APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 7, 413–420 (1993)

WORKING METHODS PAPER

Improvements in methylmercury determination prior to the certification of two tuna fish materials

Ph Quevauviller,* I Drabaek,†‡ H Muntau§ and B Griepink*
*CEC, Measurements and Testing Programme (BCR), rue de la Loi 200, B-1049 Brussels, Belgium,
† Kemiteknik, Teknologisk Institut, Gregersensvej, DK-2630 Taastrup, Denmark, and
§ CEC, Joint Research Centre, I-21020 Ispra, Italy

An account is given of a systematic, collaborative investigation to detect problems and sources of error in the determination of methylmercury (MeHg) in biological samples. The work was done by a group of analytical laboratories under the auspices of the Community Bureau of Reference (BCR) of the Commission of the European Communities (EC). The paper presents the organization of three intercomparisons on organic mercury in solution and solid matrices, and the results obtained by the participants. The different analytical steps of the methods used (extraction, clean-up, separation, final detection) are compared and assessed.

Keywords: Methyl-mercury, intercomparisons, improvement, certification, biological materials

INTRODUCTION

Methylmercury (MeHg) may be directly released in the environment (e.g. in MinaMata, Japan, from polyvinyl wastes) or originate from the biomethylation of inorganic mercury in biological tissues.[1,2] This highly toxic compound accumulates in the food chain and affects biota and humans. Therefore MeHg has to be determined accurately in environmental matrices and food. In some countries, legislation on MeHg in food is considered to be preferable to legislation on total Hg; so far such legislation has been impossible because of the lack of reliability and accuracy of existing methods. Hence, there is an urgent need for validating analytical methods for MeHg determination. Many methods have been described but a systematic collaborative investigation of their performance for the analysis of fish and mussel tissues has shown that, although the results were considered to be acceptable by the participants,[3] the coefficient of variation between laboratories was in the range 20–25%, which is not sufficient to allow an accurate comparison of data to be made. There are various ways to determine MeHg, the majority of these consisting of an extraction, a separation and an identification/quantification step. Extraction is often performed with a lipophilic solvent or the organic sample is destroyed in an alkaline solution followed by a selective reduction of inorganic mercury (e.g. with $SnCl_2$). The separation and identification can be carried out by gas chromatography, ion exchange or high-pressure liquid chromatography. Techniques such as cold-vapour atomic absorption spectrometry (after selective separation), electron capture detection and mass spectrometry are generally used for the detection and quantification. Radiochemical methods and headspace gas chromatography are also applied. The complexity of the methods and the multiplicity of analytical steps are the reasons why errors are easily made.

In view of the urgent need for the improvement of the quality of the analyses, a project for MeHg has been discussed and designed with a group of analysts in the framework of the BCR programme (Community Bureau of Reference) of the Commission of the European Communities (EC). The programme of work was set up in analogy to the work of the BCR group on chlorinated biphenyls (CBs).[4] In particular, the various steps of the analytical methods, e.g. extraction, clean-up and separation, were studied individually by each of the participants. To do so, three intercomparisons were organized of which the contents, the main

* Present address: dkTEKNIK, 15 Gladsaxe Møllevej, DK-2860 Søborg, Denmark.

0268–2605/93/060413–08 $09.00

Received 26 March 1993
Accepted 15 May 1993

issues, the conclusions and the planned continuation are presented in this paper.

AIM OF THE PROJECT

The aim of the BCR programme of the EC is to assist in the improvement of measurement in the EC member states. A significant part of this programme (now the Measurements and Testing programme) includes the organization of series of intercomparisons and certification exercises with appropriate laboratories.

In May 1987, the BCR invited a number of analytical laboratories to identify current needs to improve the quality of the determination of MeHg and to discuss the possibility of undertaking a series of analytical exercises which, if successful, would lead to certification.

One of the most powerful tools in detecting and removing sources of error due to a particular technique or a lack of quality control (QC) within a laboratory is to participate in intercomparisons.[5,6] In general, besides the sampling error, the following main sources of error can be detected in speciation analyses:[7]

(a) instability of compounds during storage and sample drying (volatilization, degradation);
(b) sample pretreatment (e.g. incomplete extraction, change of original speciation, losses in clean-up);
(c) derivatization (inhibition, incomplete transformation, decomposition);
(d) separation (decomposition of the species, adsorption on the column, peak overlap);
(e) final measurement (e.g. calibration errors, spectral interferences, background corrections);
(f) laboratory conditions (e.g. training and educational level of workers, care applied to the work, awareness of potential problems, management, clean room or bench facilities).

When different laboratories participate in an intercomparison, different sample pretreatment methods and different techniques of final determination are compared and discussed as well as the laboratory's performance. If results of such an intercomparison are in good and statistical agreement, the collaboratively obtained value is likely to be the best approximation of the actual value.

An intercomparison can be held (1) to detect the problems of a commonly applied method and to ascertain its performance in practice, (2) to measure the quality of a laboratory or a part of a laboratory (e.g. audits for accreditation of laboratories), (3) to improve the quality of a laboratory in collaborative work in a mutual learning process and (4) to certify the contents of a reference material. The programme described here is of type (3).

PARTICIPATING LABORATORIES

The preparation of the fish extracts and the verification of their homogeneity and stability were carried out by Danish Isotope Centre and the National Food Agency (Søborg, Denmark). The aqueous solutions were prepared at the Kernforschungsanlage (Jülich, Germany) and the mussel and tuna samples were prepared at the Joint Research Centre (Ispra, Italy).

The following laboratories participated in the intercomparison (in alphabetical order):

Bundesforschungsanstalt für Fischerei, Hamburg, Germany
Danish Isotope Centre, Copenhagen, Denmark
IFREMER, Nantes, France
Institut Jozef Stefan, Ljubjana, Slovenia
Kernforschungsanlage, Jülich, Germany
Leicester Polytechnic, School of Applied Physical Sciences, UK (now De Montfort University, Leicester, UK)
MRC Toxicology Unit, Carshalton, UK
National Food Administration, Uppsala, Sweden
National Food Aency, Søborg, Denmark
Presidio Mutlizonale di Prevenzione, La Spezia, Italy
Presidio Multizonale di Prevenzione, Venezia, Italy
RIKILT, Wageningen, The Netherlands
Universidad Complutense, Madrid, Spain
Universidad de Santiago de Compostella, Spain
Universita di Genoa, Italy
Vrije Universiteit Brussel, Lab. voor Anal. Scheikunde, Belgium

THE PROGRAMME AND TIMETABLE

From the beginning it was clear that it was essential to examine critically each step in the methods for MeHg determination. To do so, series of samples were prepared (1) to test the detection methods with solutions of known composition of mercury compounds, (2) to test the performance of the separation using cleaned extracts, (3) to verify the clean-up procedures by performing analysis of raw extracts and (4) to test the total analytical procedure by analysing real samples. These evaluations were carried out in three intercomparisons, the first of which started in 1987 (simple solutions) and the second in 1988 (cleaned and raw extracts); the third "round-robin" exercise was carried out in 1989 (extracts and real samples). Youden plots[8] in some cases supported the technical conclusions.

PREPARATION OF THE MATERIALS

First intercomparison

In the first intercomparison three solutions were studied.

(1) Solution 1 contained about 10 mg kg^{-1} of CH_3HgCl in toluene, and solution 2 was a mixture of *ca* 10 mg kg^{-1} of each of CH_3HgCl, C_2H_5HgCl and C_6H_5HgCl. These solutions were prepared by the Danish Isotope Centre, Denmark, by dissolving the above-mentioned mercury compounds in 5 ml of dimethyl sulphoxide (DMSO) and in 10 litres of toluene. Samples were provided in 250-ml bottles protected against light.

(2) Solution 3 was an aqueous solution containing 2 mg kg^{-1} of CH_3HgCl and $HgCl_2$. The optimal NaCl and HCl concentrations to avoid adsorption were studied. This sample was prepared in the KFA in Jülich, Germany.

Second intercomparison

Approximately 4 kg of flounder was purchased at Sletten Havn located in the Niva Bay, 30 km north of Copenhagen, where high levels of total mercury in fish tissues had been reported previously. The fish sample was mixed with redistilled water, homogenized and stored at −20 °C. Six subsamples of homogenate, each of 0.2 g were analysed for total mercury by neutron activation analysis with radiochemical separators (RNAA). The total content was found to be 191 ± 20 ng g^{-1} (as Hg) on a wet mass basis. Extracts were then prepared by the Danish Isotope Centre, Denmark and the stability of MeHg was verified by the National Food Agency, Denmark). Another batch of aqueous solutions as described under the first intercomparison protocol was prepared by KFA in Jülich, Germany, and sent to the participants. The samples were prepared according to the following routine.

Raw extract
Subsamples of 30 g of fish homogenate were mixed with 80 ml HCl and 20 ml $CuSO_4$. This mixture was shaken, left to react for 15 min and extracted three times with toluene to obtain *ca* 37 litres of extract which was dried by addition of anhydrous Na_2SO_4 and stored at 5–10 °C. The samples were bottled in 500-ml light protected borosilicate bottles with PTFE gaskets in the screw cap.

Raw extract spiked with MeHg
Approximately 2500 ml of the raw extract was spiked with CH_3HgCl and dissolved in DMSO to obtain a concentration of about 0.011 µg/ml. Samples were bottled in 250-ml light protected borosilicate bottles.

Cleaned extract
Subsamples of the raw extract were extracted twice with 150 ml of cysteine acetate. After acidification with HCl, the mixtures were back-extracted twice with toluene. This cleaned extract was dried by addition of anhydrous Na_2SO_4 and samples were distributed in 100-ml bottles.

Aqueous solution
Portions (1 mg) of each of CH_3HgCl and $HgCl_2$ were dissolved in water containing 30 g l^{-1} NaCl and 25 ml l^{-1} HCl. Samples were bottled in 250-ml borosilicate glass bottles with screw caps and stored in the dark at ambient temperature.

All the samples were provided with solid CH_3HgCl calibrants (purity >99.9%).

Third intercomparison

Cod homogenate (4.6 kg) was prepared as described before from samples fished in the Koege Bay, south of Copenhagen. The total mercury

concentration determined by cold-vapour atomic absorption spectroscopy three times in the homogenate (CV AA) was *ca* 190 ng g^{-1}

Raw extract
Portions of 30 g of fish homogenate were treated with 80 ml HCl and 20 ml $CuSO_4$ solution, shaken and left to react for 15 min. This mixture was extracted three times with about 80 ml of toluene per extraction. Portions were bulked to 1500 ml of toluene extract and were then extracted with 400 ml of cysteine acetate solution. After separation, the cysteine acetate solution was acidified and extracted twice with 95 ml of toluene. The volume of toluene produced by bulking up was 4000 ml.

Method and reagent blank solution
This was produced alternately with the actual extraction, using the same glassware and reagent.

Calibrant solution
MeHgCl was dissolved in toluene in order to obtain a stock solution with a concentration of 44.43 μg g^{-1}. This solution (10.5 g) was diluted with toluene to obtain a calibrant solution of 266.9 ng g^{-1}.

Spiked extract
Stock solution (4.4 g) was added to the toluene extract (raw extract) to obtain a concentration in the spiked extract of $(0.9972x + 123.77)$ ng g^{-1} of MeHgCl, where x is the concentration in the toluene extract.

The four toluene extracts were bottled in 50-ml light-protected borosilicate bottles with PTFE gaskets in the screw cap.

In addition to these samples, mussel and tuna samples were prepared at the Joint Research Centre of Ispra, Italy. The mussel flesh (wet weight) was collected and minced, whereas the tuna fish was filleted; the samples were frozen, freeze-dried, ground and homogenized. The two samples were bottled in brown borosilicate bottles each containing 15–20 g.

HOMOGENEITY AND STABILITY TESTS

First intercomparison

The stability of solutions 1 and 2 was verified on the content of ten bottles stored at 4 °C in the dark over a period of eight weeks at the National Food Agency, Denmark. Analyses were performed by packed-column gas chromatography followed by electron capture detection. Determinations were performed in three replicates on each of three bottles.

Stability tests of solution 3 (aqueous solution) were performed at the KFA (Jülich, Germany) by ion exchange/CV AA. In a first stage, storage experiments at 0 °C and at ambient temperature were carried out but no measurable effects of temperature were observed. However, significant losses of mercury were observed after a 100-fold dilution of the solution containing approximately 2 mg l^{-1} of MeHgCl and inorganic mercury; therefore it was recommended that the solution should be diluted only shortly prior to the determination. The stability was verified over three months on the content of one bottle and no significant changes were observed for either MeHg or inorganic mercury ambient temperature. All determinations were performed on seven replicates.

Second intercomparison

The stability of the extracts stored at 4 °C in the dark was verified five months after the preparation at the National Food Agency, Denmark. Analyses were performed by packed-column gas chromatography followed by electron capture detection either by direct injection (cleaned extract) or after clean-up with cysteine/toluene (raw and spiked raw extracts) in three replicates in each of three bottles.

Stability tests of the aqueous solution were performed at the KFA, Germany. The stability was verified over two months on the content of one bottle (ten replicate analyses) and no significant changes were observed for MeHg.

Third intercomparison

The stability of the calibrant solution and extracts was verified over a period of five months at the National Food Agency, Denmark. Analyses were performed by packed-column gas chromatography followed by electron capture detection in five replicates on each of three bottles.

Stability of freeze-dried fish sample

It is important to know the stability of MeHg in freeze-dried biological samples when preparing a certification campaign. Preliminary experiments

Table 1. Summary of techniques used in the intercomparison

Pre-treatment/extraction	Separation	Detection
HCl or $Na_2S_2O_3$, UV irradiation	Ion-exchange chromatography	CV AA
Toluene, cysteine acetate	Packed GC or capillary GC	ECD
Cysteine/$Na_2S_2O_3$	Packed GC	MIP
$Na_2S_2O_3$	None (total organic Hg)	ET AA
Westöö extraction	None (total organic Hg)	RNAA

have been performed at the Danish Isotope Centre on a laboratory reference material (RM) of freeze-dried tuna fish. No instability of the MeHg content in the RM stored at ambient temperature could be observed after three years. Both total organic mercury and methyl/phenylmercury were determined at regular intervals by RNAA.[9,10]

ANALYTICAL METHODS

Table 1 summarizes the different techniques of separation and final determination used by the 16 laboratories from ten European countries which participated in the programme (see above). The extraction techniques were based on solvent or acid/solvent extraction (e.g. HCl/toluene, H_2SO_4/toluene, toluene/cysteine/toluene). Separation was generally performed by packed-column gas chromatography (e.g. 5% DEGS/PS on Supelcoport 100–120 mesh, 5% PDEAS on Chromosorb W AWDCMS) or capillary gas chromatography (e.g. polar cyanophenylsilicone phase OV-275, methylsilicone HP-1, CP SIL 8CB). The final determination was made by neutron activation analysis with radiochemical separation (RNAA), electron capture detection (ECD), cold-vapour atomic absorption spectrometry (CV AA) or electrothermal AA.

RESULTS OF THE INTERCOMPARISONS

The results submitted in the different intercomparisons were discussed amongst all participants in technical meetings. Each laboratory which participated in each of the intercomparisons was requested to make a minimum of five independent replicate determinations. The results were presented in the form of bar-graphs indicating the laboratory codes along with the methods used, the means of the individual laboratories and the mean of the laboratory means with the corresponding standard deviations; Figs 1(a) and 1(b) give examples of bar-graphs used in the technical discussion (MeHg in mussel and tuna fish, respectively).

First intercomparison

The first intercomparison on solutions did not reveal any major discrepancies in the results and hence in the final methods of determination used. The mean of laboratory means was in all cases very close to the value expected upon preparation ($10.1 \pm 0.08\ \mu g^{-1}$ as MeHgCl for solution 1; $12.7 \pm 1.2\ \mu g\ g^{-1}$ as MeHgCl for solution 2; $2.13 \pm 0.26\ \mu g\ g^{-1}$ as MeHgCl for solution 3). Table 2 lists the coefficient of variation (CV) obtained between laboratories: both for solutions 1 and 2, the CVs obtained (8.0 and 8.9% respectively) were considered to be acceptable. In the case of the aqueous solution (solution 3), a CV of 12.3% was found to be too high for the present state of the methodology. On the basis of these results, it was decided to organize a second intercomparison on fish extracts and to repeat the exercise on aqueous solutions.

Second intercomparison

Analyses of extracts led to difficulties mainly because of a lack of good long-term reproducibility for many laboratories. Capillary GC was found to offer good possibilities but its use was hampered by the absence of commercially available columns. Furthermore, sources of error were probably attributable to losses of MeHg. A Youden plot of raw and spiked extract demonstrated systematic errors (Fig. 2) which were illustrated by the high CVs found between laboratories (Table 2: 16.6 and 17.4% for raw and spiked raw extracts, respectively). Better results were obtained for the cleaned extract (12.5%) but the

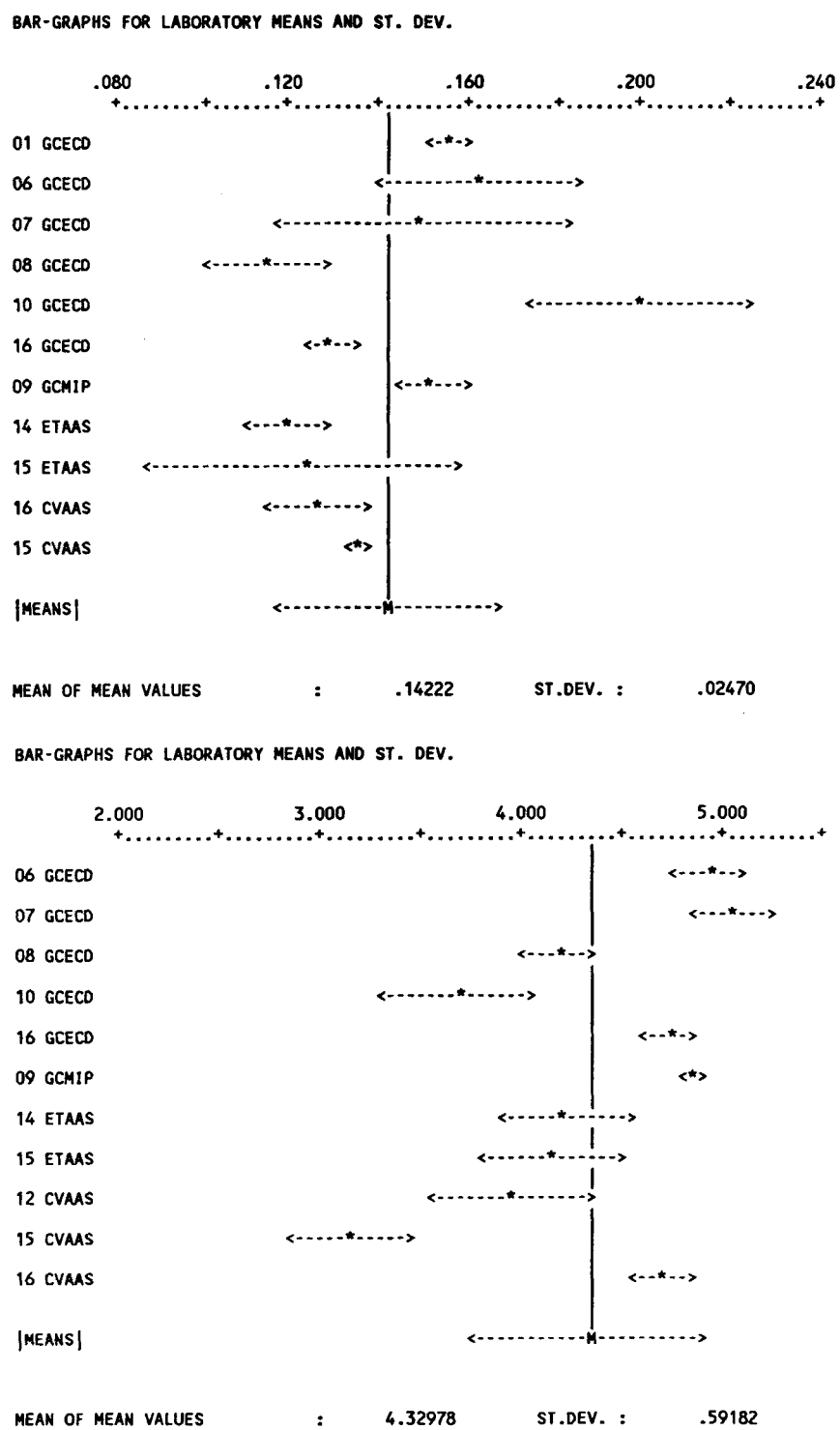

Figure 1 Bar-graph of MeHg in (a) mussel tissue and (b) tuna fish. The laboratory codes are given along with the methods used (abbreviations as in Table 1). The results plotted correspond to five replicate determinations. *M* is the mean of the laboratory means.

Table 2. Results of the three intercomparisons

First round

Solution 1		Solution 2		Solution 3	
CV[a] (%)	Range[b]	CV (%)	Range	CV (%)	Range
8.0	1.3	8.9	1.2	12.3	1.4

Second round

Raw extract		Spiked extract		Cleaned extract		Aqueous solution	
CV (%)	Range	CV (%)	Range	CV (%)	Range	CV (%)	Range
16.6	1.6	17.4	1.6	12.5	1.5	8.4	1.3

Third round

Raw extract		Spiked extract		Mussel tissue		Tuna fish	
CV (%)	Range	CV (%)	Range	CV (%)	Range	CV (%)	Range
11.3	1.7	8.8	1.4	17.4	1.7	13.7	1.6

[a] Coefficient of variation (%) between laboratories. [b] Ratio (higher value/lower value).

spread was still considered to be too high. However, a consequent improvement was obtained for the aqueous solution analysis (CV 8.4%).

Owing to the high spread of results obtained for the MeHg determinations in the extracts, it was

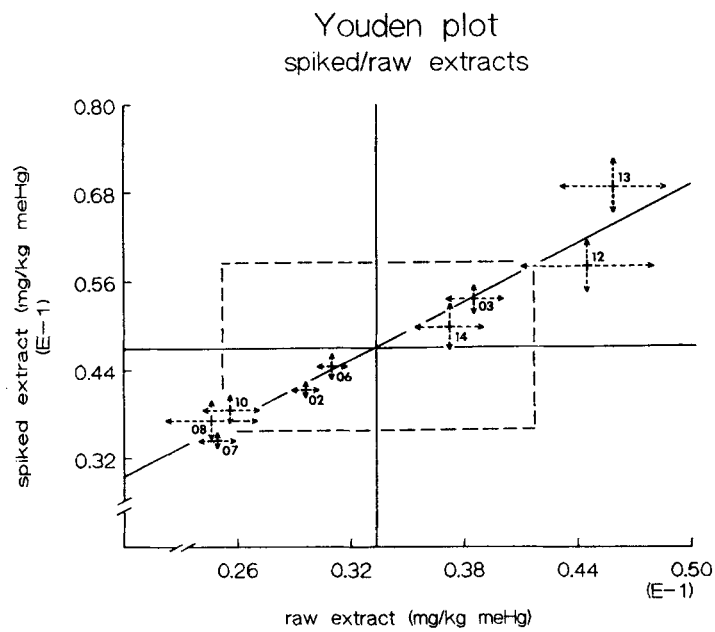

Figure 2 Youden plot. MeHg in spiked extract versus MeHg in raw extract. The horizontal and vertical continuous lines are the means of the laboratory means, the broken lines being the standard deviations of these means. The lengths of the bars are equal to the standard deviation (five replicates) of the laboratories.

decided to organize a third "round-robin" exercise to attempt to improve the position.

Third intercomparison

Sources of discrepancies were found for the analysis of the matching calibrant, the most important one being the inadequacy of the packed chromatographic columns. The CV obtained for raw data was 13.7% (Table 2); however, the results improved to 6.3% after outliers had been removed (on technical grounds). The results obtained with CP-SIL 8 capillary columns appeared in most cases better than the ones using packed columns. It was stated that a precision (as CV) of *ca* 3–4% can be achieved, with CP-SIL 8 capillary columns lasting for one to two years using proper optimization. Extensive work was carried out to evaluate the CP-SIL 8 columns;[11] the results showed that the use of an on-column insert is recommended to avoid losses of mercury due to contact with hot metal surfaces in the injector. The experiments suggested also that it is important to use capillary columns with a thick film, considering that such film reduces the contact between the volatized mercury and a silica column that is possibly not entirely deactivated.

An additional source of error was calibration, which should be done systematically using the compound to be determined (e.g. with MeHgCl and not with $HgCl_2$).

The CVs between laboratories (Table 2) showed, however that the results were improved in comparison with the second round-robin exercise (11.3% instead of 16.6% for the raw extract, and 8.8% instead of 17.4% for the spiked raw extract).

The mussel and tuna analyses were used to test the long-term reproducibility of the laboratories [Figs 1(a) and 1(b)]. It was noted that interferences were systematically higher with mussel tissue but the higher CV obtained (17.4% in comparison with 13.7% for tuna fish) could also be due to the fact that the MeHg content of mussel was much lower that that of tuna fish ($0.14 \pm 0.01\ \mu g\ g^{-1}$ as MeHgCl in mussel and $4.33 \pm 0.11\ \mu g\ g^{-1}$ as MeHgCl in tuna fish). The use of cysteine paper was also recommended in order to remove impurities by washing repeatedly with toluene while MeHg is immobilized on the cysteine paper.

FURTHER DEVELOPMENT

This series of interlaboratory exercises enabled the identification of some sources of error occurring in the determination of MeHg in fish extracts and biota samples (mussel and tuna) which in turn allowed an improvement of the state of the art. Considering this improvement and the need for supporting good-quality control in MeHg determination in fish tissues, the BCR decided to organize a certification campaign of two tuna fish materials; this was successfully concluded in March 1993. the materials were collected in the Adriatic Sea and frozen; the dorsal muscles were taken, ground, freeze-dried and homogenized, and the homogeneity and stability were verified. It is expected that the two CRMs, containing *ca* $3\ \mu g\ g^{-1}$ (as MeHg) and $5.4\ \mu g\ g^{-1}$ (as MeHg) respectively, will be available before the end of 1993. The results of the certification will be published in due course.

Acknowledgements The collaboration with C Vestergaard, J H Petersen and M Stoeppler for the preparation of the fish extracts and aqueous solutions is gratefully acknowledged. The authors wish to thank the participants in the programme for all their effort, which enabled a considerable improvement of the state of the art of MeHg analysis to be made.

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