Elemental Analysis Manual

for Food and Related Products

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U.S. Department of Health & Human Services

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4.8 High Performance Liquid Chromatographic-Inductively Coupled Plasma-Mass Spectrometric Determination of Methylmercury and Total Mercury in Seafood

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GLOSSARY

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4.8.1 SCOPE AND APPLICATION

This method describes procedures for using high performance liquid chromatography (HPLC) and inductively couple plasma-mass spectrometry (ICP-MS) to determine methylmercury and total mercury in seafood. Total mercury in this method is calculated as the sum of inorganic and methylmercury determined in analytical solution. Other matrices may be analyzed by these procedures if performance is verified in the matrix of interest, at the concentration levels of interest. The limits listed in 4.8 Table 1 are intended as a guide and actual limits are dependent on the sample matrix, instrumentation and selected operating conditions.

4.8 Table 1. Analytical Limits

Analytical parameter	Abbreviation	ASDL[a] (µg/L)	LOD[b] (µg/kg)	LOQ[b] (µg/kg)
Methylmercury	MeHg	0.039	3.8	28
Total mercury	TotHg	0.065	6.5	47

[a]Based on fortified method blanks.
[b]Based on 0.5 g analytical portion.

This method should be used by analysts experienced in the use of HPLC and ICP-MS, including the identification of chromatographic and matrix interferences, and procedures for their correction; and should be used only by personnel thoroughly trained in the handling and analysis of samples for determination of trace elements in food products.

4.8.2 SUMMARY OF METHOD

Hg species are isolated from 0.5 g non-dried, finely comminuted seafood or 0.2 g dried reference material by extracting with 50 mL aqueous solution of 1% (w/v) L-cysteine•HCl•H_2O for 120 minutes at 60 °C. The seafood-cysteine mixture is cooled to room temperature and filtered to remove particles > 0.45 µm diameter. Fifty µL portions of filtered extract are injected onto a C-18 column where Hg species are separated by HPLC using a mobile phase of aqueous 0.1% (w/v) L-cysteine•HCl•H_2O + 0.1% (w/v) L-cysteine. Hg at mass-to-charge ratio 202 vs. time is recorded and analyte peak areas are measured. Hg species are identified by retention times of peaks. Methylmercury and inorganic Hg concentrations are calculated using response factors determined for standard solution prepared in aqueous 1% (w/v) L-cysteine•HCl•H_2O and peak areas measured in extracts. Total Hg is calculated as the sum of methyl and inorganic Hg concentrations determined in extracts. Quality control procedures include (a) determination of methylmercury and inorganic Hg in check solutions with known analyte concentrations, in independent check solutions, and in method blanks, (b) determination of percent recovery of analyte added to portions of samples, (c) determination of analyte concentrations in certified reference materials, and (d) determination of inorganic Hg contamination in methylmercury stock standard solution.

4.8.3 EQUIPMENT AND SUPPLIES

Disclaimer: The use of trade names in this method constitutes neither endorsement nor recommendation by the U. S. Food and Drug Administration. Equivalent performance may be achievable using apparatus and materials other than those cited here.

(1) Inductively coupled plasma-mass spectrometer—Capable of measuring mass-to-charge ratio 202 in time resolved (chromatographic) mode. Equipped with PEEK™ Mira Mist nebulizer (Burgener Research), and quartz, Scott-type, double-pass spray chamber maintained at 2 °C. Instrument should electronically interface with or can be configured to remote start by standard HPLC instruments for integrated operation.

(2) High performance liquid chromatograph—An integrated or modular system consisting of an analytical pump and autosampler capable of delivering aqueous mobile phase through analytical column isocratically and programmed injection of acidic aqueous solutions.

(3) HPLC analytical column—Phenomenex Synergi Hydro-RP (C-18), 150 x 4.6 mm, 4 µm particle size (Phenomenex cat. no. 00F-4375-E0).

(4) Glass vials for extracting analytical samples—Amber, borosilicate glass vials, 60 mL capacity, with screw caps (I-Chem cat. no. S246-0060 or equivalent).

(5) Heated water bath—Capable of temperature control with sufficient water and thermal capacity to allow immersion of extraction vials to cap level and maintain water temperature at 60 ± 4 °C for 120 minutes.

(6) Syringe for filtering extracts—Disposable, general use and non-sterile.

(7) Syringe filters for filtering extracts—Disposable, 0.45 µm polypropylene membrane with polypropylene housing.

4.8.4 REAGENTS AND STANDARDS

Use reagents with sufficiently high purity and low mercury contamination to ensure that results are accurate and that quality control criteria can be met. Reagents should be checked for contamination before use. **Prepare all solutions (except stock standard solutions) daily and as close in time as possible to actual time of use.** Hold solutions in tightly sealed containers when not in use.

Safety Note: Reagents should be regarded as potential health hazards and exposure to these materials should be minimized as much as possible.

(1) Reagent water—Water that meets specifications for ASTM Type I water[1].

(2) Methylmercury(II) chloride—CH_3HgCl crystals, purity ≥ 95%, formula wt. 251.08.

(3) Mercury(II) chloride—$HgCl_2$ crystals, ACS grade, formula wt. 271.50.

(4) L-cysteine hydrochloride monohydrate (L-cysteine•HCl•H_2O)—Purity > 98.5%, formula wt. 175.64.

(5) L-cysteine (free base)—Purity ≥ 99.8%, formula wt. 121.16.

(6) Extraction solution, aqueous 1% (w/v) L-cysteine•HCl•H_2O—Dissolve 10 ± 0.1 g L-cysteine•HCl•H_2O crystals in 1000 ± 10 mL reagent water.

(7) Cysteine solution for preparation of standard solutions, aqueous 10% (w/v) L-cysteine•HCl•H_2O—Dissolve 5 ± 0.05 g L-cysteine•HCl•H_2O crystals in 50 ± 0.5 mL reagent water.

(8) Mobil phase, aqueous 0.1% (w/v) L-cysteine + 0.1% (w/v) L-cysteine•HCl•H_2O—Dissolve 0.5 ± 0.01 g L-cysteine and 0.5 ± 0.01 g L-cysteine•HCl•H_2O in 500 ± 5 mL reagent water.

(9) Methylmercury stock solution, CH_3HgCl in H_2O that may contain up to 20% (v/v) methanol, Hg=1000 mg/L—Tare 100-mL volumetric flask on analytical balance in chemical fume hood. Weigh 0.1252 g CH_3HgCl (FW=251.08) in flask with stopper in place. Add ≤ 20 mL methanol and swirl stoppered flask to dissolve CH_3HgCl. Dilute to 100.0 mL with reagent water. Discard solution in which inorganic Hg is > 3% of the theoretical methylmercury concentration.

(10) Inorganic Hg stock solution, $HgCl_2$ in 0.1% (v/v) HCl, Hg = 2000 mg/L—Tare 50-mL polypropylene centrifuge tube. Weigh 0.1354 g $HgCl_2$ (FW = 271.50) in tube. Add 5.0 ± 0.1 mL 1% (v/v) HCl and swirl to dissolve. Dilute to 50.0 ± 0.5 mL with reagent water.

(11) Multi-analyte intermediate solution, Hg due to CH_3HgCl = 1000 µg/L and Hg due to $HgCl_2$ = 1000 µg/L in 0.02% (w/v) L cysteine•HCl•H_2O—Mix approximately 40 mL reagent water and 0.1 mL 10% (w/v) L-cysteine•HCl•H_2O in 50-mL polypropylene tube. Add 50.0 µL methylmercury stock solution and 25.0 µL inorganic Hg stock solution. Dilute to 50.0 ± 0.5 mL with reagent water.

(12) Multi-analyte working standard solution, Hg due to CH_3HgCl = 1 µg/L and Hg due to $HgCl_2$ = 1 µg/L in 1% (w/v) L-cysteine•HCl•H_2O—Mix approximately 40 mL reagent water and 5.0 ± 0.05 mL 10% (w/v) L-cysteine•HCl•H_2O in 50-mL polypropylene tube. Add 50.0 µL multi-analyte intermediate solution. Dilute to 50.0 ± 0.5 mL with reagent water. Mix and immediately transfer a portion to glass HPLC autosampler vial(s) for storage before use.

(13) Check solution—Use multi-analyte working standard solution for the check solution.

(14) Independent check solution (ICS)—Prepare independent inorganic and methylmercury stock solutions, and independent multi-analyte intermediate and working standard solutions according to steps (9) – (12) from a different starting material than that used to prepare the primary stock solutions. Use of a commercial source material with a different lot number is acceptable, but a source material from a different manufacturer is preferred.

4.8.5 EXTRACTION PROCEDURE

Note: Methylmercury in extraction solution decomposes over time. To ensure accurate quantification of methylmercury, extracts must be analyzed within 8 hours of preparation.

Note: To assist homogenization of the analytical sample, reagent water ≤20% of the mass of seafood may be added if its addition provides a more visually homogenous and easier-to-manipulate material. If reagent water is added to assist homogenization, record to 4 significant figures the weights of edible portion and reagent water that are combined to prepare the analytical sample and apply mass correction factor (MCF) in calculation of concentration of analyte in analytical portion. Reserve a portion of reagent water used for homogenization to prepare method blanks.

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(1) Weigh analytical portion into 60-mL amber glass extraction vial and determine mass of analytical portion. Generally, weigh 0.5 ± 0.1 g edible portion of seafood. Use 0.2 ± 0.01 g for reference materials.

(2) Add 50.0 ± 0.5 mL extraction solution (aqueous 1% (w/v) L-cysteine•HCl•H_2O) to extraction vials, cap tightly, and shake vigorously by hand.

(3) Heat extraction vials 120 ± 5 min in water bath at 60 ± 4 °C. Shake each vial vigorously by hand after 60 minutes of heating and again after 120 minutes of heating.

(4) Remove extraction vials from water bath and allow cooling to room temperature.

(5) Filter a portion of extract through 0.45 µm filter directly into HPLC autosampler vial.

4.8.6 DETERMINATION PROCEDURE

The determination procedure was developed using a Waters Alliance 2690 Separations Module (HPLC) and Agilent 7500c ICP-MS. 4.8 Table 2 is an example of operating conditions used with this analytical system. The optimum operating settings and conditions must be determined for the equipment used.

4.8 Table 2. Typical HPLC-ICP-MS Operating Conditions

Conditions for Agilent 7500c ICP-MS and Waters Alliance 2690 Separations Module				
ICP-MS Conditions	setting		HPLC Conditions	setting
RF Power (W)	1550		Mobile phase flow rate (mL/min)	1.0
Plasma gas flow rate (L/min)	15		Injection volume (µL)	50
Auxiliary gas flow rate (L/min)	0.9		Degasser	OFF
Nebulizer gas flow rate (L/min)	1.2		Column temperature	not controlled
Sampling depth (mm)	8			
Peristaltic pump speed (rps)	0.2			
Spray chamber temperature (°C)	2			
Isotope (mass-to-charge ratio)	202			
Integration time (sec/point)	1			
Total acquisition time (sec)	300			
Reaction/collision cell mode	OFF			

Instrument Setup

(1) Setup and configure HPLC and ICP-MS separately before connecting equipment together. Follow instrument standard operating procedures for startup and initialization.

- Tune ICP-MS normally. Ensure instrument performance meets default specifications for sensitivity, precision, stability, and/or other established system suitability requirements.
- Set ICP-MS data acquisition for mass-to-charge ratio 202 in time resolved mode with 1 replicate (read) per point and use an initial dwell (integration) time of 1 second per point.
- Purge and condition HPLC and analytical column with mobile phase.

(2) Connect HPLC to ICP-MS.

- Enable communication between instruments to synchronize ICP-MS data acquisition with HPLC injection start.

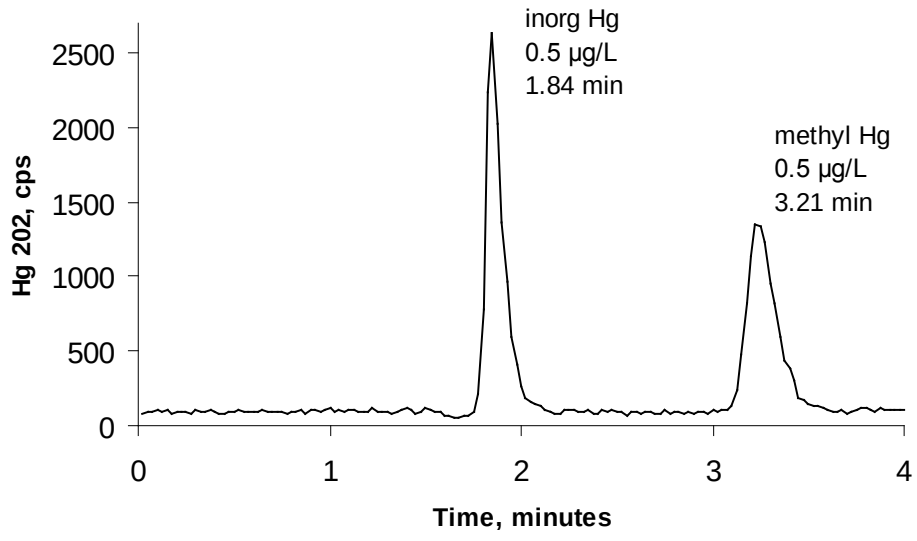
- Stop HPLC flow and connect column output directly to ICP nebulizer using HPLC PEEK™ tubing and fittings.

(3) Optimize operating conditions.

- Start HPLC flow and ensure proper liquid flow through ICP nebulizer and drainage of spray chamber.
- Analyze a multi-analyte standard solution and adjust acquisition parameters to obtain 10-20 data points across narrowest analyte peak.
- Monitor instrument conditions to ensure operation is stable and within normal functioning range.

(4) Check instrument performance.

- Verify baseline resolution between inorganic and methylmercury peaks and that peaks are not tailing excessively. A typical chromatogram can be seen in 4.8 Figure 1.
- Analyze a multi-analyte standard solution 3 or more times and verify short term precision is less than 5% relative standard deviation (peak area) for all analyte(s) of interest.
- Verify absence of instrument carry-over.



4.8 Figure 1. Typical HPLC-ICP-MS chromatogram of multi-analyte Hg standard solution.

Determination of Analyte Concentration Using Response Factor

(1) Analyze a multi-analyte standard solution (or single analyte standard solutions separately) and extraction solution 2 or more times each.

(2) Calculate response factors and check accuracy of working standard(s).

- Analyze independent check solution(s). Acceptance criteria: recovery within 100 ± 5%.

(3) Analyze analytical solutions and quality control solutions. A typical sequence for an analytical run is listed in 4.8 Table 3.

4.8 Table 3. Typical Analytical Sequence

Solution	Purpose	QC Criteria
standard solution(s), analyze 2 or more times	standardize instrument	
extraction solution, analyze 2 or more times		
independent check solution(s)	verify standardization	95-105% of expected
check solution	verify standardization	90-110% of expected
extraction solution	verify absence of carry-over	< ASDL
MBK #1	verify absence of contamination	< MBK_C
MBK #2		
sample #1	determine Hg conc.	≤LDR
sample #2		
sample #3		
sample #3 FAP	spike recovery	80-120% recovery
RM	accuracy	80-120% recovery
check solution	verify standardization	90-110% of expected
extraction solution	verify absence of carry-over	< ASDL

(4) Check instrument measurement performance

- Check solution analyzed at a frequency of 10% and at end of the analytical run has a recovery of 100 ± 10% (continuing calibration verification).
- Extraction solution analyzed following each check solution analysis is < ASDL (verify absence of carry-over).
- Measurements do not surpass the LDR. Dilute analytical solution with extraction solution if necessary to comply with criteria.
- Retention time of analyte peaks of analytical solution is comparable to standard solution.

4.8.7 CALCULATIONS

Calculate response factor of analyte, RF (cps-s/µg/L)

$$RF = \frac{A_{std\text{-}ave} - A_{es\text{-}ave}}{C_{std}}$$

where

$A_{std\text{-}ave}$ = average peak area of $n \geq 2$ injections of standard solution(s) (cps-s)
$A_{es\text{-}ave}$ = average peak area of $n \geq 2$ injections of extraction solution (cps-s) (0 if no peak is detected)
C_{std} = analyte concentration (µg/L) in standard solution(s)

Calculate concentration of analyte (inorganic mercury or methylmercury) in analytical solution, S (µg/L)

$$S = \frac{A_{as} - A_{es\text{-}ave}}{RF}$$

where

A_{as} = peak area of analyte in analytical solution (cps-s)
$A_{es\text{-}ave}$ = average peak area of analyte in extraction solution (cps-s) (0 if no peak is detected)
RF = response factor of analyte (cps-s / µg/L)

Calculate concentration of total Hg in analytical solution, S_T (µg/L)

$$S_T = S_{inorg} + S_{methyl}$$

where

S_{inorg} = concentration of inorganic Hg in analytical solution (µg/L)
S_{methyl} = concentration of methyl Hg in analytical solution (µg/L)

Calculate the concentration (mass fraction) of analyte in the analytical portion according to the formula

$$\text{Concentration } (\mu g/kg) = \left[(S_T \times DF) - MBK_L\right] \times \frac{V}{m \times MCF}$$

where

S_T = concentration of analyte (S or total Hg, S_T) in analytical solution (or diluted analytical solution) (µg/L)
MBK_L = laboratory MBK (µg/L)
V = volume (L) of analytical solution (0.050 L)
m = mass of analytical portion (kg)
DF = dilution factor (1 if analytical solution not diluted)
MCF = mass correction factor (1 if water or other solvent not added to aid homogenization)

Round calculated concentration to at most 3 significant figures. Concentration may be converted to other convenient units (*e.g.*, mg/kg, ng/kg).

4.8.8 METHOD VERIFICATION

The following is the minimum number of quality control samples to be analyzed with each batch of samples: 1 reference material (RM), 1 fortified analytical portion (FAP), and 2 method blanks (MBKs). Replicate analytical portions should be analyzed for each sample whenever analyte nonhomogeneity may be an issue.

A fortified method blank (FMB) checks the accuracy of the fortification procedure without any matrix effects and is an optional quality control sample. Use same fortification level as the FAP.

Reference Material

Control limits for RM Recovery are 100 ± 20% or within concentration uncertainty (converted to percent relative uncertainty) supplied on certificate, whichever is greater. The z-score procedure, which allows for greater deviation and is discussed in §3.5.3, may also be used, although it requires additional calculations. If three or more RMs are analyzed then only two-thirds of an

element's RM recovery results must meet the control limit.

FAP Recovery

Control limit for FAP recovery is 100 ± 20%.

Method Blanks (MBK)

Minimum of 2 MBKs analyzed and concentration of both MBKs are $\leq MBK_C$. If 3 or more MBKs are analyzed then at least two-thirds of MBKs are $\leq MBK_C$.

Relative Percent Difference (RPD) of Two Replicate Analytical Portions

Control limit for RPD is 10%.

FMB Recovery (optional)

Control limit for FMB recovery is 100 ± 10%.

4.8.9 REPORT

Report results only when quality control criteria for a batch have been satisfactorily met. Report results that are ≥LOQ as the mass fraction determined followed by the units of measurement. Report results that are ≥LOD and <LOQ as the mass fraction determined followed by the units of measurement and the qualifier that indicates analyte is present at a trace level that is below the limit of reliable quantification (TR). Report results that are <LOD as 0 followed by the units of measurement and the qualifier that indicates analyte is below the level of reliable detection or is not detected (ND).

Example: MeHg LOQ = 28 µg/kg; MeHg LOD = 8 µg/kg. Levels found for three different samples were 50 µg/kg, 10 µg/kg and 5 µg/kg.

50 µg/kg is ≥LOQ; report 50 µg/kg

10 µg/kg is ≥LOD but also <LOQ; report 10 µg/kg (TR)

5 µg/kg is <LOD; report 0 µg/kg (ND)

4.8.10 METHOD VALIDATION

In-house validation. The method was validated by analyses of reference materials, recovery of analyte addition, precision measurements, and comparison of total mercury results from an independent method[2]. For seafood containing 0.013–2.78 mg/kg methylmercury and 0.016–2.92 mg/kg total Hg, precision of analyses for 3 analytical portions was ≤5% relative standard deviation. Recovery of added analyte was 94% for methylmercury and 98% for inorganic Hg. Limit of quantification for methylmercury was 0.007 mg/kg in seafood. Methyl and total Hg results for reference materials agreed with certified values and total Hg results determined by this method were equivalent to results determined independently by cold vapor atomic absorption spectrometry.

Uncertainty. A result above LOQ has an estimated combined uncertainty of 10%. Use of a coverage factor of 2 to give an expanded uncertainty at about 95% confidence corresponds with the reference material recovery control limit of ± 20%. A result above LOD but below LOQ is considered qualitative and is not reported with an uncertainty.

A detailed discussion of method uncertainty is presented in §3.3. This method conforms to the information contained in that discussion. Derivation of an estimated uncertainty specific to an analysis is also discussed §3.3.2.

Interlaboratory trial. [*Under development*]

REFERENCES

(1) ASTM International (2006) ASTM D 1193-06, "Standard Specification for Reagent Water". ASTM.

(2) Hight, S. C., and Cheng, J. (2006) Determination of Methylmercury and Estimation of Total Mercury in Seafood Using High Performance Liquid Chromatography (HPLC) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS): Method Development and Validation, *Anal. Chim. Acta* **567**, 160-172.