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WORKING METHODS PAPER

An Attempt to Certify Phenyltin Compounds in a Mussel Reference Material

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Organotin contamination of the marine environment can be considered one of the main environmental problems in the last 20 years. Starting from the 'Arcachon case', monitoring campaigns have been carried out worldwide in order to evaluate the concentration levels of these compounds in the marine environment. In 1987 the EC Standards, Measurements and Testing Programme (formerly BCR) started a 'tin speciation' project to assess and improve the quality of measurements in this field and to make available reference materials. Ten years of study within the framework of this project have produced results in the fields of method development and production of reference materials, particularly for butyltins. The quality of the phenyltin analytical results was lower than for butyltins and further efforts are still needed. To overcome this shortfall, the last certification campaign was aimed towards the certification of phenyltins, as well as butyltins, in a candidate mussel certified Reference Material. The results of the homogeneity and stability tests for phenyltins and of the certification campaign are presented here. The preparation of the material and the analytical methods used in the certification campaigns are also described. The certification of phenyltins was hindered by their lack of long-term stability; nevertheless, the exercise provided information about the state-of-the-art of phenyltin measurements in biological samples. © **1998 John Wiley & Sons, Ltd.**

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INTRODUCTION

The toxic impact on marine organisms of tributyltin (TBT) leached from antifouling paints has become well known since the first identification of its effect on oysters in Arcachon Bay (France) in the 1980s.[1] Other tin compounds are known to be very toxic to marine life, e.g. triphenyltin, and these compounds, along with their degradation products, are currently monitored routinely by some laboratories to control the levels of environmental contamination. These analyses are particularly called for in support of some EC Directives.[2] A wide variety of analytical techniques has been developed within the last decade for the determination of organotins; these methods involve several analytical steps, such as extraction, derivatization, separation and final detection, which enhance the risks of analytical errors.[3] A programme for evaluating the performance of these methods has been organized by the Community Bureau of Reference (BCR; now the Standards, Measurements and Testing Programme) of the European Commission.[4] In the preliminary stage, interlaboratory studies aimed to evaluate and improve the state-of-the-art of TBT determinations in solutions and TBT-spiked sediment; this was followed by two certification campaigns on butyltin content in sediment. The first candidate material, harbour sediment (RM 424),[5] could not be certified because of analytical difficulties and low TBT levels, whereas the second material (coastal sediment, CRM 462) was certified for its content of DBT and TBT[6] and it is now available on the market as well as the PACS-I material [from the

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National Research Council of Canada (NRCC)] that was the first sediment reference material certified for butyltin species. At this stage, a group of European laboratories expressed the need for biological materials to be certified for their contents of butyl- and phenyl-tin compounds. The only material available (in limited stock) at present is the fish material NIES No. 11 from the National Institute for Environmental Studies in Japan,[7] which was not considered to be sufficient to meet the demand. Consequently, the BCR decided to launch a project designed to certify butyl- and phenyl-tins in a candidate reference material (CRM 477). While the butyltin content could be considered for certification, the phenyltin content could not be certified owing to a large spread of results and difficulties of material stabilization. This paper describes the preparation of the mussel reference material and the results obtained in checking the homogeneity and stability for phenyltins; a short discussion is given on the technical discussion of the interlaboratory study for mono-, di- and triphenyltin.

EXPERIMENTAL

Preparation of the mussel reference material

The harbour of La Spezia (Liguria, Italy) was selected for the collection of the mussel reference material, owing to its intensive maritime traffic and to the presence of one of the most important Italian mussel farms. Analyses performed on mussels collected during these campaigns showed high contents of organotin compounds in mussel tissues as a consequence of maritime activities.[8] A 1200 kg consignment of mussels (*Mytilus edulis*) was purchased directly from the La Spezia mussel farm in July 1991. After collection, the mussel samples were washed with fresh water to eliminate matrix salts which could interfere in the preparation process or the analysis. The samples were immediately frozen by immersion in liquid nitrogen. Shelling could not be performed by cooking or using a vapour stream since this treatment could have caused degradation of organotin compounds. It was preferred to shell the frozen materials directly by using special mussel knives. The edible part of the animal was placed in polyethylene bags which were heat-sealed (*ca* 4 kg per bag) and immediately stored at −25 °C. The frozen material (*ca* 325 kg) was transported to the Biostarters Company (Parma, Italy) where it was ground, using a PTFE-coated mill, and spread on sterilized flat trays for the freeze-drying treatment. The process involved dividing the homogenate into batches, resting the material at *ca* −55 °C for 6 h, then drying under vacuum for 48 h. Analyses were performed at the end of each freeze-drying process on samples collected from the top, intermediate and bottom levels of the trays, to evaluate the suitability of the process. The results showed that a moisture content of less than 4% was achieved. The freeze-dried material (final amount *ca* 35 kg) was put into polyethylene bags, heat-sealed, stored at −25 °C and transported to the Joint Research Centre at Ispra, Italy.

The freeze-dried material was ground for 15 days in a zirconia ball mill, taking all precautions to avoid contamination. It was then sieved to pass a 125 μm mesh with a titanium sieve in order to separate the fibrous part of the bulk material and mixed for 15 days under an argon atmosphere in a special polyethylene-lined mixing drum. The argon atmosphere was renewed after 5 and 10 days. The material was bottled under an argon atmosphere into brown Pyrex-glass bottles, remixing the sample for 30 min after 40 bottles had been filled. Bottles were set aside during the bottling procedure for the homogeneity and stability studies. One thousand bottles, each containing *ca* 15 g, were obtained.

Analytical procedure

Analyses for the homogeneity and stability studies were carried out as follows.[9,10]

The subsample (500 mg) was placed in a Pyrex vial (20 ml) and about 500 ng (as Sn) of internal standard (tripropyltin chloride in methanolic solution) was added. A mixture of 14 ml of a methalonic tropolone solution (0.03%, w/v) and 1 ml of concentrated HCl was then added and the vial, capped with a Teflon-lined screw-cap, was placed in an ultrasonic bath at a temperature lower than 40 °C for 15 min. The procedure was repeated twice, the supernatant being collected after centrifugation and transferred to a 250 ml cylindrical separatory funnel. Dichloromethane (25 ml) was added and the solution was shaken with 200 ml of a NaCl solution. This operation was repeated with another 25 ml dichloromethane aliquot. The organic phase was collected through anhydrous sodium sulphate, 1 ml of iso-octane was added and the liquid was concentrated down to few millilitres by

Table 1 Operating parameter for GC-MS analyses

Electron impact ionization mode (70 eV)	
Carrier gas	Helium
Head pressure	120 kPa
Column	HP-5 (0.20 mm i.d., 0.11 μm film thickness, 25 m length)
Temperature program	80°C × 2 min, then 10 °C min^{-1} to 280 °C
Injector	splitless, 240 °C;
Transfer line temperature	280 °C;
SIM (selected ions monitoring) operation	
Dwell time	100 ms for all ions

	Start time (min)	*m/z*
TPrT	8	277, 275, 273
TBT	10	305, 303, 301
DBT	12	319, 317, 315
MBT	12.9	319, 317, 315
Sn(IV)	13.8	333, 331, 329
MPhT	15	339, 337, 335
DPhT	16.5	345, 343, 341
TPhT	18.2	351, 349, 347

Rotavapor (Heidolph VV2000, Kelheim, Germany), then transferred to a 15 ml reaction vial. Iso-octane (2 ml) was added and the solution was brought to-near dryness under a moderate flow of nitrogen. An ethereal solution (1 ml) of 2 mol l^{-1} pentylmagnesium bromide was added and the vial was sonicated for 1 min, then placed in a 50 °C water bath under mechanical agitation; the reaction was allowed to proceed at least for 30 min. Hexane (1 ml) was added and the vial was put in a beaker half-filled with cold water. Distilled water (2 ml) was added carefully dropwise followed by 6–7 ml of 1 mol l^{-1} H_2SO_4. Derivatized organotins were extracted with 2–3 ml of hexane; the extraction was repeated twice. The organic phase was put in a vial and concentrated under a moderate flow of nitrogen to *ca* 0.5 ml. The extract was transferred to the top of a silica-gel column [3 g in a glass column (30 cm long, 8 mm i.d.)] previously wetted with 0.5 ml of hexane–benzene (1:1). Hexane–benzene (1:1) mixture was passed through the column until 5 mL were collected in a vial. Finally, the solution was concentrated under a moderate flow of nitrogen to *ca* 0.5 ml of which 1 μl was injected for GC–MS determination.

Instrumentation

GC–MS analyses were performed on an HP 5890 GC/HP 5970B MSD system with the operating parameters listed in Table 1.

The timings reported in Table 1 are only indicative and should be adapted to the particular instrumental conditions in use.

Peak identification was based on the matching of retention times (±0.5%) and isotopic mass ratios (±20%) for the diagnostic ions. The relative response factors were controlled by injecting standard mixtures on a regular basis (one injection every 3–4 samples) to follow the tuning conditions of the MS system.

With these chromatographic settings, the limit of detection (LOD) for TBT, DBT and MBT at a signal-to-noise ratio of 3 was approx. 8 pg injected. Phenyltin detection was somewhat more sensitive, particularly for TPhT (LOD = 1.5 pg), owing to the peculiar fragmentation pattern of these species.

Quality control

As mentioned above, quality control of organotins in biological materials is limited, owing to the lack of appropriate CRMs available with the exception of NIES 11, certified for TBT (the TPhT concentration was provided as an indicative value only). To ensure the best traceability of measurements, pentylated organotin calibrant solutions were prepared at the beginning of the study and stored at −20 °C in the dark[11] to serve as independent control solutions, since the stability of fully derivatized organotins far exceeds that of the starting compounds, particularly for phenyltins. Freshly prepared stock and working calibrant solutions were checked for degradation products using GC–MS after pentylation, and analysed with respect to the stored pentylated calibrant solutions. In order to establish the performance of the method, organotin working calibrant solutions were run

Table 2 Within- and between-bottle homogeneity (CV $\pm$ U_{cv}) at the 500 mg level of intake

Compound	Homogeneity, CV $\pm$ U_{cv} (%)[a]		
	Between-bottle[b]	Within-bottle[c]	Method of final determination[d]
MPhT	4.4 $\pm$ 0.7	3.4 $\pm$ 0.8	3.0 $\pm$ 0.7
DPhT	11.0 $\pm$ 1.8	8.3 $\pm$ 1.9	5.2 $\pm$ 1.2
TPhT	6.5 $\pm$ 1.0	3.5 $\pm$ 0.8	3.0 $\pm$ 0.7

[a] Uncertainty on the CVs: $U_{cv} = CV/(2n)^{1/2}$.
[b] Single determination on the content of each of 20 bottles.
[c] Ten replicate determinations on the content of one bottle.
[d] Seven replicates of an extract solution.

regularly throughout the whole analytical procedure. Single analytical steps were carefully checked for performance problems or deterioration of the materials used (e.g. clean-up for new batches of silica gel).

Recovery tests

Recovery tests were performed using freeze-dried mussel samples analysed before and after spiking with *ca* 150 ng (as Sn) of each compound.[9,10] Organotins, as methanol solutions, were added to the samples, which were previously wetted with distilled water; after the addition, the mussel samples were shaken for at least 30 min and allowed to equilibrate overnight. The recoveries were calculated with respect to the sum of the contents of the endogenous compounds and the spikes. Recoveries were 82 $\pm$ 17% for monophenyltin, 85 $\pm$ 14% for diphenyltin and 92 $\pm$ 9% for triphenyltin.

Recovery tests performed five years later, during the certification campaign, according to the guidelines reported below in the text, showed recoveries of 88% for MPhT, 104% for DPhT and 78% for TPhT.

Homogeneity study

The between-bottle homogeneity was verified by the determination of phenyltin compounds on subsamples of 500 mg taken from 20 bottles which were set aside at regular intervals during the whole bottling period. The within-bottle homogeneity was assessed by 10 replicate determinations on the well-mixed contents of one bottle. Each bottle was shaken manually for 5 min to eliminate segregation of particles which might have occurred during transport and storage.

The uncertainty of the method of separation and final determination was assessed from seven replicate determinations of each organotin compound on one extract solution; the coefficient of variation (CV) of the method, therefore, does not comprise the CV introduced by the extraction procedure.

As described elsewhere,[12] the results obtained for butyltins demonstrated a good homogeneity for these compounds. For phenyltins, the CVs obtained for MPhT, DPhT and TPhT are presented in Table 2. An *F*-test at a significance level of 0.05 did not reveal significant differences between the within- and between-bottle variances. The within-bottle

Table 3 Within- and between-bottle homogeneity (CV $\pm$ U_{cv}) at the 100 mg level of intake

Compound	Homogeneity, CV $\pm$ U_{cv} (%)[a]		
	Between-bottle[b]	Within-bottle[c]	Method of final determination[d]
MPhT	46.6 $\pm$ 11.7	32.0 $\pm$ 7.5	4.1 $\pm$ 0.9
DPhT	48.2 $\pm$ 12.1	29.0 $\pm$ 6.8	5.1 $\pm$ 1.1
TPhT	33.5 $\pm$ 8.8	5.2 $\pm$ 1.2	4.4 $\pm$ 1.0

[a] Uncertainty on the CVs: $U_{cv} = CV/(2n)^{1/2}$.
[b] Single determination on the content of each of 8 bottles.
[c] Nine replicate determinations on the content of one bottle.
[d] Seven replicates of an extract solution.

Table 4 Stability tests for phenyltins at −20, +4, +20 and +40 °C

Compound	Time (months)	Temperature (°C)	R_t[a]	U_t
MPhT	1	−20	0.99	0.02
	3	−20	0.98	0.04
	6	−20	0.97	0.13
	12	−20	0.35	0.02
	24	−20	0.31	0.07
	36	−20	0.27	0.13
	44	−20	0.23	0.10
	1	+4	0.85	0.03
	3	+4	0.59	0.04
	6	+4	0.54	0.03
	12	+4	0.22	0.02
	1	+20	0.77	0.04
	3	+20	0.50	0.08
	6	+20	0.60	0.08
	12	+20	0.17	0.03
	1	+40	0.39	0.13
	3	+40	0.17	0.04
	6	+40	0.14	0.02
	12	+40	0.03	0.03
DPhT	1	−20	0.80	0.13
	3	−20	0.99	0.08
	6	−20	1.10	0.10
	12	−20	nd[c]	nd[c]
	24	−20	4.33	0.18
	36	−20	7.67	0.37
	44	−20	7.13	0.31
	1	+4	0.82	0.05
	3	+4	0.92	0.12
	6	+4	1.31	0.07
	12	+4	nd[c]	nd[c]
	1	+20	0.99	0.24
	3	+20	0.89	0.10
	6	+20	0.82	0.18
	12	+20	nd[c]	nd[c]
	1	+40	1.72	0.65
	3	+40	0.39	0.05
	6	+40	0.23	0.10
	12	+40	nd[c]	nd[c]
TPhT	1	−20	0.96	0.09
	3	−20	0.95	0.03
	6	−20	1.02	0.02
	12	−20	0.71	0.04
	24	−20	0.65	0.04
	36	−20	0.68	0.23
	44	−20	0.53	0.07
	1	+4	0.95	0.10
	3	+4	0.88	0.11
	6	+4	1.00	0.03
	12	+4	0.59	0.09
	1	+20	0.99	0.10
	3	+20	0.76	0.05
	6	+20	0.75	0.04
	12	+20	0.44	0.10
	1	+40	0.60	0.05
	3	+40	0.47	0.02
	6	+40	0.31	0.03
	12	+40	0.19	0.02

[a] $R_t = X_t/X_0$, where X_t = mean of 3 replicates on each occasion of analysis (at +4, +20 or +40°C); X_0 = mean at start of stability study.
[b] $U_t = (CV_t^2 + CV_0^2)^{\frac{1}{2}}$. R_t; where CV_t = coefficient of variation of 3 replicates at time t; CV_0 = coefficient of variation at X_0.
[c] nd, Not determined.

CV is very close to the CV of the method and, therefore, no inhomogeneity of the material was suspected for these compounds at the preparation stage. However, instability problems (see below) made it advisable to repeat the homogeneity study before the certification exercise. Data obtained from the second homogeneity study (Table 3), performed four years after the first one, showed higher inhomogeneity, even though the two studies are not easily comparable since the level of intake of the second study was 100 mg instead of 500 mg. The second homogeneity study was performed at the 100 mg level of intake because many laboratories used a 100 mg sample intake during the certification analyses.

Stability

The stability was verified for butyltins and phenyltins. Bottles were kept at −20, +4 +20 and +40 °C, respectively, over a period of 12 months and the compounds were determined at regular intervals during the storage period. Tests were made at the beginning of the storage period and after 1, 3, 6 and 12 months; the tests were repeated after 24, 36 and 44 months of storage at −20 °C. Samples were analysed using the procedures detailed above for the homogeneity study; the compounds were determined by performing on each occasion of analysis one replicate determination in each of three bottles stored at different temperatures. Any change in the content of an analyte with time indicates an instability, provided that a good long-term reproducibility of the analytical method is obtained. Instability may be detected by comparing the contents of different analytes in samples stored at different temperatures on various occasions of analysis with the $t = 0$ values (at the start of the storage period). The results obtained on the samples at $t = 0$ were used as references for the results obtained on each occasion of analysis ($t = 1$, 3, 6 and 12 months for the samples stored at +4 +20 and +40 °C, and $t = 1$, 3, 6, 12, 24, 36 and 44 months for the samples stored at −20 °C) respectively.

Table 4 gives the ratios (R_t) of the mean values (X_t) of three measurements made after a period t at the different temperatures and the mean (X_0) obtained at the start of the stability study:

$$R_t = X_t / X_0 \qquad [1]$$

The uncertainty U_t has been obtained from the CV of three measurements obtained after the respective periods at each temperature:

$$U_t = (\mathrm{CV}_t^2 + \mathrm{CV}_0^2)^{1/2} R_t \qquad [2]$$

CV_t and CV_0 are the coefficients of variation of X_t and X_0, respectively.

In the case of ideal stability, the ratios R_t should be 1. In practice, however, there are some random variations due to the error on the measurement. Butyltins were shown to degrade drastically at +40 °C and to a lesser extent at +20 °C; the three butyltin compounds were, however, found to be stable at +4 °C and −20 °C.[12] With respect to phenyltins, none of the three compounds tested was found to be stable at the different temperatures tested; it is worth stressing, however, that TPhT and MPhT degradation in samples stored at −20 °C seemed to reach a plateau after a high degree of degradation occurred after six months of storage (Table 4).

The results of the homogeneity and stability studies hampered, for phenyltins, the possibility of certification. Anyway, the participating laboratories were invited to determine phenyltins in the candidate CRM 477 in order to provide information regarding the state-of-the-art of these measurements in biological samples.

Design of the interlaboratory study

Each laboratory that took part in the interlaboratory study was requested to make a minimum of five independent replicate determinations on at least two different bottles of the mussel reference material on different days.

Laboratories were furthermore asked to perform a recovery test, for the evaluation of the extraction efficiency and the derivatization yields. The participants were provided with procedures for blank, extraction efficiency and derivatization yields evaluation. In particular, for recovery evaluation, a strict protocol for the experimental conditions in which spiking experiments should be carried out was provided. The protocol, discussed in the first meeting, established the equilibration time, the spike solvent, the spike amount, the temperature and light conditions, and so on. Further details are provided later in the text.

One of the most critical aspects of speciation analysis is the lack of calibrants of suitable purity and stoichiometry for calibration and/or verification of derivatization yields. The purity of commercially available organotin compounds is often not adequate, while many of the alkylated derivates need to

be prepared in-house. It was felt that by providing ultrapure calibrants, synthesized and purified in large quantities by an expert laboratory, some errors related to calibration could be avoided. In order to ensure the best conditions for achieving accurate results in the certification of CRM 477, it was hence decided to prepare a set of organotin calibrants to be distributed to the certifying laboratories for quality control checks. The task was performed by the Department of Organic Chemistry of the Free University of Amsterdam; the purpose was to prepare highly purified butyltin and phenyltin compounds (in the form of salts) as well as their ethylated and pentylated derivatives for use as calibration and recovery tests. A detailed description of the preparation of calibrants, their purity etc. is reported elsewhere.[12]

Recovery tests

During the initial meeting of the project it was decided that a test for recovery evaluation was to be considered mandatory.

Participants using pentylation or ethylation as the derivatization technique were obliged to check the derivatization yield by using the derivatized compounds (ethylated and pentylated provided).

The evaluation of recovery had to be checked by spiking experiments. They were advised that underivatized spiking compounds should be used, and given the limited stability of the organotin salts, that the spike solution should be prepared fresh. Further advice was given as follows.

Preparation of the spiking solutions and the addition to the sample should be measured by weight rather than volume.

For a recovery estimate the participants should carry out a standard addition at three different levels (approximately once, twice, and four times the amounts already present in the sample).

A preliminary analysis should be performed in order to obtain an idea of the organotin concentration levels in the sample.

The same amount of sample should be taken for all spiking experiments.

Methanol (1–2 ml) should be added to each aliquot (100–500 mg) for sample rewetting. Then, 1 ml of methanol containing the spiking compounds should be added drop wise to the aliquots.

All the aliquots should be left overnight under mechanical agitation. All the aliquots should be analysed in the same day.

Participants using chromatographic techniques should submit chromatograms of the blank, of the calibration solution, of the internal standard if used, of the sample (CRM 477), and of the spiked sample for recovery evaluation.

For atomic absorption and other techniques with a short linear range there must be more than a single calibration point; at least bracketing should be used.

Linearity ranges and a calibration curve should be reported.

Results of recovery tests and their technical discussion highlighted the fact that experimental conditions in which spiking experiments are carried out strongly influence the recovery itself and that further efforts are needed in this field.

It is worth emphasizing that the best operating conditions and choice of suitable matrices on which spiking experiments should be carried out are still a controversial matter.

A good approach could be to exploit organotin-free matrices, in such a way that the presence of compounds bound to the matrix in different ways from spiked compounds does not affect the recovery evaluation. However, it is very difficult to find organotin-free matrices and when available (for instance, from remote areas such as Antarctica) they are usually too different in physicochemical composition from the samples to be analysed.

In any case, the guidelines aimed to provide a common protocol for spiking experiments rather than the best one (much efforts is still necessary to identify the best spiking procedure).

Analytical techniques

Techniques used by the participants were as follows.

Ethylation/GC–AAS

The sample (ca 0.1 g) was digested with 10 ml of 20% tetramethylammonium hydroxide (TMAH), buffering with 1 ml of HCl and 2 ml of sodium acetate/acetic acid (pH 4). Extraction was carried out by addition of 10 ml of methanol (with ultrasonic shaking for 1 h and mechanical shaking for 1 h). Recoveries were evaluated by spiking the mussel matrix with the different phenyltin compounds: MPhT (95%), DPhT (94%) and TPhT (98%). Derivatization was performed with 2% $NaBEt_4$ in 2 mol l^{-1} sodium acetate/acetic acid mixture (shaking for 1 h). Clean-up was with an alumina column. Separation was by capillary gas chromatography (CGC) using a column 30 m long, 0.32 mm i.d.; DB-5 as stationary phase, 0.25 mm film thickness; helium as carrier gas at 5 ml min^{-1}; and air and H_2 as make-up gases at 90 and 350 ml

min^{-1}, the column temperature ranged from 80 to 250 °C. Detection was by AAS (detector temperature 750 °C). Calibration was by standard additions, using organotin chloride calibrants in methanol.

Ethylation/CGC–FPD

First method

The sample (0.5 g) was extracted with 2.5 ml of methanol (mechanically shaking for 2 h) and 12.5 ml of 0.12 mol l^{-1} HCl (ultrasonic mixing for 1 h). A 0.5 ml portion of the methanolic extract was buffered with 100 ml ethanoate buffer (pH 4.8), derivatized with 0.2 ml of 2% $NaBEt_4$ solution in deionized water, and back-extracted with 0.3 ml of iso-octane (extraction into the organic solvent at 420 rpm for 45 min). Separation was by CGC using a column 30 m length, 0.25 mm i.d.; DB-1 as stationary phase (polydimethylsiloxane), 0.25 mm film thickness; N_2 as carrier gas at 0.7 ml min^{-1} and as make-up gas at 30 ml min^{-1}; an injector temperature of 290 °C; and a column temperature ranging from 80 to 270 °C. Detection was by FPD (detector temperature 290 °C). Recoveries were assessed by wetting 0.25 g of sample with 2 ml of methanol followed by addition of 1 ml of methanol solution containing the spiking compounds (four additions); results were 75% for MPhT and 77% for TPhT. Calibration was by standard additions, using organotin chloride compounds in methanol.

Second method

The sample (0.2 g) was extracted with 8 ml of acetic acid/H_2O. Ethylation was performed with 0.75% $NaBEt_4$ solution, simultaneously extracting with 1 ml of nonane during a 3 min microwave exposure (40 W). TPrT was added as internal standard. The derivatization yield was verified with ethylated compounds. Clean-up was performed with an alumina column followed by elution with diethyl ether. Separation was by CGC (25 m column length, dimethylpolysiloxane as stationary phase, 0.17 mm film thickness; N_2 as carrier gas; injector temperature 250 °C; column temperature ranging from 120 to 280 °C). Detection was by FPD (detector temperature 350 °C). Recoveries were evaluated by spiking the reference material at three different levels; results were $81 \pm 8\%$ for TPhT. Calibration was by standard additions.

SFE/ethylation/CGC–FPD

The sample (ca 1 g) was extracted by supercritical fluid extraction with CO_2 and a mixture of acetic acid and 0.2% tropolone with hexane as solvent (pressure 50 atm, temperature 50 °C). Ethylation was carried out with 2 mol l^{-1} ethylmagnesium chloride. TPeT was added as internal standard. Separation was by CGC (column 30 m long, 0.25 mm i.d.; DB-17 as stationary phase, 0.25 mm film thickness; H_2 as carrier gas at 5 ml min^{-1}; N_2 as make-up gas at 30 ml min^{-1}; injector temperature 250 °C; column temperature ranging from 60 to 280 °C). Detection was by FPD (detector temperature 300 °C). Recoveries were assessed by standard additions; results $65 \pm 3\%$ for TPhT. Calibration was by calibration graph and standard additions, using the calibrants provided by SM&T.

Pentylation/CGC–FPD

First method

The sample (*ca* 0.2 g) was pre-treated with 50 ml HBr/H_2O mixture for 1 h and extracted into 50 ml of 0.05% tropolone in dichloromethane for 2 h under manual and mechanical shaking. Pentylation was carried out with 1 mol l^{-1} pentylmagnesium chloride for 1 h; the derivatization yield ranged from 85 to 119% (verified with pentylated compounds). Clean-up was performed with Florisil, the resulting extract being evaporated to dryness by rotary evaporation and under a gentle flow of N_2; redissolution was carried out in a Pe_2Me_2Sn solution in hexane. Separation was by CGC (column 15 m long, 0.53 mm i.d.; SPB-1 as stationary phase, 1.5 mm film thickness; He as carrier gas at 1.2 ml min^{-1}; injector temperature 250 °C; column temperature ranging from 80 to 250 °C). Detection was by FPD (detector temperature 300 °C). Recoveries were evaluated by three-level spikings of the mussel material; results were 166% for MPhT, 77% for DPhT and 99% for TPhT. Calibration was by standard additions.

Second method

The sample (ca 0.1 g) was extracted with 15 ml of 0.03% tropolone solution in methanol and 1 ml of 12 mol l^{-1} HCl by ultrasonic shaking for 15 min and centrifuging at 3000 rpm for 10 min; this was followed by addition of 15 ml dichloromethane and 100 ml of 5% NaCl, with manual shaking for 3 min. The resulting extract was reduced to 1 ml by rotary evaporation and to near-dryness under an N_2 flow. Pentylation was performed with 1 ml of 2 mol l^{-1} pentylmagnesium bromide in ethyl ether; the derivatization yields ranged from 85 to 119% (as verified with pentylated compounds). Tripropyltin was added as internal standard. Separation was by CGC (column 30 m long, 0.53 mm i.d.; methyl-

phenylsilicon as stationary phase, 1.5 mm film thickness; He as carrier gas at 9 ml min^{-1}; injector temperature 240 °C; column temperature ranging from 80 to 280 °C). Detection was FPD (detector temperature 240 °C). Recoveries were evaluated by three-level spikings; results were 88% for MPhT, 104% for DPhT and 78% for TPhT. Calibration was by calibration graph, using the calibrants supplied by SM&T.

Ethylation/CGC–MIP AES

First method

The sample (0.1 g) was digested with 5 ml of 25% tetramethylammonium hydroxide (TMAH) with stirring magnetic for 4 h at 50 °C. Derivatization was by addition of 20 ml of 0.1 mol l^{-1} acetate buffer (pH 5), 1.3 ml of acetic acid, 1 ml of 0.6% $NaBEt_4$ solution and 2 ml of hexane containing Pe_3EtSn as internal standard, shaking for 5 min and centrifuging at 3500 rpm for 3 min; the derivatization yield was verified by analysis of a spike (without mussel) and found to be 100% for all compounds except DPhT (44% only). Clean-up was performed with a column filled with alumina and elution with 0.5 ml hexane and 1 ml diethyl ether. The combined eluate was reduced to 0.5 ml using a gentle stream of N_2. Separation was by CGC equipped with programmed temperature vaporization (HP-1 column 25 m long, 0.32 mm i.d.; 0.17 mm film thickness; He as carrier gas; injector temperature 15–20 °C; column temperature ranging from 45 to 280 °C). Final detection was by MIP AES at 303.42 nm. Recoveries were assessed by spiking 0.1 g tissue with methanol solution, leaving it overnight and evaporating under an N_2 flow (three-level spiking); results were $68 \pm 8\%$ for MPhT and $75 \pm 8\%$ for TPhT. Calibration was by calibration graph, using ethylated butyl- and phenyl-tin compounds as organic salts in methanol.

Second method

The sample (*ca* 0.2 g) was digested with 15 ml of 25% TMAH by microwave leaching in pressurized vessels at 120 °C for 3 min. The pH was adjusted to 5 by addition of 10 ml of 1 mol l^{-1} acetic/acetate buffer, 3.8 ml of glacial acetic acid, 2 ml iso-octane and 2 ml of 1% $NaBEt_4$ (shaking for 5 min); this was followed by centrifugation at 2500 rpm for 5 min. Tripropyltin was used as internal standard. Clean-up was performed with alumina. Separation was by CGC, followed by MIP AES detection.

Ethylation/CGC–MS

The sample (*ca* 0.3 g) was extracted by addition of 15 ml of methanol, 1 ml of concentrated acetic acid and 10 ml of hexane. Derivatization was by addition of 5% $NaBEt_4$, with simultaneous extraction into hexane for 15 min. A small, constant flow of reagent was added continuously during the 15 min extraction; water as added to facilitate the transfer of the derivatized organotins into the organic phase. Clean-up was carried out with alumina oxide (10% water) and elution with hexane. The eluate was washed with 6 mol l^{-1} to remove by-products of the reaction, and then evaporated to near-dryness. Tripropyltin was added as internal standard. Separation was by CGC (column of 50 m long, 0.20 mm i.d.; HP–methylsilicon as stationary phase, 0.33 mm film thickness; He as carrier gas; injector temperature 250 °C; column temperature ranging from 70 to 270 °C). Calibration was by calibration graph.

Pentylation/CGC–MS

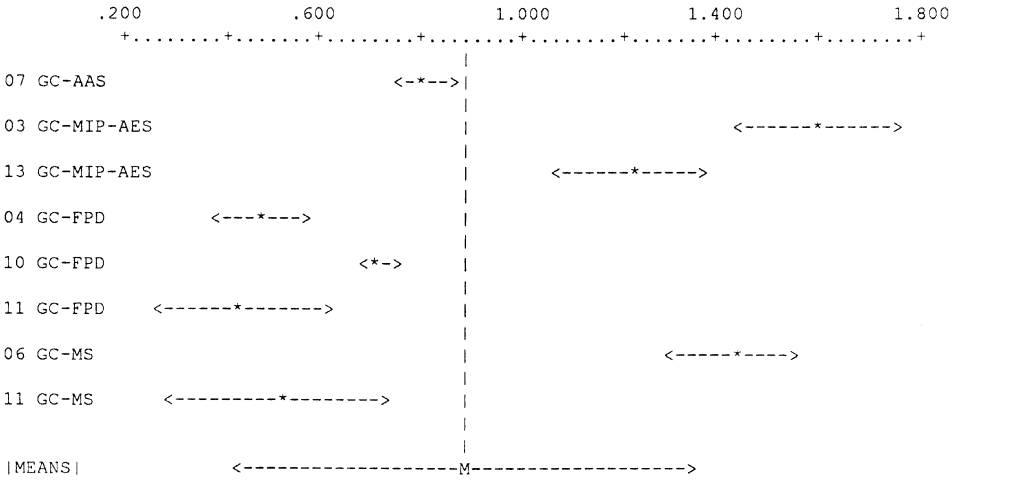
The sample (*ca* 0.2 g) was digested with 5 ml of dilute HCl, 12 ml of diethyl ether and 0.3 g NaCl. Extraction was by adding 2×12 ml of diethyl ether with 0.25% tropolone, followed by evaporation under an N_2 flow and drying with Na_2SO_4. Derivatization was performed with 2 mol l^{-1} pentylmagnesium bromide in diethyl ether. Clean-up was carried out with 5 g of 100% active alumina, eluting with 6 ml hexane/diethyl ether mixture. Ph_2SnEt_2 and Ph_3SnEt were added as internal standards. Separation was by CGC (column 30 m long, 0.25 mm i.d.; 5% phenyl/methyl-polysiloxane as stationary phase, 250 mm film thickness; injector temperature ranging from 60 to 200 °C; column temperature ranging from 60 to 280 °C). Detection was by MS (ion-trap MS). Recovery experiments were performed by spiking 0.2 g tissue at three levels; results were $78 \pm 6\%$ for MPhT, $80 \pm 26\%$ for DPhT and $58 \pm 4\%$ for TPhT. Calibration was by calibration graph, using pentylated organotin compounds.

HPLC–ICP MS

The sample (*ca* 1 g) was digested enzymatically with 0.05 g of lipase and 0.05 g of protease in a 0.1 mol l^{-1} citrate/phosphate-buffered medium (*ca* 40 ml) at pH 7.5; the mixture was kept overnight at 37 °C under mechanical shaking. Extraction was performed by three additions of 10 ml of dichloromethane. The extract was preconcentrated by rotary evaporation to dryness. Dilution of the dried extract was performed with 66% methanol and 33%

DATA SET	NUM	REPLICATES					MEAN	ST.DEV
07 GC-AAS		.80400	.72900	.82400	.88200	.78500		
	6	.80200					.80433	.04995
03 GC-MIP	5	1.84600	1.54600	1.47800	1.45000	1.64300	1.59260	.15999
13 GC-MIP		1.15600	1.41200	1.07400	1.23800	1.08000		
	6	1.39300					1.22550	.14963
04 GC-FPD	5	.44000	.39400	.51500	.41300	.63300	.47900	.09761
10 GC-FPD	5	.67900	.74400	.73400	.66600	.69400	.70340	.03416
11 GC-FPD	5	.62500	.29500	.56100	.45500	.22400	.43200	.17063
06 GC-MS		1.18000	1.18900	1.46700	1.47600	1.55400		
		1.49500	1.51500	1.49600	1.45900	1.40400		
	12	1.47900	1.40400				1.42650	.12049
11 GC-MS	5	.71600	.28100	.69300	.26300	.59200	.50900	.22141

BAR-GRAPHS FOR LABORATORY MEANS AND ST. DEV.



MEAN OF ALL INDIVIDUAL VALUES : .97708 ST.DEV. : .45197

MEAN OF MEAN VALUES : .89654 ST.DEV. : .45665

Figure 1 Monophenyltin (mg kg^{-1} as MPhT) in CRM 477.

mobile phase (70% MeOH and 30% of 0.03 mol l^{-1} aqueous citrate buffer). Separation was by HPLC (gradient elution, cation exchange with Partisil-10 SCX, 2 × 25 cm long, 4.6 mm i.d.; 10 mm particle size). Final detection was by ICP MS. Recoveries were assessed by spiking the CRM; the result was 27 ± 3% for TPhT. Calibration was by calibration graph, using TPhTCl as calibrant.

HPLC–fluorimetry

The sample (*ca* 0.25 g) was digested with 20 ml of 0.6 mol l^{-1} HCl and 2.5 mol l^{-1} NaCl in aqueous

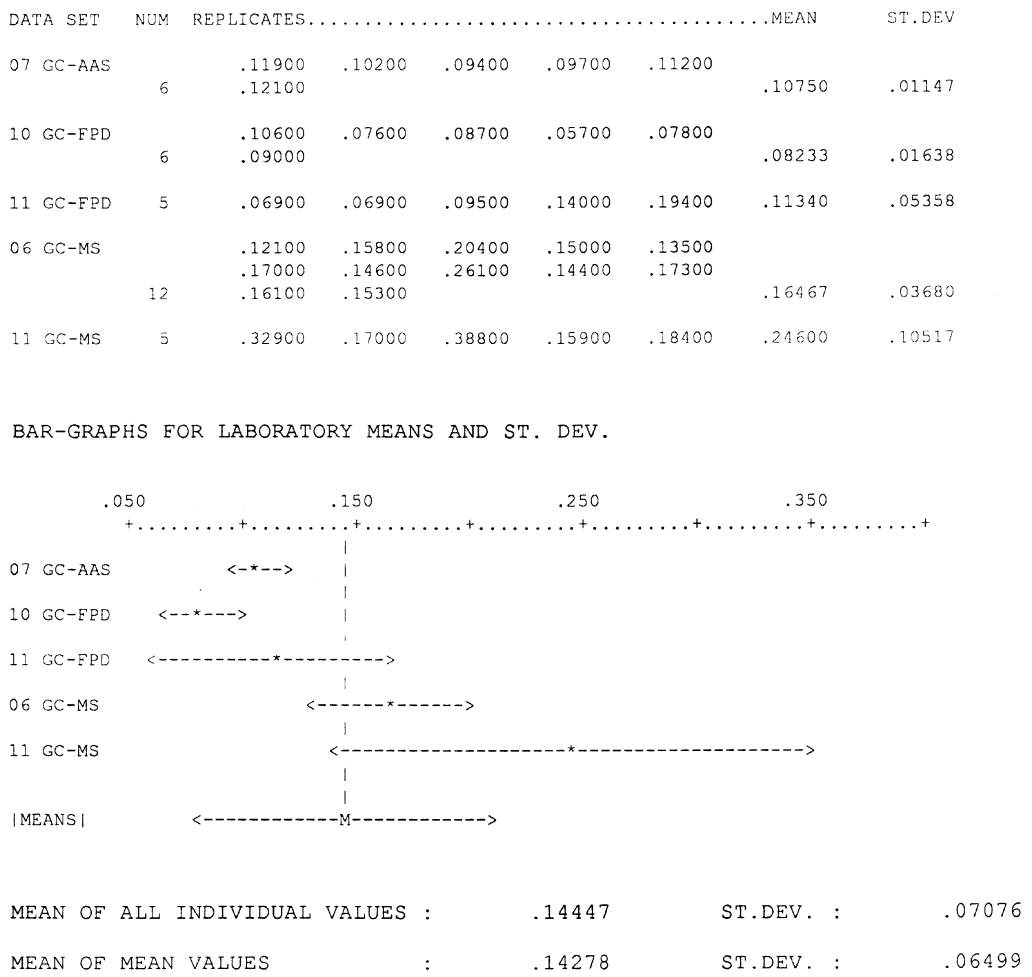


Figure 2 Diphenyltin (mg kg^{-1} as DPhT) in CRM 477.

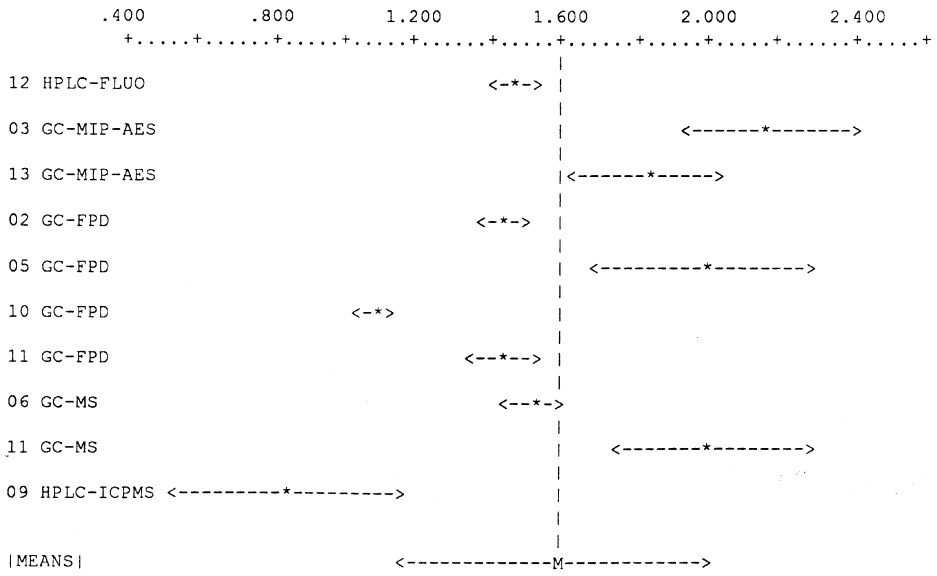
solution, and addition of 2 × 10 ml of ethyl acetate, followed by mechanical shaking for 30 min and centrifugation at 10000 rpm for 20 min. The combined extracts were washed with 10 ml of 0.5 mol l^{-1} $NaHCO_3$ and 1 mol l^{-1} NaCl, shaken manually for 2 min and centrifuged at 2000 rpm for 5 min. Ethyl acetate (5 ml) was added to the washing aqueous phase and the organic phase was evaporated to dryness by rotary evaporation at 35 °C, followed by addition of 2.5 ml of methanol. The filtered methanolic phase was injected into the HPLC column (cation exchange, Partisil SCX, 25 cm long, 4.6 mm i.d.; 10 mm particle size). Post-column derivatization was carried out with 0.02 mol l^{-1} Triton X-100 and 3,3′,4′,7-tetrahydroxy-flavone at a flow rate of 3 ml min^{-1}. Final detection was by fluorimetry. Recoveries were assessed by spiking the CRM at one level (three replicates); the result was 89 ± 3% for TPhT.

Analytical results

Laboratories using gas chromatography coupled with atomic absorption spectrometry (AAS), flame photometric detection (FPD) and mass spectrometry (MS) were able to provide results for all the phenyltin compounds. Laboratories using microwave inductively coupled plasma–atomic emission spectroscopy (MIP AES) after gas-chromatographic separation provided results for mono- and tri-phenyltin; diphenyltin results were not provided because of the extremely poor recovery (less than 30%) found in at least one out of two laboratories using this technique. Finally, the laboratories using

DATA SET	NUM	REPLICATES					MEAN	ST.DEV
12 HPLC-FLUO		1.40000	1.36000	1.46000	1.43000	1.55000		
	9	1.47000	1.49000	1.49000	1.50000		1.46111	.05711
03 GC-MIP	5	2.54300	2.23300	2.13000	1.92400	2.02700	2.17140	.23751
13 GC-MIP		1.81300	2.08800	1.53200	1.72400	2.01700		
	6	1.83700					1.83517	.20087
02 GC-FPD		1.52100	1.31700	1.43300	1.47400	1.44000		
	6	1.47000					1.44250	.06893
05 GC-FPD		1.71000	1.62000	2.10000	2.32000	2.33000		
	6	1.91000					1.99833	.30262
10 GC-FPD		1.06100	1.09600	1.14000	1.07200	1.09300		
	6	1.00800					1.07833	.04386
11 GC-FPD	5	1.42200	1.36900	1.52200	1.56100	1.33900	1.44260	.09604
06 GC-MS		1.47000	1.47000	1.66000	1.56000	1.63000		
		1.50000	1.54000	1.34000	1.53000	1.48000		
	12	1.52000	1.52000				1.51833	.08156
11 GC-MS	5	2.25500	1.70000	2.32700	1.80900	1.97600	2.01340	.27300
09 HPLC-ICPMS		.63000	.69000	.65000	.61000	1.02000		
	6	1.42000					.83667	.32383

BAR-GRAPHS FOR LABORATORY MEANS AND ST. DEV.



MEAN OF ALL INDIVIDUAL VALUES : 1.55535 ST.DEV. : .41458

MEAN OF MEAN VALUES : 1.57978 ST.DEV. : .42657

Figure 3 Triphenyltin (mg kg^{-1} as TPhT) in CRM 477.

liquid chromatography coupled with inductively coupled plasma–mass spectrometry (ICP MS) and fluorimetry were able to determine triphenyltin only.

Technical discussion

The results, presented in the form of bar-charts giving the code number of the laboratory and the abbreviation of the technique used, are reported in Figs 1–3.

For triphenyltin, the effect of the use of methanol and drying (blowing down) the sample in the derivatization step (Grignard reaction) was discussed. It seemed clear that, under certain conditions, degradation products could be formed which would lead to significant analytical uncertainties, particularly if the extraction/derivatization were too long. Degradation could be photo-induced.

A set of data obtained by SFE–GC–FPD was withdrawn since the technique was optimized for TBT but not for TPhT.

The relatively large standard deviation obtained in HPLC–ICP MS was discussed; difficulties in optimization of the HPLC conditions would have contributed to this poor precision. In order to safeguard against contributions from nearby peaks, peak heights had been used as the basis for quantification.

The relevance of the requested spike recovery test, practical aspects of the spiking experiments (e.g. choice of matrix, equilibration time, the spike solvent, the spike amount, the temperature and light conditions, etc.) and the problems of degradation of calibrants were discussed and will be the subjects of further studies.

Owing to the large spread of results and the demonstration of instability risks of this compound, it was decided not to give any value for TPhT. The between-laboratory CV after removing the sets mentioned above was 25%.

A large spread of results, a poor between-bottle homogeneity (CV *ca* 48%) and a lack of stability precluded the certification of DPhT, for which no indicative value is proposed (between-laboratory CV 43%).

The same situation was observed for MPhT (between-laboratory CV 56%) in comparison with DPhT, and a similar decision was taken.

CONCLUSION

The stability study of phenyltins in the candidate CRM 477, performed over 44 months, showed high degradation of these compounds even when they were stored at −20 °C. Consequently (owing to the different degradation from bottle to bottle), the inhomogeneity of the material increased over the same period.

Despite the fact that the certification was hindered by a lack of long-term stability of these compounds, the exercise provided information about the state-of-the-art of phenyltin measurements in biological samples. Furthermore, the technical discussion helped laboratories to identify possible sources of error, and provided a support for future improvements in the quality of these measurements.

An attempt to certify phenyltins, together with other organotin compounds, in sediment material is in progress.

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