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Comparative Tests on the Efficiency of Three Methods of Methylmercury Extraction in Environmental Samples

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Mercury is used in gold mining in the Brazilian Amazon and released in significant amounts to the environment as Hg^0. After its oxidation it may be methylated, mainly in bottom sediments and in the rhizosphere of floating aquatic macrophytes. Methylmercury (MeHg) is highly bioavailable and subject to biomagnification.

The objective of this work was to evaluate the efficiency of three methylmercury extraction techniques, applied to replicates of environmental samples (stream sediments from Floresta da Tijuca, Rio de Janeiro; and roots of the floating macrophyte *Eichhornia crassipes* from Lagoinha, Rio de Janeiro) previously incubated with $^{203}Hg^{2+}$. Method A is based on acid leaching and extraction of $Me^{203}Hg$ in toluene. Method B uses alkaline digestion, extraction in dithizone–benzene and separation of organic and inorganic ^{203}Hg dithizonates by thin-layer chromatography (TLC). Method C consists in separating the $Me^{203}Hg$ from the sample matrix by distillation. Total ^{203}Hg and $Me^{203}Hg$ were detected by gamma spectrometry or liquid scintillation.

For both matrices, $Me^{203}Hg$ extraction efficiency was better for smaller samples (range: 0.08–0.5 g for sediment, 0.1–0.5 g for *E. crassipes* roots). For the sediment samples, the three selected methods presented similar $Me^{203}Hg$ extraction efficiencies: 7–13, 5–14 and 4–17% of total added ^{203}Hg was found as MeHg for procedures A, B and C, respectively. For *E. crassipes* roots, on the other hand, a lower extraction efficiency was obtained for the procedure C (11–28% of total ^{203}Hg present as $Me^{203}Hg$) than for methods B (22–36%) and A (20–44%). In all the root samples prepared with procedure B, a strong and durable chemiluminescence effect was observed, which required measuring the final $Me^{203}Hg$ extracts by gamma spectrometry only. In the specific conditions we used, extraction via distillation required re-extraction of $Me^{203}Hg$ in the distillate, due to the presence of traces of inorganic ^{203}Hg in the latter. **Copyright © 1999 John Wiley & Sons, Ltd.**

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INTRODUCTION

In the last saw decades, the release of important amounts of metallic mercury to the environment by informal gold mining, in Brazil and other developing countries, has stimulated interest in the mercury cycle. Similarly methylmercury (MeHg) concentrations have been found in fish and human hair in gold-mining regions[1] and in pristine areas[2] and the neurotoxic consequences of low levels of MeHg in the local human populations have been documented[3]. MeHg is formed from Hg^{2+} by chemical and microbiological activity in a variety of environmental sites. Surface sediments and wetland soils are considered important methylation sites but Guimarães *et al.*[4], Lemos *et al.*[5] and Mauro *et al.*[6] found higher methylation potentials in the rhizosphere of floating aquatic macrophytes than in the underlying sediments in a variety of sites in Brazil.

Natural MeHg in environmental samples can be determined using numerous different extraction and measurement methods, several of which have been adapted for use with $Me^{203}Hg$. The objective of this work was to compare the efficiency of three

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methods for the extraction of $Me^{203}Hg$ formed from added ^{203}Hg in sediments and roots of the water-hyacinth *Eichhornia crassipes*. Samples were incubated with ^{203}Hg for production of $Me^{203}Hg$, and the different methods were applied to replicates of these homogenized samples.

EXPERIMENTAL

Sampling and sample incubations

Fine surface (0–1 cm) sediment was aspirated from the bottom of a stream in Floresta da Tijuca National Park, Rio de Janeiro. After sieving through 1-mm sieves, samples were kept in a refrigerator for up to three days if not incubated on the same day with ^{203}Hg. The sediment was mostly <74 μm, containing 7% volatile organic matter and 90 ng g^{-1} total Hg, determined by Cold-vapor atomic absorption Spectroscopy (CVAAS) (see Ref. 7 for more details).

Roots of the water-hyacinth *Eichhornia crassipes* were sampled at Lagoinha freshwater lagoon, in Parque Ecológico Municipal Chico Mendes, Rio de Janeiro, and transported to the laboratory in sealed polyethylene bags. Before incubation with ^{203}Hg, the roots were separated from coarse debris and cut into 0.5–2 cm pieces.

Incubations with ^{203}Hg

Sediment and macrophyte root samples were added to previously prepared ^{203}Hg solutions in 1-litre borosilicate beakers and then thoroughly homogenized. Table 1 gives more details of incubation parameters. Incubations were performed at room temperature (23 ± 2 °C) and in the dark. After 48 h, incubation was stopped by addition of 4 M HCl to pH $\simeq 1$ and the samples were again carefully homogenized. Aliquots were separated in sealed polyethylene bottles and frozen until $Me^{203}Hg$ was extracted.

Methylmercury extraction

The methods selected for this study were:

(A) acid leaching and extraction into toluene;[4,8,9]
(B) alkaline digestion, extraction into dithizone–benzene (Dz:Bz) and separation by thin-layer chromatography;[10] and
(C) acid leaching and separation by distillation.[11]

Method A was adapted from the method described by Furutani and Rudd.[8] It has been thoroughly tested in our laboratory[9] and used routinely in methylation experiments.[4] Other variants of method A have been used in similar experiments by other authors.[12,13] Method B was developed at the National Institute for Minamata Disease, Japan, a WHO Collaborating Center. It is regularly used there in the certification of reference materials (with ECD–gas chromatography instead of TLC) and is also in routine use in our laboratory for natural MeHg measurements in different environmental samples.[14] Method C is being used currently in the certification of reference materials[15,16] and is applied widely to the determination of MeHg in a variety of environmental samples including water, when combined with aqueous-phase ethylation and cold-vapor atomic fluorescence spectrometry (CVAFS).[17]

Acid leaching and extraction in toluene

$Me^{203}Hg$ was leached from the samples by shaking them vigorously for 1 min with 2 ml of 3 M NaBr and 0.5 ml of 0.5 M $CuSO_4$ in 11% H_2SO_4. After centrifugation, at 2000 rpm for 2 min, the supernatant was shaken for 15 min in separation funnels with a scintillation cocktail prepared from toluene and the scintillation salts POP (2,5-diphenyloxazole) and POPOP (1,4-bis[5-phenyl-2-oxazolyl]-benzene). The organic phase was centrifuged for 5 min at 2000 rpm and its supernatant shaken with 1 g of anhydrous Na_2SO_4 to remove any trace of

Table 1 Sample incubation conditions

Sample type	Incubated material (g wet wt)[a]	Total added ^{203}Hg activity (Bq)	Total added Hg (ng g^{-1} dry wt)
Sediment	46	5.6×10^5	520
Macrophyte roots	500	2.6×10^4	7.1

[a] Wet weight/dry weight ratio = 12.7 for macrophyte roots and 3.02 for sediment.

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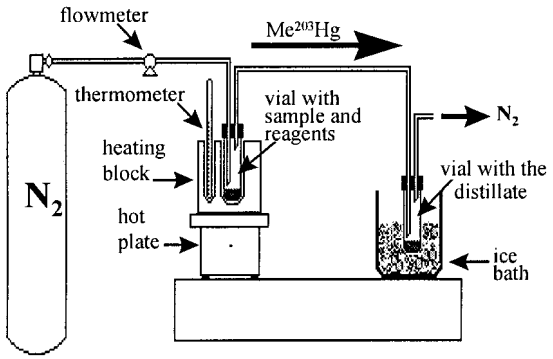

Figure 1 Schematic view of the distillation system.

water containing inorganic mercury. The samples were then transferred to another vial and $Me^{203}Hg$ was quantified by liquid scintillation.

The validity of the assumption that all activity in the final extract is $Me^{203}Hg$ was confirmed by back-extraction in Na_2S and then in benzene, followed by submission to TLC.[10]

Alkaline digestion, extraction in dithizone–benzene and separation by TLC

Sediment and macrophyte root samples were measured for total ^{203}Hg by gamma spectrometry in glass tubes and digested at room temperature in the same vials by shaking for 20 min with 8 ml of 1 M KOH in ethanol. After being slightly acidified by addition of 5 ml of 2.4 M HCl, the samples were shaken for 5 min with 5 ml of 0.1% dithizone in benzene (Dz:Bz), and centrifuged at 1000 rpm for 5 min. Next, 1–2 ml of the Dz:Bz layer was dried and cleaned by percolation through a column made in a disposable Pasteur pipette, containing 0.5 g of Florisil® topped by 0.5 g of anhydrous Na_2SO_4. The eluate was collected in another vial, shaken twice with 10 ml of 2.5% ammonia to remove the excess of free dithizone, and centrifuged at 1000 rpm for 5 min. A precise volume of the Dz:Bz (usually 0.5 ml) was collected in a scintillation vial and measured for total ^{203}Hg by gamma spectrometry to check the recovery. The sample was evaporated to dryness under a stream of nitrogen, dissolved in 2–3 drops of acetone, and applied with glass capillaries to a TLC sheet (silica-gel Polygram Cell, Macherel–Nagel, Germany), together with similarly prepared standards of non-radioactive Hg^{2+} and MeHg. The plates were developed using benzene/hexane (1:1) as developing agent and the stripes corresponding to inorganic mercury dithizonate and methylmercury dithizonate (respectively visualized as pink and yellow spots, with R_f values of 0.6 and 0.25) were cut and measured by gamma spectrometry, as well as those corresponding to the application point and solvent front.

Comparison of total mercury in the initial samples and Dz:Bz extracts indicated recoveries close to 100% or higher; the recoveries over 100% were attributed to the loss of benzene during extraction by evaporation and dissolution in the aqueous phase, leading to a higher mercury concentration in the final extracts than in the initial sample. As the activity of the stripes corresponding to the application point and the solvent front was negligible, the proportions of inorganic mercury and MeHg in each sample was calculated from the total activity of the corresponding TLC stripes.

Acidification and separation by distillation

After initial determination of total ^{203}Hg by gamma spectrometry, the samples were introduced in 12-ml glass tubes with Teflon®-lined screw-caps, with 5 ml of deionized water, 15 µl of 20% KCl and 250 µl of 8 M H_2SO_4. The tubes were placed in a heating block at 145 °C and 85% of the original sample volume was distilled under a flux of 7 ml min^{-1} nitrogen in other glass tubes placed on ice (Fig. 1). Gas-tight connections between the glass tubes were made of Teflon® tubing. To verify the presence of inorganic ^{203}Hg, the distillate was measured for total ^{203}Hg before and after re-extraction using method A. The residue in the distillation flasks was submitted to the same treatment to check for incomplete distillation.

Radioactivity measurements

$^{203}HgCl_2$, lot number 102A purchased from Amersham International UK, presented a specific activity of 1.14 mg Hg mCi^{-1} and was diluted in distilled water before use. Samples were measured by gamma spectrometry on a Wallac LKB Compugama 1282 with a 2×2 NaI detector or by liquid scintillation on a Wallac LKB Rackbeta 1214. All radiometric data were converted to disintegrations per minute. In measurements by liquid scintillation, quenching and chemiluminescence were routinely controlled and corrected. All radioactive samples were manipulated in well-ventilated fume hoods. The counting error was kept below 1% and data

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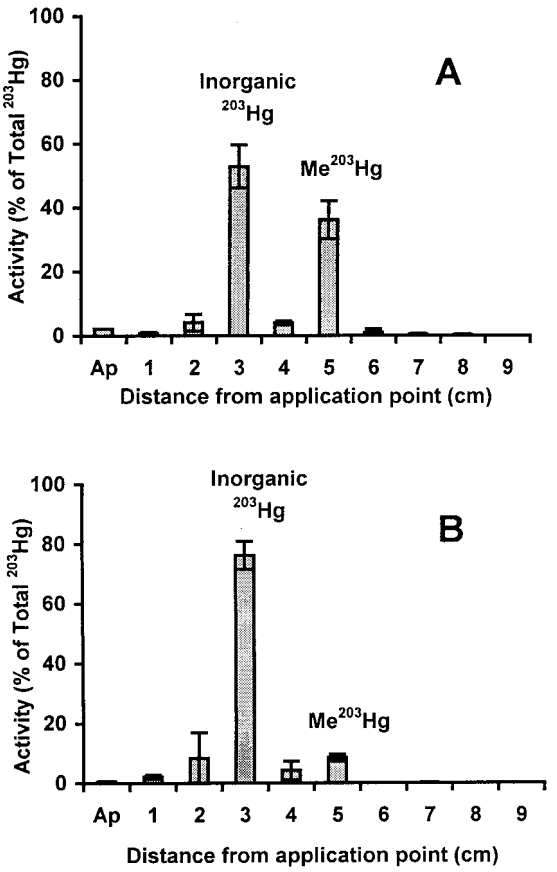

Figure 2 Distribution of ^{203}Hg along TLC plates of samples of *E. crassipes* roots (A) and sediment samples (B). Stripes 3 and 5 correspond respectively to the spots of stable inorganic mercury and monomethylmercury dithizonate standards applied with the samples. Error bars represent 95% confidence intervals from triplicate samples. Ap, application point.

were corrected for decay. All chemicals were of analytical grade.

Preliminary tests on methods B and C

To verify the distribution of activity along the TLC sheets obtained with method B, these sheets were cut into 1-cm stripes, the first including the application point. Figure 2 shows the data obtained for TLC plates of replicate sediment and macrophyte samples ($n = 3$). For both types of sample, only a negligible fraction of total initial activity is retained at the application point and the peaks in activity correspond exactly to the pink–red spot of stable inorganic dithizonate standard and the yellow spot of the MeHg dithizonate standard. A much greater MeHg production in the macrophyte root samples (36.2 ± 5.9 %) than in the sediment samples (8.4 ± 1.1%) is evident.

To verify the stability of the inorganic and organic mercury retained on the TLC sheets, the stripes corresponding to inorganic mercury and MeHg were stored in the dark at room temperature and their activity was measured after nine and 20 days. The results (Fig. 3) suggest a greater stability of inorganic mercury than of MeHg.

In the TLC stripes from extracts of macrophyte roots observed by liquid scintillation there was a strong and durable chemiluminescence effect, while for extracts of sediment samples no such effect was observed. Hence, ^{203}Hg measurements in TLC stripes from macrophyte samples could be made by gamma spectrometry only. Alternatively, chemiluminescence may be avoided with the use of solid scintillators, which were not available during the present study.

For method C, the preliminary tests aimed at verifying whether some MeHg remained in the distillation tube and whether some inorganic mercury was present in the distillate. To that end, the contents of both tubes were re-extracted using method A. The results of these tests are illustrated in Figs 4 and 5. It can be observed that some MeHg remained in the distilled samples, specially those of macrophyte roots, from which 2.9–6.0% of the total ^{203}Hg is obtained as MeHg after re-extraction with method A. This is probably due to the small size of the distillation tubes in comparison with the volume of the root samples, a problem not

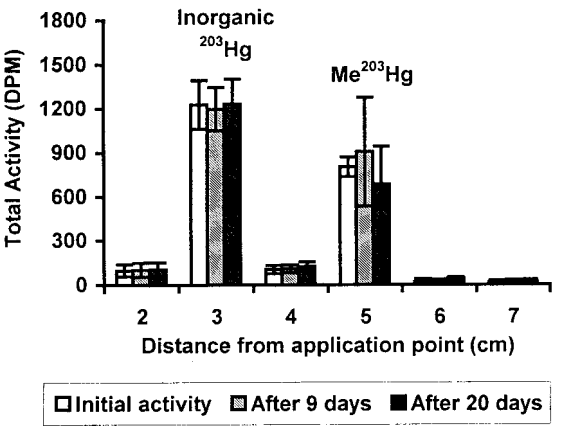

Figure 3 Influence of storage time (22 ± 2°C in the dark) on ^{203}Hg activity of TLC stripes of *E. crassipes* root samples. Inorganic mercury and MeHg respectively are in stripes 3 and 4.

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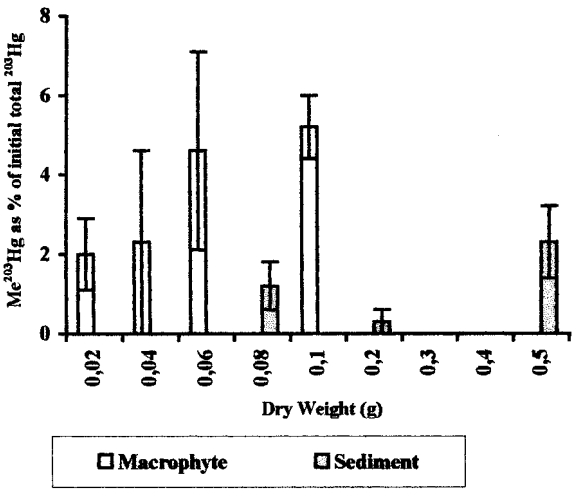

Figure 4 Percentage of total initial activity found as MeHg after re-extraction of distilled sediment and macrophyte root samples using method A.

experienced with sediment samples. This necessitated the use of different sample weight ranges for each matrix.

Some carry-over of inorganic mercury to the distillate was observed, especially for sediment samples. Figure 5 shows the estimated MeHg percentages in sediment (B) and macrophyte root samples (A), assuming that all the ^{203}Hg in the distillate is $Me^{203}Hg$ (white bars) or calculating the MeHg% only after re-extraction of the distillate with method A (shaded bars). The higher proportion of inorganic mercury carry-over for the sediment samples of higher weight may be a result of accidental transfer of sediment particles to the distillate and suggests again that optimum distillation was not reached with the combination of glass tube capacity and distillation time used herein. The use of anti-foaming agents could help in the optimization of this technique in our specific working conditions.

Recent evidence has suggested that artifactual MeHg can be formed in various types of samples submitted to distillation.[18] In checks with water samples spiked with $^{203}Hg^{2+}$ immediately before distillation, we found negligible activity in the distillate. Though we did not make similar tests with sediment and root samples, we believe that artifactual MeHg formation would not be relevant in our experimental conditions, due to the similarly high percentage of MeHg found in the same samples when using two other methods for which no such artifact has been documented to date.

Comparison of MeHg extraction efficiency of selected methods

Figure 6 shows the results of extractions of triplicate samples of sediment and macrophyte roots, in different weight ranges. For sediment samples, method A tends to yield higher percentages of MeHg than method B over the whole weight range. Furthermore, for all the methods a higher percentage of MeHg is obtained at smaller weights, suggesting a decrease in extraction efficiency with increasing sample weight. A sharper decrease in recovered MeHg is seen for method C (distillation with re-extraction by method A). For macrophyte root samples, a similar yield was obtained for methods A and B, with no pronounced

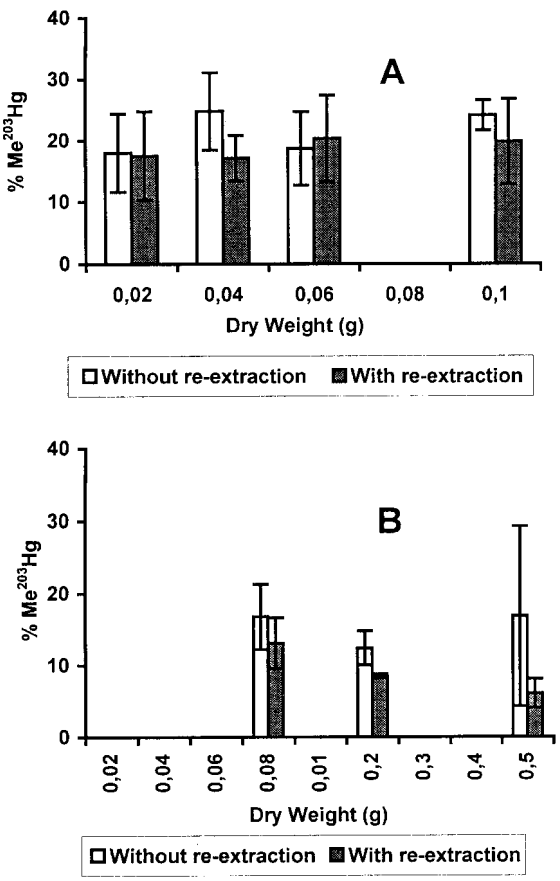

Figure 5 MeHg (% of added total Hg) in samples of *E. crassipes* roots (A) and sediment (B), assuming that all the ^{203}Hg in the distillate is $Me^{203}Hg$ (white bars) and after re-extraction of the distillate by method A (shaded bars).

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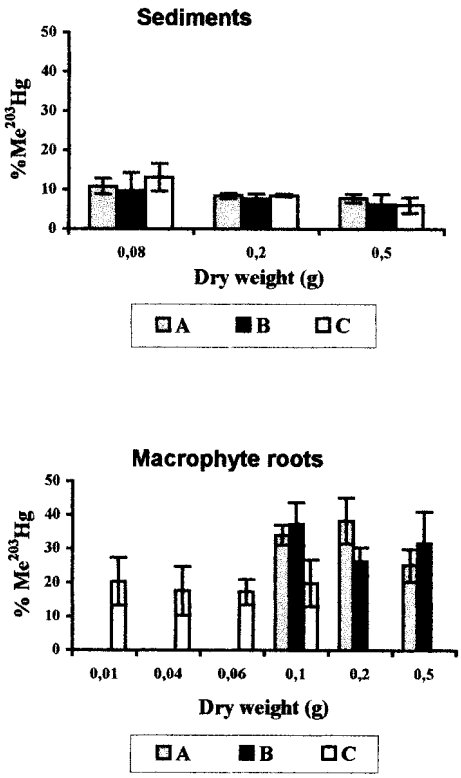

Figure 6 MeHg (% of added total Hg) in triplicate samples of *E. crassipes* roots and sediment of different weights, extracted by methods A (acid leaching and extraction in toluene), B (alkaline digestion and extraction in dithizone/benzene) and C (acidification and distillation). Sediment and roots were spiked by soaking for two days in a ^{203}Hg solution, after which they were acidified to pH $\sim$ 1 with HCl and carefully homogenized. Aliquots of the homogenized samples were kept at -18°C for 11 days (sediment) and 28 days (macrophyte roots) before extraction tests with the different procedures.

effect of sample weight in the range 0.1–0.5 g dry weight. For method C (distillation), similar percentages of MeHg are found in the range 0.01–0.1 g dry weight.

For macrophyte roots, the efficiency of the methods could be compared only with samples normalysed at 0.1 g, due to the different weight ranges for each sample type. For these samples, the results obtained by the three methods could be considered as equivalent, particularly if the residual MeHg in the distillation tubes is accounted for (see Fig. 4). An additional factor that may have decreased the recovery of MeHg in samples extracted by distillation is the degradation of $Me^{203}Hg$ during the storage period between the end of incubation and extraction, which was longer for the samples used to test this procedure than for those used in tests with procedures A and B. Guimarães *et al.*[9] showed that storage of sediment samples at pH$\simeq$1 and -18 °C leads to some $Me^{203}Hg$ degradation, and based on their data we estimate that approximately 10% of the $Me^{203}Hg$ initially present in the samples could be degraded during the 11-day storage of sediment samples and 28-day storage of macrophyte root samples.

DISCUSSION

The experiments described here were not specifically designed to compare the methylation potential of sediment and macrophyte roots, but it is worth noting that the latter converted a higher proportion of added $HgCl_2$ to MeHg than did bottom sediment. This corroborates with observations from similar studies in different sites in Brazil — with a variety of floating macrophyte species — in which the same pattern was observed.[4–6] However, lower MeHg formation was found in those other studies than here, because for the present study we selected samples taken from sites here we had previously found higher methylation potentials, which would be more useful to the specific objectives of this work. It should be noted, however, that radiochemical methylation measurements, except when using ^{203}Hg of high specific activity, require addition of Hg(II) in higher concentration and availability than is found in natural environments; and MeHg percentage levels as high as found here are not expected to be found in the latter. However, this approach is a good predictor of preferential mercury methylation sites in natural environments, as shown recently[19] (M. Roulet, unpublished results) in floodplain lakes in the Brazilian Amazon, where seasonal and spatial variations of natural MeHg and of ^{203}Hg methylation potentials showed similar patterns, and floating macrophyte mats were among the sites of higher natural MeHg concentration in water.

The decreasing MeHg yield with increasing sediment sample weight, observed even in the narrow range we worked on, suggests that in some studies, in which sediment samples of up to 10 g were extracted with the same proportions of reagents as used here, the mercury methylation potentials have probably been largely underestimated.

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The selected extraction methods presented similar efficiencies, and differed more in other characteristics such as cost, safety and labor demand.

Method B offers the advantage of a direct identification of MeHg, but it is time-consuming and requires more skill. It also requires higher initial mercury additions. Method A is as reliable as method B, but it is faster and uses toluene instead of benzene, which presents higher toxicity. Method C (distillation) is an elegant way of separating MeHg by transferring it from the sample matrix to a clear aqueous phase. If properly optimized, distillation allows a very convenient single-step $Me^{203}Hg$ extraction, the distillate requiring no further treatment if the measurement is made by gamma spectrometry.

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