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ARTICLE

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Certification of a fish otolith reference material in support of quality assurance for trace element analysis†

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Production and certification activities relating to the development of a new Certified Reference Material (FEBS-1) based on a fish otolith matrix are described. The material was prepared from 4.5 kg of dried saggital otolith harvested from the red snapper (*Lutjanus campechanus*), ball milled and sieved to <200 mesh, homogenized, irradiated, bottled into 1 g aliquots and certified for major, minor and trace element composition using inductively coupled plasma mass spectrometry (ICP-MS), isotope dilution-ICP-MS, neutron activation, ICP-optical emission spectrometry and X-ray fluorescence. Several laboratories participated in this activity, providing for a robust estimate of each measurand. Certified values for seven elements (Ba, Ca, Li, Mg, Mn, Na and Sr) are reported, accompanied by full GUM uncertainty estimates. Reference values for Cd, Cu, Ni, Pb and Zn are also provided; these elements were considered to be not certifiable because of the large component of uncertainty contributed by homogeneity. This CRM will be of interest to those laboratories requiring quality assurance of measurements of bulk elemental composition of otoliths and other marine aragonites.

Introduction

Aggressive management of fisheries resources has become important to many nations. As tools available to marine ecologists and fisheries scientists for this purpose diversify, the use of fish otoliths as a means of delineating species populations and even the environmental history of individual fish appears to occupy a useful niche.[1–3]

Otoliths, or fish "ear bones", consist of three pairs of small carbonate bodies located in the head of teleostean (bony) fish. The largest of these, the sagittae, are the most frequently studied. Otoliths are precipitated crystals containing varying amounts of proteinaceous organic matter (otoline) and calcium carbonate (principally aragonite). These microlaminate structures exhibit ring-like patterns as the larvae grow and incorporate constituent elements from their environment. Otolith composition is influenced by the water mass into which larvae are initially released (core regions), the water mass where the larva resided during the pelagic phase, and the water mass at the point of settlement. Campana[4] espouses that, to the extent that populations or stocks of fish inhabit different environments, the elemental composition of the otolith should serve as a proxy for population identity. Such compositional signatures have been used to identify the routes that larvae have traveled, from source population to settlement. As noted by Campana,[4] two assumptions underlie the use of the otolith elemental composition as a stock discriminator: material deposited on the otolith is metabolically inert after deposition and is not susceptible to resorption; and the physical and chemical environment influences the rate of trace element incorporation into the growing otolith surface. Elements under strong physiological regulation (*e.g.*, Na, K, S, P, Cl) probably do not meet the second assumption, and thus are of limited value for stock identification studies. However, both assumptions appear to be met with respect to elements such as Sr, Ba, Mn, Fe and Pb (and perhaps Li, Mg, Zn, Cu and Ni), in which both ambient element and Ca concentrations, as well as temperature, produce significant effects on otolith composition. Environmental responses such as these, recorded permanently in the otolith, imply that the concentrations of selected elements in the otolith can be used as a biological tag to discriminate among groups of fish which have spent at least part of their lives in different environments. Discriminatory power is increased greatly by treating the selected elements as a group, using multivariate statistics, rather than individually, leading to characterization by "elemental fingerprint". Campana[4] provides an extensive review of the physiology and use of otolith chemistry for these purposes. A comprehensive review of the field of otolith composition and chemistry is provided in ref. 5. An in-depth examination of the value of elemental fingerprints as natural tags for fish is presented in ref. 1.

Two forms of such elemental fingerprinting are in general use: one based on whole dissolved otoliths, and the other based on analysis of the otolith core. Advantages of whole-otolith assays include ease of preparation, absence of error associated with sampling or identifying growth increments, and the

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† Availability: This CRM is available from the NRC INMS (http://inms-ienm.nrc-cnrc.gc.ca/calserv/chemical_metrology_e.html). Address correspondence to R. Sturgeon.

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availability of accurate and precise assay protocols. The major disadvantage is associated with the inability to take advantage of the chronological growth sequence recorded in the otolith. Thresher *et al.*[6] and Edmonds *et al.*[7] were amongst the first to highlight stock identification through a comparative study of the trace element composition of otoliths. Elemental analysis of otoliths presents a challenging problem as many elements of interest are present at trace or ultra-trace levels and the calcium matrix introduces additional difficulties unless some form of chemical separation is undertaken. Currently, isotope dilution inductively coupled plasma mass spectrometry appears to be a method of choice and several successful reports of its use for the accurate and precise characterization for otolith material have appeared.[7–11] However, research in this area has generally been impeded by a lack of certified reference materials for analytical quality assurance. The National Institute for Environmental Studies (NIES), Environment Agency of Japan, recently released a fish otolith reference material certified for Na, Mg, K, Sr and Ba for this purpose,[9] but there remains a need not only for complementary materials, but for materials which are certified for additional trace heavy metals that can serve as additional markers to extend the usefulness of otolith composition for fisheries studies. The preparation of a new fish otolith Certified Reference Material to support fisheries research is detailed in this work.

Experimental

Sourcing of raw material

Saggital otoliths were procured from adult (0.5–10 kg) red snappers (*Lutjanus campechanus*) captured in the Gulf of Mexico by commercial fishermen and landed at Cameron, Louisiana, USA, during 1999. At Old Dominion University, the otoliths were extracted from the fish through the opercular cavity by first exposing the otic capsule with a stainless steel chisel and then removing the otoliths from the head with stainless steel tweezers. After removal, otoliths were immediately rinsed in water to remove adhering tissue and then returned to the lab where they were rinsed in DI water, air-dried and then stored, in bulk, in polyethylene bottles. Surface contamination was removed by initially rest-rinsing all otoliths in 2% ultrapure HNO_3 for 5 minutes, followed by triple-rinsing in ultrapure water, in a class 100 cleanroom. Otoliths were then sonicated in ultrapure water for 10 minutes, triple-rinsed again in ultrapure water and allowed to dry under a laminar flow hood for 72 hours. Finally, otoliths were transferred to acid-washed polyethylene bottles for storage.

Otolith preparation

Approximately 4.5 kg of the dried otoliths were forwarded to facilities at the National Institute for Standards and Technology (NIST) for further processing. As received, the otoliths were intact, measuring ~2–3 cm and composed almost entirely of calcium carbonate. Due to the presence of discrete annuli in the otoliths, it was important to produce a material of sufficiently small grain size so that sample-to-sample variations could be minimized. The otoliths could not be jet milled nor crushed with a Teflon puck mill as significant amounts of Teflon were eroded from the mill during the process. Ultimately, a new ball mill assembly (stainless steel shell lined with alumina ceramic and alumina balls) was used for the crushing. The otoliths were processed in small lots, being ground for 90 minutes to avoid heating of the sample during crushing. The resulting powder was sieved through 100 and 200 mesh stainless steel sieves (150 μm and 75 μm particle sizes, respectively). The 100+ and 200+ fractions were returned separately to the mill and ground until all material passed the 200 mesh sieve. Each lot, subsequent to sieving, was remixed by hand rolling after all the material was finer than 200 mesh.

A total of 4.518 kg of <200 mesh otolith powder was produced and placed into two large glass jars. The material was then subjected to irradiation for sterilization (Neutron Products), wherein a minimum dose was 25 kGy and the measured absorbed dose was between a minimum of 30.3 kGy and a maximum of 38.1 kGy. A portion of the total mass was then bottled into 650 units containing 1 g aliquots in 13 ml screw-capped brown glass bottles. Each of the bottles was then heat sealed in a trilaminate foil pouch to ensure no exchange of atmosphere with its immediate environment and placed into storage at room temperature.

With the exception of Li and Mn, homogeneity studies were conducted by ICP-OES and based on an ANOVA of data generated from three replicate 0.25 g sub-samples drawn from each of 13 randomly selected bottles of material. For Li and Mn, measurements were completed by ICP-MS based on results from 8 independent bottles. A collaborative analysis approach to the certification was selected, involving NRC and expert laboratories from NIST and Old Dominion University. The analytical techniques applied included isotope dilution ICP-MS, ICP-OES, INAA and XRF.

A correction factor for moisture content was determined by drying separate test samples in an electric oven for 3 hours at 105 °C. The dry weight correction factor was 0.99684 ± 0.00050 (*i.e.*, $<1\%$ moisture content).

Sample preparation and analysis

ID-ICP-MS at NRC. Samples were prepared for analysis by isotope dilution-ICP-MS in a class-10 or class-100 clean room environment. Twelve sub-samples of otolith, approximately 0.25 g each from different bottles, were weighed into individual pre-cleaned screw capped conical polypropylene centrifuge tubes. After addition of suitable amounts of enriched isotopic spikes, 1 ml of nitric acid and 0.2 ml of H_2O_2 were added. Similarly, three sample blanks were prepared. The tubes were heated under an IR lamp overnight to ensure complete dissolution and then the samples were diluted to 50 ml with DIW. For most elements, a further 10-fold dilution of the digests with 0.1 M HNO_3 was implemented prior to *direct* analysis. The reference/spike isotope pairs used were $^{7}Li/^{6}Li$, $^{26}Mg/^{25}Mg$, $^{60}Ni/^{61}Ni$, $^{63}Cu/^{65}Cu$, $^{66}Zn/^{68}Zn$, $^{88}Sr/^{86}Sr$, $^{114}Cd/^{111}Cd$, $^{138}Ba/^{137}Ba$ and $^{208}Pb/^{207}Pb$. In all cases, calibration of enriched spikes was achieved by reverse spike isotope dilution referenced against two independently prepared solutions of natural abundance material. Enriched isotopic standards were obtained from Isotec Inc. (Miamisburg, OH, USA) in the form of the carbonate salt (for Li, Sr and Ba), oxide (Mg and Ni) or pure metal (Cd, Cu, Zn and Pb); high purity natural abundance materials were obtained from Alfa Aesar. All single element stock solutions were gravimetrically prepared by acid dissolution of the metal or its salt followed by appropriate dilution with DIW.

To efficiently separate spectroscopic matrix interferences observed on selected isotope pairs, sample solutions were diluted 20-fold with DIW and then analyzed for Cu, Ni, Zn, Cd and Pb following on-line matrix removal using FIAS-400 flow injection column separation of the analytes. Raw data were processed off-line using in-house software for peak area quantitation based on $^{60}Ni/^{61}Ni$, $^{63}Cu/^{65}Cu$, $^{66}Zn/^{68}Zn$, $^{114}Cd/^{111}Cd$ and $^{208}Pb/^{207}Pb$ ratios, from which the analyte concentrations in the otolith were calculated.[11] Manganese [^{55}Mn] was quantitated using standard additions calibration.

A PerkinElmer SCIEX ELAN 6000 (Concord, Ontario, Canada) ICP-MS equipped with a Gem cross-flow nebuliser and an alumina sample injector tube was used. A double pass Ryton® spray chamber was mounted outside the torch box and maintained at room temperature. Optimization of the ELAN 6000 and implementation of dead time correction were performed as recommended by the manufacturer. A PerkinElmer (Norwalk, USA) FIAS-400 MS flow injection accessory was used for on-line matrix separation of several analytes based on use of Toyopearl AF-Chelate 650M iminodiacetate resin (Tosohaas, Montgomeryville, PA, USA). Details of the procedure have been presented elsewhere.[11]

External calibration ICP-MS at Old Dominion University

Dilute nitric acid was prepared from the Seastar ultra-pure concentrated acid (Sydney, BC, Canada) using Milli-Q 18 MΩ cm DIW (Millipore, Bedford, MA, USA). Natural abundance multi-element calibration standard solutions (10 μg g^{-1}) for all elements except Ca and Sr were prepared from high purity single-element stock solutions at (High-Purity Standards, Charleston, SC, USA). Calcium and Sr standard solutions (10 μg g^{-1}) were prepared by dissolving carbonates of the enriched isotopes of ^{48}Ca and ^{86}Sr (Oak Ridge National Laboratory, Oak Ridge, Tennessee). Since the enriched ^{48}Ca and ^{86}Sr carbonates contain trace amounts of impurity elements (particularly Zn), the concentrations of all elements in the multi-element calibration standards were quantitated against single element standards before use.

Six replicates along with a method blank were prepared for each individual sample. For each replicate, a 100 mg sub-sample was accurately weighed into a 15 ml Savillex Teflon jar and dissolved overnight using 4 ml of concentrated HNO_3. The dissolved sample solutions were transferred to 125 cm^3 Nalgene polyethylene bottles and diluted to 100 g with 1% HNO_3 (v/v). All calibration standards and sample solutions were spiked with 2 ng g^{-1} In, which served as an internal standard with which corrections for instrument drift and sample matrix effects were facilitated.

A Finnigan Element 2 double focusing sector field (SF)-ICP-MS (Bremen, Germany) was used, operating under upgraded Version 2.354 software. Sample introduction was achieved using a self-aspirating PFA microflow nebulizer (50 μL min^{-1}). Following optimization of the instrument, calibration curves were obtained and samples were run in the following cycle sequence: acid calibration blank (0.15 M HNO_3), control standard, samples 1–8, acid calibration blank, control standard, samples 9–16, *etc*. The isotopes ^{7}Li, ^{25}Mg, ^{55}Mn, ^{85}Rb, ^{89}Y, ^{111}Cd, ^{137}Ba, ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{157}Gd, ^{163}Dy, ^{165}Ho, ^{167}Er, ^{208}Pb and ^{238}U were measured in low resolution mode, whereas ^{23}Na, ^{31}P, ^{48}Ca, ^{45}Sc, ^{51}V, ^{52}Cr, ^{56}Fe, ^{63}Cu, ^{66}Zn and ^{86}Sr were measured at medium resolution. No on-line concentration calculations were made. Count rates (ASCII text format) were collected using the instrument software and all subsequent data manipulation was performed off-line using commercial spreadsheet software on a personal computer. Background correction for a particular sample was based on the average response from the nearest two acid calibration blanks run before and after the sample. Instrument drift and matrix effects were corrected for using In as an internal standard. Sample solution concentrations were derived from the external calibration curve.

External calibration and standard additions by ICP-OES at NRC

Sample preparation was performed in a clean laboratory providing a class 100 environment. Nominal 0.25 g sub-samples were weighed into Pyrex beakers and slurried with 5 ml of DIW. A 10 ml volume of 20% high-purity HNO_3 was then added to each sample and three blanks. The beakers were covered with watch glasses and the samples refluxed overnight under an IR heat lamp. After cooling, samples and blanks were transferred to pre-cleaned polyethylene screw capped bottles using DIW and stored until analysis.

Quantitation of Ba, Ca, Mg, Na and Sr was performed by ICP-OES using a PerkinElmer OPTIMA 3000XL (radial view). Both external standard calibration and the method of additions were employed. Method validation was ensured by inclusion of NIES 022 fish otolith certified reference material (National Institute for Environmental Studies, Ibaraki, Japan) as a quality control sample. Stock solutions of calibration standards were prepared gravimetrically from high purity carbonates of Ca, Ba and Sr (99.99%, Alfa Aesar), NaCl (99.999%, Alfa Aesar) and high purity Mg metal (characterized in-house by GD-MS). Dissolution was achieved using sub-boiled distilled high purity nitric acid, prepared in-house, and high purity distilled deionized water (DIW, 18 MΩ cm) obtained from a NanoPure mixed bed ion exchange system fed with reverse osmosis domestic feed water (Barnstead/Thermolyne Corp, IA, USA).

INAA at NIST

INAA was used for the determination of Al, Ca, Co, Fe, Hf, Mn, Na, Sb, Sc, Se, and Zn, based on 100 mg sub-samples. NIST SRM 1566b Oyster Tissue and SRM 1577b Bovine Liver served as quality control materials. All samples were manipulated in glass bottles. For the assay of Al *via* (^{38}Al), Ca *via* (Ca), Mn *via* (^{56}Mn) and Na *via* (^{24}Na), samples, controls, and standards were irradiated for 30 s in the NIST pneumatic tube facility, RT-1, at 20 MW reactor power. SRM 1577b was used as a control for determination of Al, Ca, Mn and Na. After a decay time of 5 min and 10 min, the samples, controls and standards were counted on the "Senem" and "Isabel" detectors at 20 cm and 14 cm. For determination of Co, Fe, Hf, Sb, Sc, Se and Zn *via* (^{60}Co, ^{59}Fe, ^{181}Hf, ^{124}Sb, ^{46}Sc, ^{75}Se, and ^{65}Zn), samples, controls and standards were irradiated for 3 + 3 hours in the NIST pneumatic tube facility RT-1 at 20 MW reactor power. The irradiation container was flipped in the reactor after the first three hours. SRMs 1566b and 1577b were used as controls. After a decay time of three weeks, bags were removed and the samples were re-weighed, if necessary. Samples, controls and standards were counted on the up-looking "Linda" detector at 2 cm for 3–8 h.

The μMCA was operated under a μVAX 3400 computer-controlled data acquisition and collection system. The ND peak search, GUI, MEAN and activation analysis software was used for peak integration and the calculation of elemental concentrations. The quantitative evaluation was accomplished by the comparator method.

Gamma energy and half-life data of the nuclides were taken from Erdman and Soyka.[12] Multi-element solutions and Fe-foil were used as standards.

XRF at NRC

Two sub-samples of nominal 250 mg mass were taken from each of three random bottles, fused in Pt–Au crucibles (5% Au) by heating to 900 °C (for 20 min) in 10 g of a flux consisting of $Li_2B_4O_7 : Na_2B_4O_7 : Na_2CO_3$ (2 : 2 : 1). A glass button of approximately 28 mm diameter and 5 mm thick was then poured and cooled.

Analyses were performed with a Brüker-AXS Model SRS3400. The instrument was operated in vacuum mode at 50 kV and 81 mA with no filter. A LiF 200 crystal was used in conjunction with a medium collimator having an aperture of 0.46 degrees. A flow counter detector was employed. The peak was measured at 113.014 degrees 2θ; background was measured at 115.067 degrees 2θ. A 23 mm diameter mask isolated the central portion of the sample button for excitation.

A four point external calibration curve was prepared, bracketing the sample concentration, by fluxing known masses of high purity $CaCO_3$ (Anachemia).

Table 1 Uncertainty components

	Ba (ppm)	Ca (%)	Li (ppm)	Mg (ppm)	Mn (ppm)	Na (ppm)	Sr (ppm)
u_{char}	0.039	0.62	0.0211	0.395	0.0060	72.9	21
u_{hom}	0.108	0.35	0.0055	0.535	0.0055	23.5	34
u_c	0.115	0.717	0.022	0.665	0.0081	80.3	40
U ($k = 2$)	0.23	1.4	0.044	1.33	0.016	161	79

Table 2 Certified values of major, minor and trace elements

Element	Certified value[a]	Technique
Ba (ppm)	5.09 ± 0.23	ID-ICP-MS, ICP-MS, ICP-OES
Li (ppm)	0.305 ± 0.044	ID-ICP-MS, ICP-MS, ICP-OES, INAA
Mg (ppm)	23.6 ± 1.3	ID-ICP-MS, ICP-MS, ICP-OES, INAA
Mn (ppm)	0.686 ± 0.016	ICP-MS, INAA
Na (ppm)	2594 ± 161	ICP-MS, ICP-OES, INAA
Sr (ppm)	2055 ± 79	ID-ICP-MS, ICP-MS, ICP-OES
Ca (%)	38.3 ± 1.4	ICP-MS, ICP-OES, INAA, XRF

[a] Mean ± expanded combined uncertainty; dry weight amount content; minimum sample size of 250 mg.

Results and discussion

Homogeneity

The homogeneity components of the uncertainty in the certified values were derived according to recommendations of Ellison *et al.*[13] ANOVA was applied to the data generated by the ICP-OES analysis of 39 sub-samples from 13 randomly selected units of the bottled material and by ICP-MS analysis of 24 further sub-samples from 8 bottles (for Li and Mn measurements). For Ba, Li, Mg, Sr and Ca, the inhomogeneity contribution to uncertainty, u_{hom}, was equated to the experimentally determined between-unit standard deviation ($s_{between}$) as the best estimate of the uncertainty due to between-unit heterogeneity. However, for Mn and Na, the situation depicted by eqn. (1) occurred:

$$s^2_{between} < s^2_{meas}/n \qquad (1)$$

wherein s_{meas} is the repeatability standard deviation for the method used in the homogeneity assessment and n is the number of replicates per unit. For these cases, u_{hom} was calculated according to:[14]

$$u_{hom} = (\mathrm{MS}_{within}/n)^{1/2}\,(2/\upsilon_{\mathrm{MSwithin}})^{1/4} \qquad (2)$$

wherein MS_{within} represents the mean squares within groups and $\upsilon_{\mathrm{MSwithin}}$ is the number of degrees of freedom. Table 1 summarizes the resulting uncertainty components for homogeneity for each element at the 250 mg sub-sample size used for the testing.

Stability

As a result of interest in only the *total* amount content of the major, minor and trace elements, coupled with the chemical and physical properties of the matrix, the need for any rigorous stability testing of this material under its conditions of storage was obviated and, as such, contributions to a specific long-term stability component of the uncertainty were not included in the evaluation of the combined uncertainty presented in Table 1.

Certification of amount content

Certification of reference materials by NRC requires that data be generated using a primary method of analysis with confirmation by other methods; through the use of two or more critically evaluated independent methods, or based on one method at NRC and different methods by collaborating laboratories. These criteria were met for Ba, Li, Mg, Mn, Na, Sr and Ca, these being the only elements deemed to be sufficiently homogeneous within the population to permit a fit for purpose certification. Certified amount contents are summarized for these elements in Table 2. These values were derived from the unweighted mean of means of the data sets obtained with each of the instrumental/calibration approaches used. The associated characterization uncertainties (u_{char}) were estimated as the standard deviations of the means.[13,15]

All uncertainties indicated are expanded uncertainty ($U_c = ku_c$) where k is a coverage factor equal to 2 and u_c is the combined uncertainty propagated from the individual components. Combined standard uncertainties on the certified results were obtained by propagating together individual uncertainty components associated with characterization and homogeneity, in accordance with the ISO/GUM guide.[16]

Significant heterogeneity was observed for many other elements, precluding their certification. For a number of such elements, summarized in Table 3, a range of concentrations is presented which simply summarizes both the highest and lowest concentrations of the element determined in any given sub-sample by the primary ID-ICP-MS technique, along with corroboration by another methodology. These data may be of interest to users/readers and hence are included in this study. Typical relative standard deviations arising from replicate analyses of sub-samples taken from various bottles range from 15% for Cu to 63% for Cd. It is not clear whether this inhomogeneity is a characteristic of the material under study or was induced by contamination as a result of its handling/preparation. It may be noteworthy that reference values provided for Cu, Zn, Cd and Pb in the NIES otolith CRM[9] are approximately one order of magnitude lower than in the

Table 3 Information values for amount content of additional elements

Element	Range[a]	Technique
Cd (ppb)	1.4–3.2	ID-ICP-MS, ICP-MS
Cu (ppm)	4.2–6.8	ID-ICP-MS, ICP-MS
Ni (ppb)	16–29	ID-ICP-MS
Pb (ppm)	0.40–0.77	ID-ICP-MS, ICP-MS
Zn (ppm)	3.2–6.3	ID-ICP-MS, INAA

[a] Extreme values of concentrations obtained during sample characterization.

material under study, possibly reflecting a differential accumulation of these elements from a different water body, or contamination due to processing.

Validation and traceability of the certified values to the SI

Quality control samples incorporated into these measurement studies included NIST SRM 1566b Oyster Tissue, SRM 1577b Bovine Liver as well as NIES 022 fish otolith. Validation of the data through application of either a primary reference ratio method such as ID-ICP-MS and/or a well characterized methodology such as INAA, coupled with full uncertainty statements calculated according to ISO guidelines, provides traceability to the SI through standards prepared at NRC and NIST.

Conclusions

This new material (christened FEBS-1), certified for several major, minor and trace constituents, will serve to complement the availability of NIES No. 022 otolith and represents the first CRM of this kind wherein value assignment of amount content of the elements of interest has been established in accordance with the GUM. FEBS-1 will be of interest to those laboratories requiring quality assurance of measurements of bulk elemental composition of otoliths and other marine aragonites.

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