APPLICATION NOTE

HPLC-ICP-MS

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Determination of Methyl- and Inorganic Mercury in Fish with the NexSAR HPLC-ICP-MS Speciation Solution

Introduction

Several forms of mercury (Hg) occur naturally in the environment, the most common being metallic mercury, mercuric sulfide (cinnabar ore), mercuric chloride, and methylmercury. However, mercury can also enter the environment from anthropogenic sources.

Although it occurs naturally, methylmercury is also formed by biological activity in aquatic environments and is bio-magnified in the food chain, resulting in much higher concentrations in larger predatory fish and mammals than in water and smaller fish. Most of the total mercury concentration in fish is in the form of methylmercury, with the highest concentrations being present in mature fish. Consumption of seafood is the primary exposure route of methylmercury for humans, emphasizing the importance of accurately measuring methylmercury in fish.

Methylmercury neurotoxicity and carcinogenicity is well documented,[1-3] with the most marked impact being on the developing brain. Methylmercury easily passes through both the placental barrier and the blood-brain barrier, making exposures during pregnancy a main concern.

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As with all analytical method setup, the determination of methylmercury content in fish has to meet the criteria of modern analytical methodology, including reproducibility, repeatability, and accuracy at low concentrations. These characteristics are facilitated by using an inert HPLC system with a metal-free flow path, which eliminates the possibility of mercury leaching into the mobile phase, thereby affecting the lowest concentrations which can be measured.

In our previous work,[4] we applied an L-cysteine hydrochloride anhydrous (L-cysteine·HCl) back-extraction from toluene into an aqueous phase. The methylmercury content in the aqueous extract was then determined using HPLC-ICP-MS. The work by Nan et al.[5] has shown that dealkylation of methylmercury into inorganic mercury does not occur, even under acidified conditions. Therefore, in this work we simplify the sample preparation step through a heated bath extraction using the HPLC mobile phase.

Experimental

Samples and Sample Preparation

Method development and validation were done with BCR-463 Tuna Fish, a freeze-dried certified reference material for methylmercury and total mercury.

Sample preparation was accomplished by weighing 0.1 g of sample and extracting with L-cysteine·HCl, resulting in a final dilution of 4000x for analysis. Post-digestion spikes of 1 and 10 ppb Hg were prepared to check spike recoveries of Hg and also evaluate whether the presence of a high concentration of inorganic Hg would have any impact on the recoveries of methylmercury during the chromatographic separation.

All measurements were made against external calibration curves. The calibration blank and standards were prepared in the mobile phase. The inorganic standards were prepared from a stock 10 ppm mercury standard. This methylmercury stock standard was prepared by dissolving methylmercury chloride (Sigma-Aldrich, Malaysia) in deionized water and adding 2-mercaptoethanol (Merck, Singapore) until methylmercury was the primary Hg species. Thereafter, calibration standards (0.5, 1, 2, 5 ppb) were prepared in the mobile phase.

Instrumental Conditions and Parameters

All analyses were performed on a PerkinElmer NexSAR™ speciation analysis ready HPLC system coupled to a PerkinElmer NexION® ICP-MS using the conditions outlined in Tables 1 and 2. The NexSAR HPLC system consists of an inert and metal-free dual-piston binary pump, autosampler with an inert needle, column oven, vacuum degasser, and post-column switching valve. The column oven incorporates an in-line pre-heater which heats the solution prior to entering the column, reducing the temperature gradient across the column. Not only does the oven with pre-heater improve chromatographic stability by negating effects of changes in lab temperature during the day, but the elevated temperature sharpens peaks, allowing for lower concentrations to be measured. The post-column switching valve diverts the LC flow to waste when analyses are not being performed, such as during column equilibration and washing, while allowing clean 1% HNO_3 to flow to the plasma, thereby minimizing mobile phase deposition on the cones and reducing maintenance. The mobile phase consisted of 98% L-cysteine and 2% methanol. This can either be prepared in one bottle or as separate bottles of 100% L-cysteine and 100% MeOH, using NexSAR's on-line mixing capability. All instrument control, data acquisition, and data reduction were accomplished with Clarity™ chromatography software for speciation analyses.

The NexION ICP-MS was run in Standard mode since no interferences were found to exist on Hg in the sample. However, the NexION can also be run in Reaction or Collision modes with HPLC-ICP-MS if interferences are present which must be removed. Standard sample introduction components and plasma conditions were used.

Table 1. NexSAR HPLC system operating conditions and parameters.

Component/Parameter	Value/Condition
Column	PerkinElmer C18
Mobile Phase	0.1% (w/v) L-Cysteine HCl (98%) + Methanol (2%)
pH	2.1
pH Adjustment	None
Flow Rate	1.5 mL/min
Separation Scheme	Isocratic
Column Temperature	35 °C
Injection Mode	Partial Loop
Injection Volume	50 µL
Loop Volume	100 µL
LC Vials	1.5 mL Polypropylene

Table 2. NexION ICP-MS operating conditions and parameters.

Component/Parameter	Value/Condition
Nebulizer	Glass Concentric
Spray Chamber	Glass Cyclonic
RF Power	1600 W
Nebulizer Flow	Optimized for < 2% Oxides
Mode	Standard
Analyte	^{202}Hg
Dwell Time	250 ms
Sampling Rate	4 points/second

Results and Discussion

To assess the separation, a series of calibration standards ranging from 0.5 to 5 ppb of both methylmercury (MeHg) and inorganic mercury (iHg) were measured. The resulting chromatograms are overlaid in Figure 1 and show that the complete baseline separation of the analytes is achieved in under three minutes. The resulting calibration curves (Figure 2) have correlation coefficients (r^2) greater than 0.9999, showing excellent linearity.

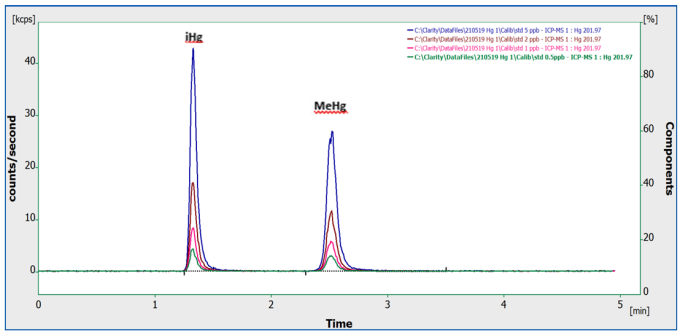

Figure 1. Separation of inorganic mercury (iHg) and methylmercury (MetHg) standards: 0.5, 1, 2, and 5 ppb of each species.

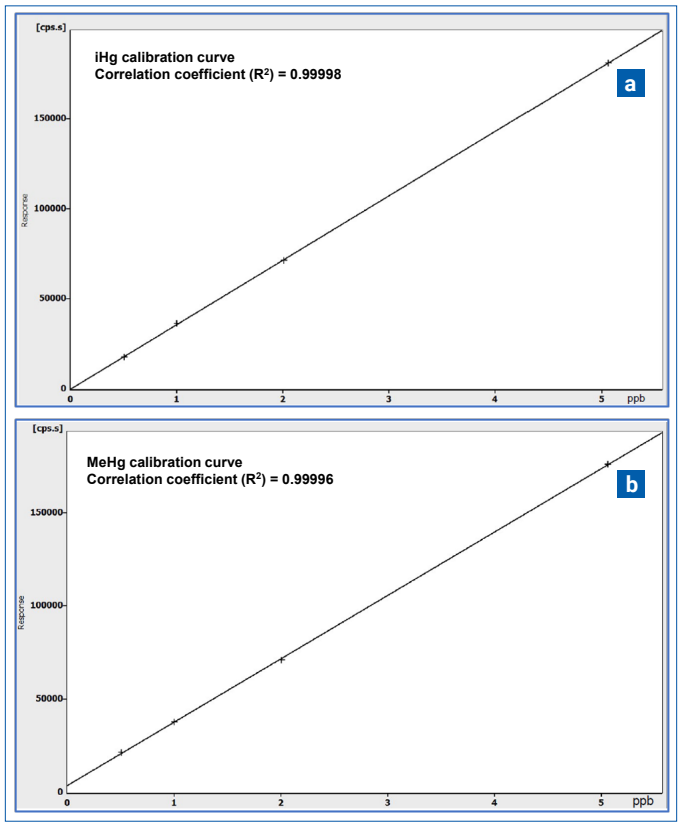

Figure 2. Calibration curves for a) inorganic mercury (iHg) and b) methylmercury (MeHg): 0.5, 1, 2, and 5 ppb of each species.

Next, the prepared reference material was analyzed five times to assess both the effect of the matrix on the chromatography and the repeatability of the methodology. The resulting chromatogram in Figure 3 shows that the tuna fish matrix does not affect the chromatography as both the peak shapes and retention times are the same as the standards. Accounting for dilution during sample preparation, the Hg concentration in the MeHg is 0.70 µg/L entering the plasma. The recoveries from all five injections are within 5% of the certified values (Figure 4), demonstrating excellent accuracy and repeatability, even at low concentrations.

As mentioned previously, to assess the accuracy for inorganic Hg, 1 ppb of inorganic Hg was spiked into five different vials of the prepared BCR-463 CRM and analyzed. As shown in Figure 5, all recoveries are within 5%, demonstrating outstanding accuracy for inorganic Hg. The accompanying chromatograms for Preparation 5 appear in Figure 6.

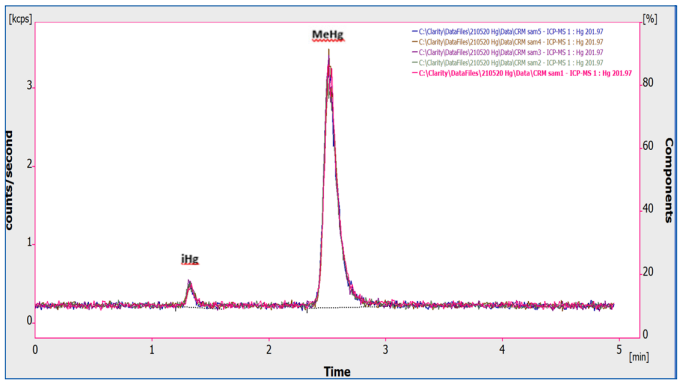

Figure 3. Chromatograms from five different sample preparations of BCR-463 Tuna Fish.

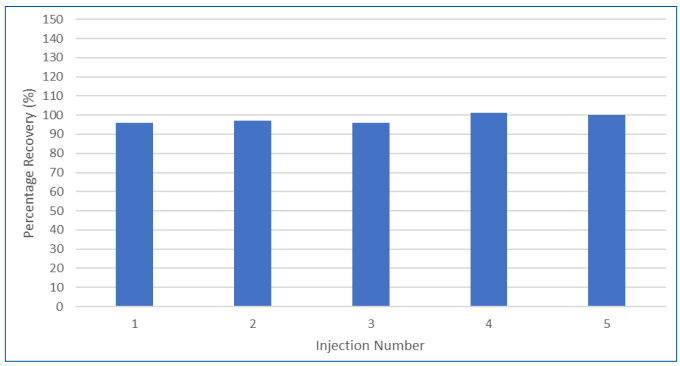

Figure 4. Methylmercury recovery in BCR-463 Tuna Fish (certified value = 3.04 µg/g).

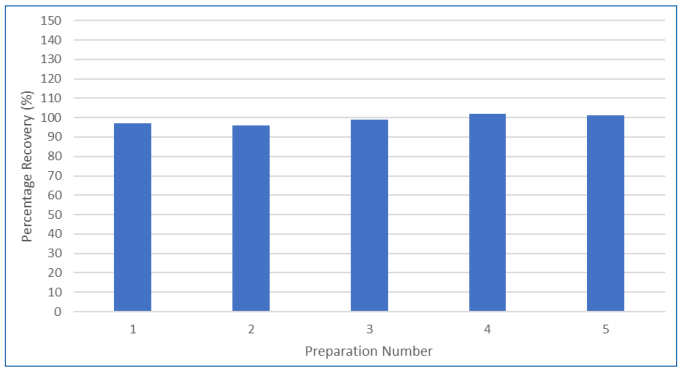

Figure 5. Recoveries of 1 ppb inorganic mercury spikes into BCR-463 Tuna Fish from five different vials.

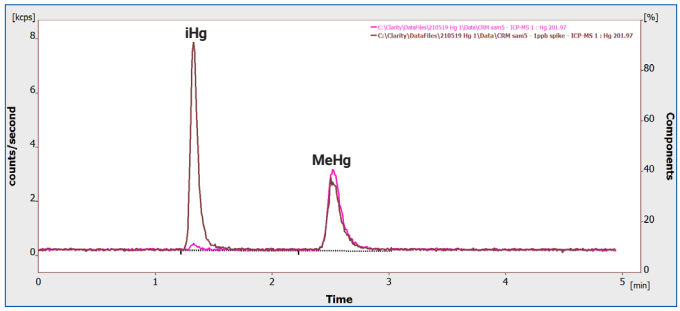

Figure 6. Chromatograms showing BCR-463 Tuna Fish both unspiked and spiked with 1 ppb inorganic mercury.

To assess the chromatographic effect of high inorganic Hg concentrations on the measurement of methylmercury, a prepared sample of the BCR-463 CRM was spiked with 10 ppb inorganic Hg. As shown in Figure 7, baseline resolution is maintained between the species, signifying that low methylmercury concentrations can be accurately measured in the presence of high inorganic Hg concentrations.

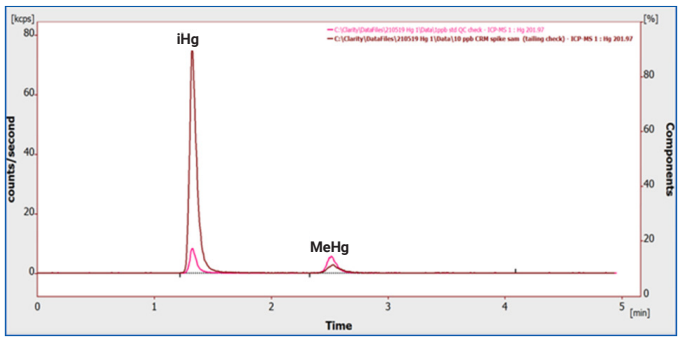

Figure 7. Chromatogram of BCR-463 spiked with 10 ppb inorganic mercury.

The stability of the methodology was evaluated by performing repeated injections of a standard containing 1 ppb of both inorganic mercury and methylmercury. As shown in Figure 8, the recoveries for both standards over four hours are within 10%. The stability is further emphasized in Figure 9, which shows the overlays of all the chromatograms acquired over the four hours.

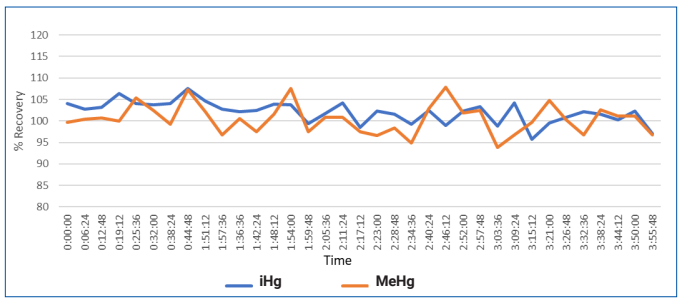

Figure 8. Recoveries of 1 ppb standard over four hours.

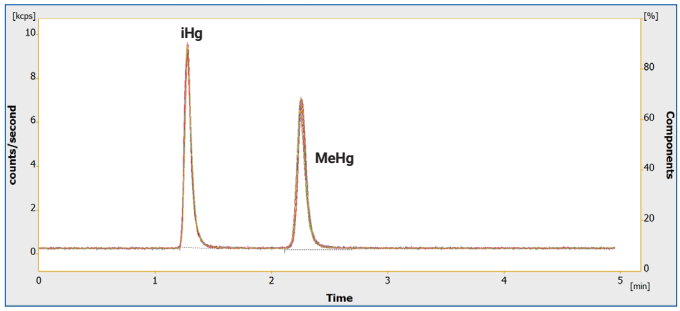

Figure 9. Overlay of chromatograms of a 1 ppb standard solution analyzed over four hours.

To explore the lowest concentrations which can be measured with this methodology, standards of successively lower concentrations were injected until a signal-to-noise ratio of ≈ 3 was attained, a common determination of detection limits with HPLC analyses. As shown in Figure 10, peaks for 0.02 ppb of both inorganic mercury and methylmercury can clearly be seen above the baseline. If lower concentrations need to be measured, larger injection volumes can be used.

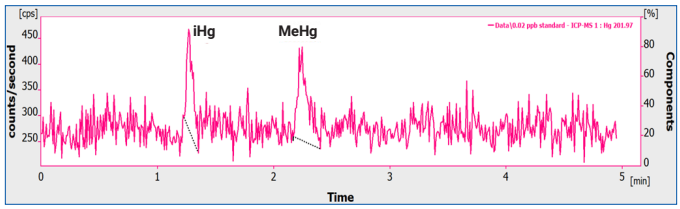

Figure 10. Chromatogram of 0.02 ppb standard of inorganic mercury and methylmercury.

Conclusion

This work has demonstrated the rapid, accurate and reproducible measurement of both methyl- and inorganic mercury in freeze-dried fish in under three minutes with the NexSAR HPLC-ICP-MS speciation solution. Sample preparation is relatively simple with an extraction of the freeze-dried and pulverized sample using L-cysteine·HCl. Moreover, the methodology and instrumentation delivered outstanding long-term stability over the four-hour analytical period, despite the fact that Hg is a notoriously "sticky" element, the result of the NexSAR's repeatable and reproducible injection volumes, heated column oven and inert, metal-free flow path. The speed and efficiency of HPLC-ICP-MS allow samples to be analyzed approximately every three minutes, with no possibility of inorganic Hg tailing into the later-eluting MeHg peak.

References

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2. Bakir, F.; Kamluji, S.F.; Amin-Zaki, L.; et al. 1973. *"Methylmercury poisoning in Iraq"*, ***Science*** 181:230-241.
3. *Kerper, L.E.; Ballatori, N.; Clarkson, T.W. (May 1992). "Methylmercury transport across the blood–brain barrier by an amino acid carrier"*, ***Am. J. Physiol.*** 262.
4. Ghanthimathi, S.; bt Ibrahima, N.; bt Nasira, Z.; Lim, L.; Ong, K. *"Speciation: Determination of Methylmercury in Fish Samples with HPLC-ICP-MS"*, ***Atomic Spectroscopy*** 32 (3), 2011, 85-89.
5. Nan, X-J.; Yu, X-P.; Guo, Y-F.; Deng, T-L. *"Function and Effects of L-cysteine on the Speciation Analysis of Mercury by High Performance Liquid Chromatography Coupled with On-line Cold Vapor Generation Atomic Fluorescence Spectrometry"*, ***Advances in Engineering Research,*** Volume 94, 2nd International Conference on Sustainable Development (ICSD 2016).

Consumables Used

Component	Part Number
Polypropylene Vials with Caps - Speciation Tested	N9301736
C18 Column	N9303546
Consumables Kit - NexSAR HPLC	N8150512
10 ppm Mercury (Hg) Pure Plus Standard, 125 mL	N9300253

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