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A Simplified Digestion Protocol for the Analysis of Hg in Fish by Cold Vapor Atomic Absorption Spectroscopy

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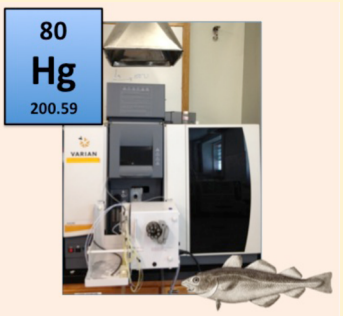
Supporting Information

ABSTRACT: Analysis of mercury in fish is an interesting problem with the potential to motivate students in chemistry laboratory courses. The recommended method for mercury analysis in fish is cold vapor atomic absorption spectroscopy (CVAAS), which requires homogeneous analyte solutions, typically prepared by acid digestion. Previously published digestion protocols require multiple acids, long heating periods or harsh conditions, and reducing agents for workup. We developed a simplified protocol for the digestion of fish that requires only nitric acid and a short heating period. This protocol was successfully implemented in an instrumental analysis lab and enabled students to perform CVAAS on commercial fish samples within the time constraints of an undergraduate lab course.



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The analysis of mercury in environmental samples is a long-standing problem of great interest to chemists[1] and the general public.[2] Elevated levels of mercury in living environments have adverse effects on the development of fish and other wildlife and can be toxic depending upon the species and concentration. A small amount of mercury contamination in fish and plants eventually affects species in the higher tiers of the food chain through biomagnification.

Concern about mercury levels in many types of seafood has been expressed by government agencies including the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA)[2a,3] as well as in mass media.[2b,c] The health effects of overconsumption of mercury are grave,[4] so much so that government agencies establish recommended consumption limits for children and pregnant women.[5] The FDA has also published the results of long-term studies of mercury levels in commonly consumed species of fish.[6]

The potential for mercury contamination and consumption[7] to pique student interest in class has been documented many times in the literature.[8] Mercury analysis methods successfully used in undergraduate laboratories to analyze environmental or food samples include complexometric titrations,[9] colorimetric or fluorescent sensing,[8c,10] and atomic spectroscopy.[11]

Despite the particular importance of mercury levels in fish and seafood, only a few procedures for use in teaching laboratories have been described. Jenkins and Rice used a direct mercury analyzer with a mercury graphite furnace to analyze canned fish and environmental samples.[11d,f] Cizdziel reports the coupling of a direct mercury analyzer with a cold vapor atomic fluorescence spectrometer for analysis of a variety of environmental samples, including fish.[11c] Niece and Hauri described an environmental chemistry lab in which cold vapor atomic absorption spectroscopy (CVAAS) is used to measure the mercury in fish.[11e]

Cold Vapor Atomic Absorption Spectroscopy for Hg Analysis

CVAAS (or CV atomic fluorescence spectroscopy) is the EPA recommended method for analysis of mercury in fish. This method has better reproducibility than graphite furnace AAS and a significantly lower cost than inductively coupled plasma (ICP). In CVAAS, mercury species in a sample are reduced to elemental mercury, which is volatilized and carried by a stream of inert gas into an absorption cell in the spectrometer. The limit of detection (LOD) is typically in the ppb range, and the required sample volume is approximately 10 mL; thus, small sample sizes and dilute standards can be analyzed, which reduces contamination risks. The use of appropriate personal protective equipment (PPE) and ventilation further limits the possibility of exposure.

The lag in the development of undergraduate laboratories to analyze mercury in fish by the EPA-recommended method could be due in part to the expense of instrumentation for CVAAS. Flame AAS is a technique used throughout the upper-level laboratory curriculum, but many departments do not assume the cost of a vapor generator for potential use in only a few CVAAS experiments. This issue was recently addressed by Niece and Hauri,[11e] who constructed a cold vapor generator through the clever use of common, relatively low-cost laboratory equipment. Their apparatus is compatible with any

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Table 1. Digestion Methods for Fish Samples for Hg Analysis

Method	Acid(s)	Digestion Time	Heat	Final $[H_3O^+]$
A	HNO_3	15 min	steam bath	60% v/v
B	$HNO_3/HCl/H_2SO_4$	15 min	steam bath	40%/4%/20% v/v
C	HNO_3	24 h	room temp.	60% v/v

flame AA spectrometer and expands the utility of this common instrumentation without a significant investment in specialized equipment.

Fish Sample Digestion Protocols

Another barrier to incorporating CVAAS in the curriculum is the sample digestion. Digestion of fish samples releases mercury ions from the organic matrix and yields a homogeneous solution. Typical digestion protocols utilize concentrated acids (HNO_3, H_2SO_4, HCl, $HClO_4$) and sometimes additional oxidizing agents ($KMnO_4$, V_2O_5, H_2O_2) to oxidize the organic compounds and release mercury species.[1,3,12] Depending on the reagent mixtures, the digestion may be improved by heating at reflux, heating under pressure, microwave irradiation, or lengthy digestion periods. After digestion, a reducing agent (often hydroxylamine hydrochloride) is added to consume any excess oxidizing agent.

The reagent combinations have been developed not only to effect complete release of mercury, but also to ensure negligible volatilization losses. The presence of solubilizing anions, along with an oxidizing environment, promotes the formation of nonvolatile mercury species. Methods requiring several of the common acids and oxidizing agents have been published, some with long heating periods. In an undergraduate lab, for which preparation and class time are often limited, these multireagent, time-consuming protocols are a barrier to performing mercury analysis.

Simplified Protocol for Fish Sample Preparation

Inspired by literature reports,[12a,e,13] we designed simplified digestion protocols and studied them using canned fish. The most promising protocol was validated through spike recovery experiments and used to measure total mercury levels in a variety of commercial fish.

The simplified digestion protocol was implemented as part of a CVAAS experiment in an instrumental analysis lab. In this course, junior and senior chemistry/biochemistry majors work in teams of four as they rotate through a variety of modules, including one covering AAS. This experiment provided students with hands-on experience in the application of atomic spectroscopy to an important problem in environmental and food chemistry. In addition to exposing students to the cold vapor technique through this lab activity, instructors can easily shift the emphasis to support different curricular goals by focusing on method validation, statistical analysis, instrument optimization and sensitivity, or environmental chemistry.

EXPERIMENTAL SECTION

Materials

Fish samples were obtained from local supermarkets and the on-campus food service. Nitric acid, sulfuric acid, hydrochloric acid, and stannous chloride (Fisher Scientific, ACS grade) were used as received. A 1000 ppm Hg standard (Fisher Scientific) in 5% HNO_3 was diluted to prepare calibration standards as described in the Supporting Information. Glassware was acid-washed prior to use.

Instrumentation

Absorbance measurements were recorded using a Varian AA240 atomic absorption spectrometer with VGA-77 cold vapor generator and a quartz cell (detailed procedure in the Supporting Information). Absorbance measurements (253.7 nm) were performed in triplicate with a preread delay of 60 s using Varian SpectrAA software.

Methods

Protocol Selection. Fish samples were homogenized and stored frozen, then defrosted prior to use.[14] Simplified digestion protocols were tested by analysis of homogenized canned tuna and salmon. As detailed in Table 1, an accurately weighed sample of approximately 2 g of fish or homogenized fish was digested in the concentrated acid(s) in an Erlenmeyer flask covered by a small watch glass or a poorly fitting glass stopper, with or without heating over a steam bath. The digested sample was cooled to room temperature, filtered if necessary, and accurately diluted with deionized water in a 50 mL volumetric flask.

Spike Recovery. An approximately 2 g sample of homogenized canned salmon was accurately weighed and added to an Erlenmeyer flask. An appropriate aliquot of a mercury standard solution was added to achieve the desired spike concentration. The samples were digested according to method A (Table 1).

HAZARDS

The acids, stannous chloride, and Hg standard solutions must be dispensed and used under a fume hood while wearing appropriate PPE, including gloves, safety glasses, and lab coat or apron. Concentrated nitric acid is corrosive, toxic, and an oxidant, and stannous chloride is corrosive, toxic, and an irritant; they should be handled while wearing gloves. During the digestion step with nitric acid, the reaction vessel must have an outlet to avoid an explosion hazard and must be under a fume hood to avoid inhalation of the evolved gases. Add acid to water when diluting digestion mixtures.

RESULTS AND DISCUSSION

Digestion Protocols

Our criteria for a simplified digestion method included (1) a minimal number of required reagents to shorten prep, (2) decreased heating temperatures and times, and (3) no specialized glassware or complicated techniques. Voegborlo has published protocols using pyrex test tubes in place of custom flasks or Teflon crucibles but requiring a mixture of nitric, sulfuric, hydrochloric, and perchloric acids at 200 °C.[12a] Evans, Johnson, and Leah describe a protocol that uses only nitric acid, with digestion at room temperature followed by reflux for 4 h.[12e] Rahimi et al. report heating samples on a steam bath until a homogeneous solution is achieved.[13] These methods were validated by spiking or comparison with standard reference materials (SRMs).

In this context, we chose to evaluate the three methods in Table 1.[15] We performed each digestion in a loosely covered

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Erlenmeyer flask to prevent loss of mercury and avoid significant concentration. For method A, the sample was digested in nitric acid with a short period of heating on a steam bath. Method B[13] utilized a mixture of nitric, hydrochloric, and sulfuric acids, with the same heating period, to determine whether additional strong acids are necessary for mercury retention. We also wondered whether adequate sample digestion could be achieved without heating if digestion time increased (method C); elimination of the heating step could be advantageous for undergraduate courses.

Samples of canned solid salmon and canned chunk tuna digested by methods A and B were generally homogeneous after heating. For some, a small amount of precipitate formed upon cooling (before dilution), which could be removed by filtration.[16] Filtration through a fritted funnel is required for method B, while filter paper is sufficient for method A. The sample prepared by method C (no heating) contained a much larger amount of very fine solid, which resulted in a tedious and always necessary filtration step. Method A is the most attractive from a procedural standpoint and was selected for further study.

Table 2 displays the measured levels of Hg in canned solid salmon and chunk tuna digested by method A. The measured mercury concentrations are consistent with the averages reported by a multiyear FDA study of many samples of these fish. The LOD for our analysis ranged from 0.05−0.5 μg L^{-1} in the analyte solution (often ∼0.1−0.2 μg L^{-1}). Assuming 2 g of fish dissolved in a total volume of 50 mL, this translates to detectable concentrations of ∼0.001−0.01 μg of Hg per gram of fish.

Table 2. Total Hg Concentration in Canned Fish Samples[a] Digested by Method A

Fish	[Hg] (μg g^{-1})	Mean from FDA[b] (μg g^{-1})
chunk tuna	0.23 ± 0.01, n = 3	0.128 ± 0.141, n = 551
solid salmon	0.15 ± 0.01, n = 3	0.008 ± 0.017, n = 34[c]

[a]Source information for fish is in the Supporting Information. [b]FDA reported mean, standard deviation, and sample size.[6] [c]FDA value for salmon reflects only methylmercury concentration.

Samples of homogenized canned salmon were spiked with concentrations of mercury between 0.0100−0.0800 mg L^{-1} and digested by method A. The average percent recovery values were greater than 91%, with standard deviations of at most 9% (most less than 5%, see Supporting Information). Digestion by method A does not result in significant loss of mercury by volatilization.

To test applicability, we digested a variety of commercial fish by method A and measured the total mercury concentrations (Table 3). All measured values were larger than the minimum measured value from the FDA for the relevant species; the average values fall within the range reported by the FDA.[6] The large standard deviations reported by FDA from the multiyear study are a useful point of discussion to engage students in careful comparison of results from different sources.

Classroom Implementation

We incorporated our digestion protocol into an experiment for instrumental analysis laboratory, which meets once a week for 4 h. Students work in pairs or groups of four to complete an experiment over the course of two lab periods. They performed the sample digestion during the first period and prepared standards and recorded measurements during the second period.

Students were encouraged to bring fish samples of interest, and the instructors provided some prehomogenized samples. Each group successfully performed the digestion and sample dilution for three different fish types, prepared Hg standards for a calibration curve, and analyzed their samples using AA spectroscopy with a cold vapor generator. The generator premixes the reducing agent and sample solutions in a reaction coil and feeds the mixture into a gas−liquid separator, where a stream of inert gas carries the vapor into the cell. Given the variables inherent to the digestion and reduction, students prepared three samples for each fish type and measured each of the three samples in triplicate; measurements were corrected with a method blank. The replicates helped students identify any measurements for exclusion due to operator error.

Figure 1 and Table 4 display typical student results. The calibration curves were linear, and the analyses had LOD values similar to those measured by the authors (∼0.3 μg L^{-1}, Figure 1). The sample concentrations were measured with acceptable

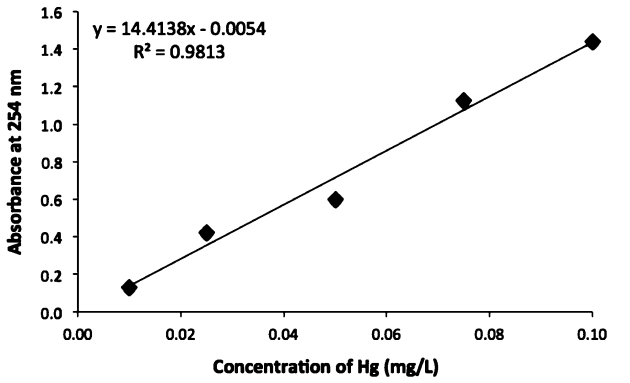

Figure 1. Calibration curve for CVAAS analysis of Hg in fish measured by students in instrumental analysis laboratory. The LOD is 0.3 μg L^{-1} in the analysis solution.

Table 3. Average Total Hg Concentration (μg g^{-1}) in Commercial Fish by CVAAS[a]

Fish Type	Origin/Harvest/Preservation	[Hg] (μg g^{-1}), sample size	Mean [Hg] from FDA[6] (μg g^{-1})
basa (swai)	unavailable/farmed/fresh	0.018 ± 0.005 n = 2	Not reported
snapper	unavailable/farmed/fresh	0.028 ± 0.001, n = 2	0.166 ± 0.244, n = 67
shark	USA/unavailable/fresh	0.025 ± 0.003, n = 3	0.979 ± 0.526, n = 356
swordfish	Indonesia/wild/frozen	0.23 ± 0.02, n = 4	0.995 ± 0.539, n = 636
tuna from sushi	unavailable/wild/fresh	0.064 ± 0.002, n = 3	0.391 ± 0.266, n = 420
tuna steak	Thailand/wild/frozen	0.52 ± 0.09, n = 2	0.415 ± 0.308, n = 120

[a]LOD = 0.3 μg L^{-1} in analysis solution; for ∼2 g of fish dissolved in 50 mL, LOD ≈ 0.008 μg g^{-1}.

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Table 4. Average Concentration of Total Hg in Fish Measured by Students in Instrumental Analysis Lab[a]

Fish Type	[Hg] (μg g^{-1}) (n = 3)	[Hg] (μg g^{-1}) from FDA, sample size
tuna from sushi	0.076 ± 0.005	0.415 ± 0.318, n = 120
albacore tuna steak	0.055 ± 0.004	0.358 ± 0.138, n = 43
swordfish steak	0.12 ± 0.09	0.995 ± 0.539, n = 636

[a]LOD = 0.3 μg L^{-1} in analysis solution; for ∼2 g of fish dissolved in 50 mL, LOD ≈ 0.008 μg g^{-1}.

precision and were consistent with the ranges reported by the FDA.

Students were generally interested in the issue of Hg in fish and were especially curious about the analysis of tuna from sushi sold in the on-campus dining facility. The analysis of a sample relevant to their lives helped them consider the public health implications of mercury pollution and, on a practical level, to consider what species of fish they choose to eat. Aside from students' comments and questions during class, their varied personal interests in this topic were reflected in the content of their lab reports. Several students discussed sources of mercury pollution in the environment; others focused on the mechanisms by which mercury damages the human body; and some focused on food safety considerations in the context of government, businesses, or society. Several of the students included reference values for mercury concentrations from source articles other than the FDA for their tested species of fish, an extra effort that helped them consider the FDA measurements and their own measurements in a deeper way. The majority of students met or exceeded the learning goals and expectations for the assignment connected to this experiment, in particular the course goal "to appreciate the role of instrumentation in solving important problems in the physical, chemical, and biological sciences" (see Supporting Information).

With small modifications, the simplified digestion protocol could be used to achieve different educational goals. To cover method validation, the students could perform the spike recovery or analyze a reference material. For statistical methods of analysis or QC, the class could pool results for the same fish and perform statistical analyses to evaluate the performance of the instrument. Sample collection from an interesting local site would enable an emphasis on environmental chemistry.

CONCLUSION

The simplified digestion protocol proved appropriate for the skill level, existing equipment, and time and safety constraints in an undergraduate lab. This faster, simpler alternative to other methods eliminates the need for all but one of the usual hazardous reagents and enables undergraduate chemistry majors to perform CVAAS for mercury analysis in upper-level laboratories. The largest challenge in implementation is the learning curve for first-time users of the vapor generator. However, with an experienced user (instructor or fellow student) present, new users were able to successfully complete CVAAS measurements during the lab period.

ASSOCIATED CONTENT

Supporting Information

Additional experimental details, laboratory handout for students, notes for instructors, learning goals and assessment rubric, and an SOP for the Varian VGA-77 vapor generator. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(16) If the precipitate settled to the bottom of the container, it was possible to avoid introducing particles into the tubing of the vapor generator attachment, so filtration was not necessary.

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