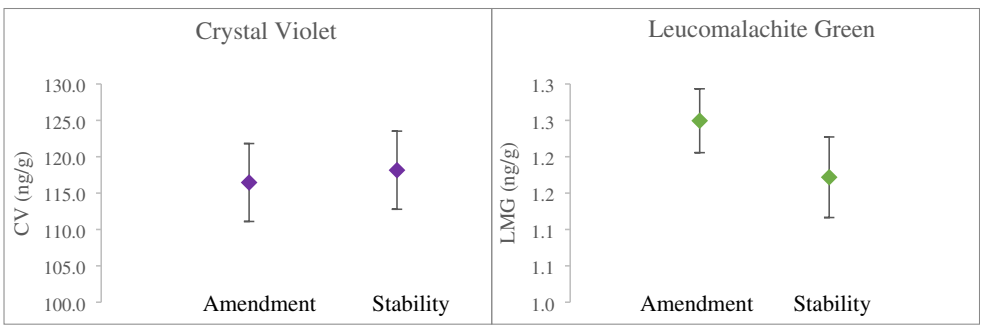

Fig. 1 Comparison of crystal violet and leucomalachite green mass fraction (ng/g) following the amendment procedure ('Amendment') and after one year of storage ('Stability'). Error bars show one standard deviation from the measured mass fraction mean

This concept could potentially be applied to larger RM production batches, particularly when naturally-occurring matrices cannot be found and there is a demand for larger amounts of material. However, production of an initial small-batch would be beneficial to determine the efficacy of the technique and to ensure the inherent properties of the matrix (moisture content, particle size, etc.) are not significantly altered as a result of the amendment. Changes in these properties may render the final RM less comparable to experimental samples.

Conclusions

The method employed here for cryogenic amendment of a biological matrix was successful based on target concentration accuracy, homogeneity and stability of the spiked analytes over a one year period, providing a promising technique for the production of small-batch, rapid-response custom RMs.

In order for this technique to have the most impact in rapid-response cases, close relationships and cooperation between experts in the given field and RM producers are imperative. Open and clear communication regarding material needs, relevant concentrations and downstream applications will provide a more robust workflow for efficient and successful production, integration of the materials into research and regulatory determinations. Advances in production like those described here are likely to initiate these types of collaborations moving forward.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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