APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 8, 451–461 (1994)

Simplified Sample Preparation for GC Speciation Analysis of Organotin in Marine Biomaterials

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Two simple sample preparation methods for the speciation analysis of triphenyltin and butyltin compounds in marine biotissues, using tetramethylammonium hydroxide (TMAH) solubilization and enzymic hydrolysis, have been developed and compared with conventional acid digestion. Derivatization was carried out *in situ* using sodium tetraethylborate ($NaBEt_4$) without prior separation of the analytes from the tissue matrix. Separation and detection was performed using capillary gas chromatography (GC) coupled to microwave-induced plasma atomic emission spectrometry (MIP AE) allowing detection limits of 2 ng g^{-1} (as tin) to be reached. The accuracy of the presented methods was demonstrated by the analysis of a fish reference material (NIES No. 11). the necessity for sample clean-up is discussed and examples of the analysis of mussel tissue are shown.

Keywords: Organotin, speciation, biological materials, sodium tetraethylborate, gas chromatography, atomic emission spectrometry, clean-up, enzymic hydrolysis, tetramethylammonium hydroxide

INTRODUCTION

Organotin compounds have been extensively used as biocides, especially in anti-fouling paints. Tributyltin (TBT) and triphenyltin(TPT), however, have been recognized as toxic not only to fouling organisms such as algae and barnacles, but also to some non-target marine biota.[1] Marine organisms such as bivalves tend to concentrate TBT from their environment and are therefore excellent sentinel organisms to monitor the environmental TBT levels available to marine organisms.[2]

The recognition of TBT toxicity was followed by environmental legislation and has stimulated the development of accurate and sensitive analytical methods for monitoring organotin concentrations. The techniques presently available combine a chromatographic separation with atomic absorption spectrometric (AA), mass spectrometric (MS), atomic emission spectrometric (AE) or flame photometric detection (FPD) and are usually termed 'hyphenated' techniques.[3–5]

Despite recent improvements in instrumentation, several limitations remain present on the level of sample preparation. Prior to GC, the organotin compounds need to be liberated from the biological matrix; this must be followed by a derivatization reaction to form species which can be separated by gas chromatography. Extraction of organotin compounds after acid leaching from the biological matrix followed by Grignard derivatization[6–8] or hydride generation[9–11] are the most common approaches. An alternative to acid leaching is the use of tissue solubilizers[12,13] [e.g. tetramethylammonium hydroxide (TMAH)] or enzymes[14,15] (e.g. lipase–protease mixture) to decompose the sample matrix. Sodium tetraethylborate ($NaBEt_4$) was recently proposed as a derivatizing reagent in the speciation analysis of organometals in environmental samples. It offers the advantage of *in situ* derivatization and extraction of the ethylated species into the organic phase and enables the development of faster and simpler sample preparation procedures.[16–21]

The different approaches for breaking down the biological matrix are revisited in the present paper. They are followed by a rapid *in situ* derivatization leading to two novel and simplified analytical methods, applied to the analysis of a reference fish tissue (NIES No. 11) and a mussel sample for nutritive consumption. An effective clean-up step is presented and the necessity of such a step in the analysis of biological materials is demonstrated.

CCC 0268–2605/94/050451–11

Received 30 October 1993
Accepted 22 December 1993

EXPERIMENTAL

Reagents

The sources and purity of the reagents and standards used have been described in detail elsewhere.[20] Samples were prepared in 60 cm^3 bottle-shaped, thin-necked (9 mm i.d.) vessels to enable easy recovery of the oranic phase for acid digestion and TMAH solubilization, and in 8 cm^3 test-tubes for enzymic hydrolysis. Sodium tetraethylborate ($NaBEt_4$) was obtained from Strem Chemicals (Bischheim, France). The reagent was kept in a desiccator and a 0.6% (w/v) solution was prepared daily. Alumina-B, Super I basic form, was obtained from ICN Biomedicals (Eschwege, Germany); TMAH (25% in water) was obtained from Fluka (Buchs, Switzerland). Protease Type XIV and lipase Type VII enzymes were obtained from Sigma (St Louis, USA). Fish tissue reference material (NIES No. 11) was obtained from Promochem (Wesel, Germany).

Citric acid/phosphate buffer solution (pH 7.5; 0.1 mol dm^{-3}; 5% ethanol) was prepared by dissolving 21.0 g citric acid monohydrate, 11.5 g ammonium dihydrogen phosphate and 64 cm^3 of ethanol in 1 dm^3 of water followed by pH adjustment with concentrated ammonia. Acetate buffer (pH 5; 0.1 mol dm^{-3}) was prepared by dissolving 8.2 g of sodium acetate in 1 dm^3 of water followed by pH adjustment with concentrated acetic acid.

Apparatus

Separation of the ethylated organotin species was performed using an HP Model 5890 Series II gas chromatograph (Hewlett–Packard, Avondale, USA) fitted with a capillary column (HP-1). The GC was equipped with a model KAS 503 programmed temperature vaporization (PTV) injection system (Gerstel, Mülheim, Germany). Injections were made by means of an HP 7673A automatic sampler for volumes up to 5 µl. Detection was performed with an HP Model 5921A atomic emission detector (AED).[22] The injection liners used were smooth deactivated glass tubes (93 mm × 1.25 mm i.d. × 2 mm o.d.). Samples were centrifuged in a Centra-CL (IEC, UK) centrifuge.

Procedures

A scheme of the different sample preparation procedures is shown in Fig. 1.

Acid digestion
To a 0.1 g tissue sample in a 60 cm^3 extraction vessel were added 2 cm^3 of concentrated hydrochloric acid and 20 cm^3 of a 0.1% solution of tropolone in hexane. The mixture was treated ultrasonically overnight. The phases were separated and the organic phase was evaporated to dryness. The residue was redissolved in 1 cm^3 of hexane containing Pe_3SnEt as the internal standard, then 50 cm^3 of the pH 5 buffer and 1 cm^3 of the 0.6% (w/v) $NaBEt_4$ solution were added and the mixture was extracted for 5 min.

Enzymic hydrolysis
A 0.1 g tissue sample was placed in a 8 cm^3 test-tube together with protease (Type XIV) and lipase (Type VII), 0.01 g each. Then 4 cm^3 of pH 7.5 buffer was added and the mixture was stirred magnetically for 4 h at 37 °C. After hydrolysis, 50 µl of acetic acid, 1 cm^3 of the $NaBEt_4$ solution and 1 cm^3 of hexane containing the internal standard were added and the mixture was extracted for 5 min.

TMAH solubilization
A 5 cm^3 portion of the TMAH solution was added to a 0.1 g tissue sample in a 60 cm^3 extraction vessel. The mixture was stirred magnetically for 4 h at 60 °C, then 20 ml of the pH 5 buffer, 1.3 cm^3 of acetic acid, 1 cm^3 of the $NaBEt_4$ solution and 1 cm^3 of hexane containing the internal standard were added and the mixture was extracted for 5 min.

Centrifugation
After 5 min of phase separation the samples were centrifuged at 3500 rpm for 3 min to enable easy recovery of the organic phase.

Clean-up
A Pasteur pipette was filled with alumina to form a plug of approximately 5 cm. Some silanized glass wool was inserted in the tip of the Pasteur pipette and on to the top of the alumina plug. The sample extract was introduced onto the clean-up column. After elution of the extract, an additional volume of 1 cm^3 of diethyl ether was put on the clean-up column. Argon gas was used to force the extract and the diethyl ether through the clean-up column. The diethyl ether was evapor-

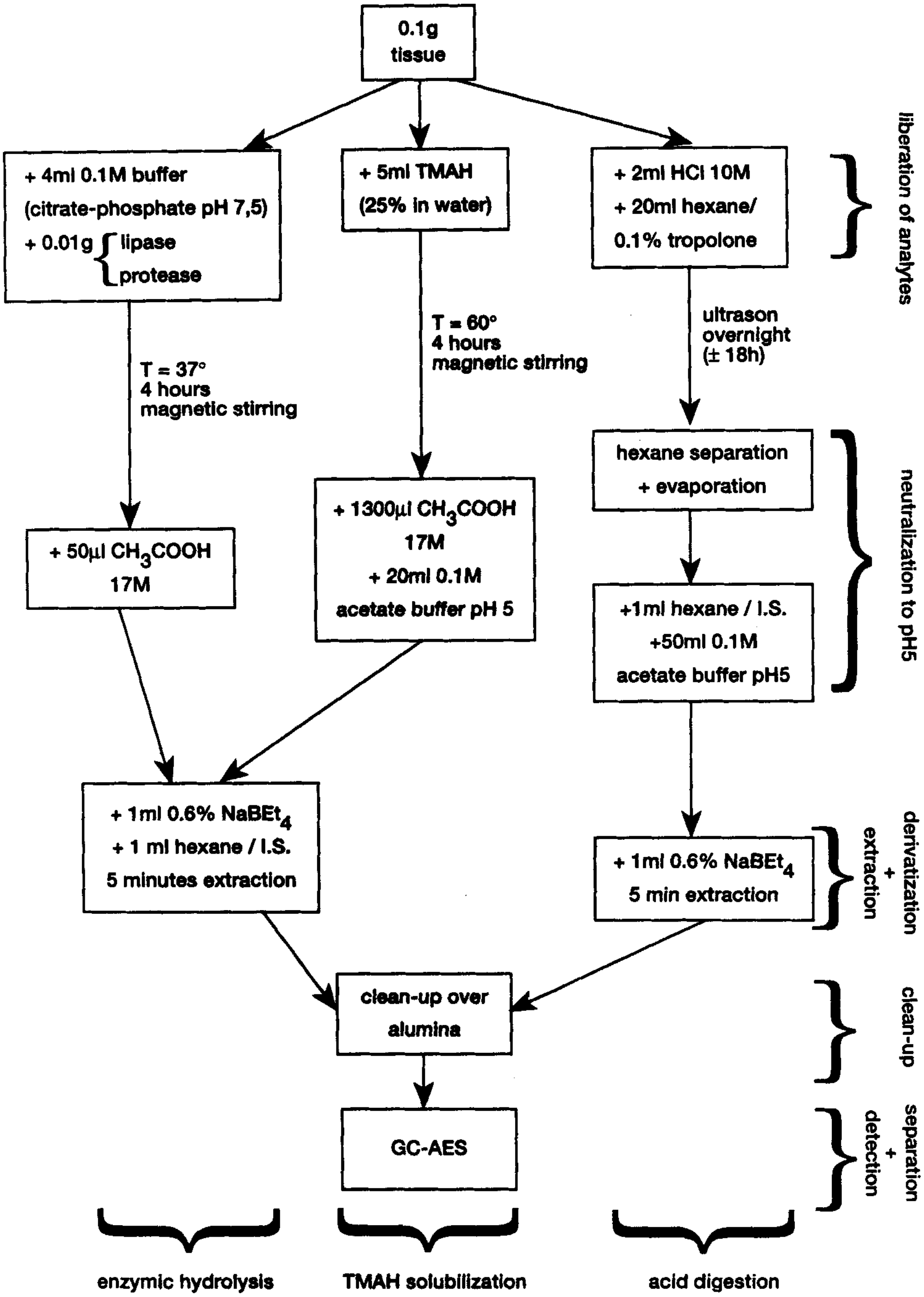

Fig. 1 Schematic representation of the different sample preparation procedures used for the isolation of organotin compounds from biological matrices.

Table 1 GC–AED operating conditions

Injector parameters (splitless without solvent venting)	
Injection volume	1 μl
Injection temperature	40 °C
Heat-up rate	12 °C s^{-1}
Retention temperature	260 °C
Retention period	60 s
GC parameters	
Carrier gas	Helium
Column	HP-1 (25 m × 320 μm × 0.17 μm)
Column head pressure	130 kPa
Oven programme	
Initial temp.	45 °C (1 min)
Ramp rate	20 °C min^{-1}
Final temp.	280 °C (1 min)
Detector block temp.	280 °C
Purge valve	Off (1.25 min)→On
Purge flow rate	100 cm^3 min^{-1}
Interface parameters	
Transfer line	HP-1 column
Transfer line temperature	280 °C
AED parameters	
Wavelength	303.419 nm
He make-up flow	240 cm^3 min^{-1}
Scavenger gases	
H_2 pressure	50 psi (345 kPa)
O_2 pressure	20 psi (138 kPa)
Spectrometer purge flow	2 dm^3 min^{-1} of N_2
Solvent vent off-time	4.5 min
Column–detector coupling	Column-to-cavity
Cavity temperature	280 °C

ated from the combined eluate using a gentle stream of argon.

Analysis

After sample clean-up, a 1 μl aliquot of the extract was injected into the GC–AED. Injector, gas chromatograph, interface and detector settings are summarized in Table 1.

RESULTS AND DISCUSSION

Acid digestion

Acetic acid and hydrochloric acid are the most common agents used in acid leaching/digestion-based sample preparation methods for organotin speciation in biological samples, and were both examined in this study. For efficient *in situ* derivatization and recovery of the organic phase, it was found in this study that a complete dissolution of the tissue is vital, and that this could only be achieved after an overnight ultrasonic treatment with concentrated hydrochloric acid. Large concentrations of acids, however, hampered the ethylation reaction and led to low extraction yields. When acetic acid or dilute hydrochloric acid was used, leaching occurred, the tissue did not undergo any physical changes and the 1 cm^3 of extracting solvent could not be recovered even after centrifugation. From this it was concluded that prior to the derivatization a separation of the analytes from the tissue matrix was necessary. This required the use of a complexing agent to extract the organotin compounds into the organic solvent. No inhibition of the ethylation reaction was observed when the derivatization was carried out in the organic phase. However, as a complexation reaction followed by solvent evaporation is an extra step in the procedure and does not offer any advantages over methods previously reported in the literature, no further attention was given to it.

Another drawback of sample preparation procedures involving acids for organometal specia-

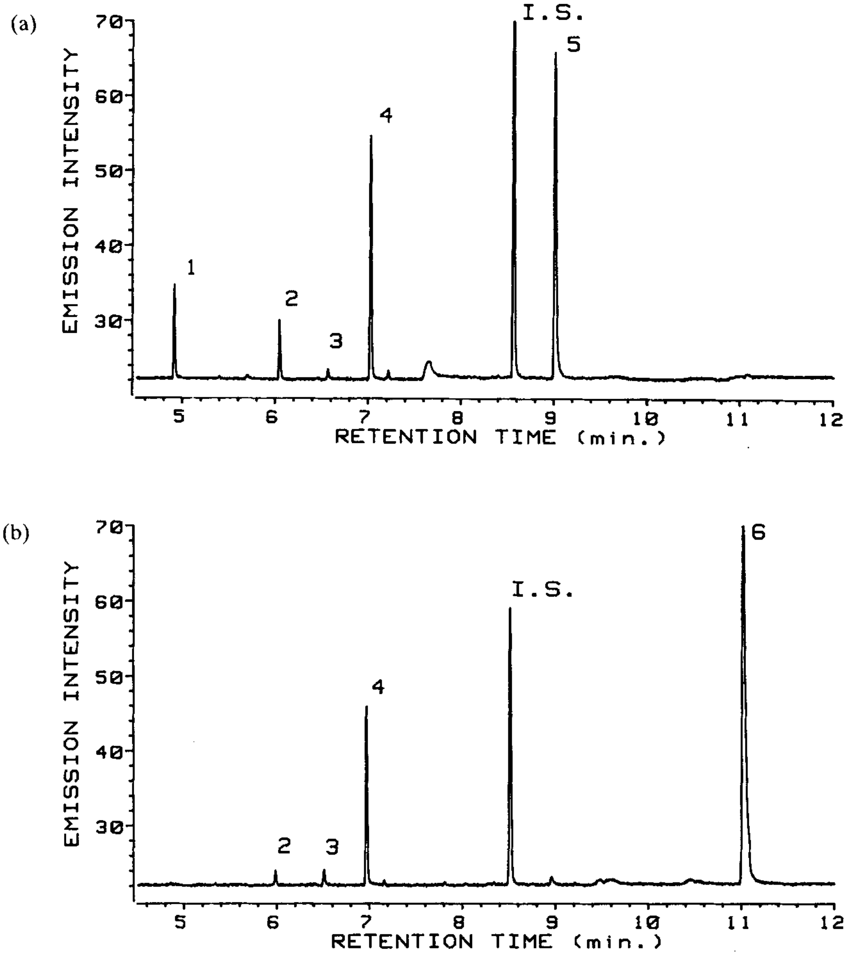

Figure 2 Chromatograms for the analysis of a sample of fish reference tissue after (a) acid digestion and (b) enzymic hydrolysis: 1, monobutyltin; 2, dibutyltin; 3, monophenyltin; 4, tributyltin; I.S. internal standard (Pe_3SnEt); 5, diphenyltin; 6, triphenyltin.

tion is that these treatments often lead to the degradation of the analytes. Figure 2(a) shows a chromatogram for the analysis of the fish reference tissue (NIES No. 11) containing TBT and TPT at $\mu g\, g^{-1}$ levels, confirming this statement. After overnight treatment of the tissue with concentrated hydrochloric acid none of the TPT could be recovered while a high DPT signal was present in the chromatogram due to the acid-induced degradation of TPT. Figure 2(b) shows a chromatogram for the analysis of the same tissue after enzymic hydrolysis, demonstrating no proof of degradation of TPT. Further, it can be seen from Figs 2(a) and 2(b) that the TBT degradation products, dibutyltin (DBT) and monobutyltin (MBT), are also present at higher concentrations when acid digestion was used as the sample preparation method.

Enzymic hydrolysis

To our knowledge, only one method for organotin speciation analysis has been reported in the literature[14, 15] which uses a protease–lipase mixture to decompose the biological matrix. The analytes were extracted in an organic solvent from the decomposed tissue–enzyme matrix as organotin–tropolone complexes, followed by Grignard derivatization.

In this study, however, in order to reduce the number of handling steps, *in situ* derivatization was performed in the hydrolysate itself. This required an optimization of the derivatization conditions to make them compatible with the hydrolysis parameters. Optimum activity of the protease and lipase enzymes occurs at pH 7.5, while the optimum pH for derivatization and extraction of the ethylated organotin compounds was found to be 5.[20] It was therefore necessary to decrease the pH after the hydrolysis and this was done by the addition of acetic acid, which proved to give better results than hydrochloric acid.

Another parameter to be optimized was the hydrolysis time. To speed up the hydrolysis, only a small amount (0.1 g) of the tissue and a relatively high concentration of the enzymes (0.01 g) were used. Using this enzyme/tissue ratio, successful liberation of all organotin occurred after incubation at 37 °C for 4 h. Further, it was found that the concentration of the derivatizing reagent ($NaBEt_4$) needed to be increased strongly, up to 0.1%, in comparison with 0.005% in the ethylation of organotin in water samples.[20] The reason for this is that a number of compounds are present in the sample matrix which may react with $NaBEt_4$ or interfere with the ethylation reaction. During the extraction an emulsion is formed, an observation which is contradictory to some reports which state that reduced emulsion formation is one of the advantages of enzymic hydrolysis.[23] However, after centrifugation the hexane phase could be recovered without any problems. The whole procedure may be carried out in a single 8 cm^3 test-tube, thereby diminishing the risk of contamination and losses of analytes during sample preparation.

TMAH solubilization

An alternative to enzymic hydrolysis is to solubilize the tissue in order to liberate the lipid- and protein-bound organotin. In accordance with reports of methods in which TMAH is used, the tissue was solubilized at an increased temperature of *ca* 60 °C. Magnetic stirring during 4 h proved to be sufficent to dissolve readily 0.1 g of the tissue and to librate all organotin present. No degradation of the organotin compounds was observed under these conditions. After solubilization, the pH of the highly basic TMAH–tissue solution needed to be lowered to 5 to optimize derivatization and extraction with $NaBEt_4$. To reduce the pH of the basic TMAH solution, 1300 µl of acetic acid and 20 cm^3 of the pH 5 buffer were added to the mixture. Derivatization with a reagent concentration of 0.025% was found not to be hampered in this matrix. As for enzymic hydrolysis, in this case also the entire procedure can be carried out in a single extraction vessel.

Clean-up

Extracts of samples rich in organic matter, such as biological tissues, contain large amounts of coextractives (fats, high-boiling hydrocarbons) which can cause rapid column contamination and background interferences, thus negatively affecting the detection limits. A clean-up of the extract can overcome this problem to a great extent. The main methods of clean-up have been based on low-resolution chromatographic separations such as (i) ion-exchange, (ii) size-exclusion and (iii) partition, adsorption or thin-layer chromatography (e.g. using alumina, silica gel or Sep-pak C-18 columns).[24]

During this study it was observed that the column needed cleaning after approximately 40 injections of tissue extracts. Column contamination was recognized by broad, tailing peaks of decreased size. A new injection liner was installed in the injector port whenever the column was cleaned. During this survey, interferences appeared at retention times of 9–12 min, seriously disrupting the baseline and TPT peak shape. The elution of carbon-containing compounds at these retention times was unambiguously demonstrated by measuring the extract on the carbon emission wavelength of 193 nm. Figure 3(a) shows a broad band eluting between retention times of 9 and 12 min, most probably being the reason for the aforementioned baseline distortion and broad TPT signals in the tin chromatogram.

The clean-up method of choice was based on adsorption on alumina. In order to recover all organotin quantitatively from the clean-up column, it was found necessary to add an additional 1 cm^3 of diethyl ether after elution of the extract. As volumetric changes are likely to occur due to the evaporation of the ether from the extract, the use of an internal standard was mandatory. Pe_3SnEt was chosen for this purpose, as it is not present in environmental samples and gives a signal well separated from the analyte peaks. No losses of analytes were observed during sample clean-up. Figure 3(b) shows the chromatogram of an extract measured on the C-193 channel after clean-up. It can be seen that the majority of the high-boiling carbon compounds are effectively removed, as may be concluded from the reduced carbon emission. On the tin chromatogram, sample clean-up results in the reduction by a factor of two of the width of the TPT signal and a flatter baseline between retention times of 9 and 12 min.

An attempt to incorporate the clean-up step *on-line* by adding 0.5 cm of alumina on top of the 2 cm Tenax plug of the injection liner was not successful.

In the case of enzymic hydrolysis it was observed that samples incubated for 24 h were completely transparent, indicating an increased decomposition of lipids and proteins. Although this prolonged hydrolysis did not result in higher recoveries, it was expected that less high-boiling hydrocarbons pass to the extract, especially as some authors claim that no additional clean-up step is necessary when samples are hydrolysed enzymically.[14] However, carbon emission spectra of these extracts were similar to those of 4 h hydrolysates.

Recovery and precision

The recovery was evaluated by the extraction of organotin spiked reference tissue and comparison of the results with those obtained from the GC–AE analysis of ethylated standards. The values found for the different compounds for enzymic hydrolysis and TMAH solubilization are presented in Table 2. The values reflect the results of four replicate analysis of a 1 μg g^{-1} (as tin) spike on the reference tissue. The reason for the large standard deviation found for TPT is most probably attributable to interferences occurring during separation and detection because of baseline and TPT peak shape which, even after clean-up, are not ideal. Further, it can be seen

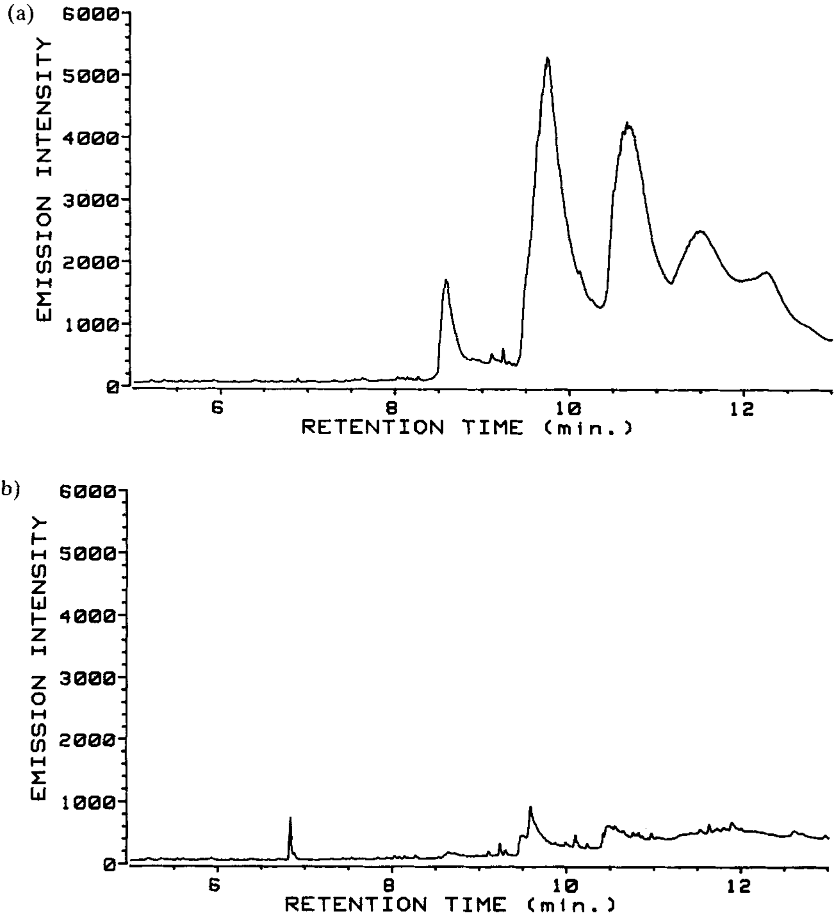

Figure 3 Chromatograms measured on C-channel ($\lambda = 193$ nm) for a sample of fish reference tissue after TMAH solubilization (a) before clean-up and (b) after clean-up of the extract.

Table 2 Mean recoveries of organotin compounds using enzymic hydrolysis and TMAH solubilization

	Recovery (%)			
	$BuSn^{3+}$	Bu_2Sn^{2+}	Bu_3Sn^+	Ph_3Sn^+
Enzymic hydrolysis	73 ± 6	89 ± 5	93 ± 5	60 ± 11
TMAH solubilization	84 ± 5	91 ± 4	94 ± 3	72 ± 9

that, for TBT and DBT, recoveries are similar for both methods while, for MBT and TPT, the procedure involving TMAH solubilization gives slightly better results. MPT and DPT could not be recovered from the matrix using these sample preparation procedures (recoveries <20%). It should be noted that the physicochemical form of the analytes in the spike is likely to be different from that in the sample, especially in the analysis of biological tissues where the organotin compounds are incorporated in the tissue and may be bonded to lipids and proteins. Therefore, the results obtained from recovery experiments may not completely reflect the amount of organotin extracted from real tissue samples.

Accuracy of analysis

The developed methods were validated by the analysis of the reference fish material (NIES No. 11). The results of 5 replicate analyses, expressed in μg g^{-1} of the compound in its chloride form, are shown in Fig. 4. Quantification was carried out by means of three-point standard addition. It can be seen that for TBT excellent agreement was found with the certified value (1.3 ± 0.1 μg g^{-1}), as well for the method based on enzymic hydrolysis (1.36 ± 0.07 μg g^{-1}) as for the procedure based on TMAH solubilization (1.34 ± 0.07 μg g^{-1}). However, for TPT large discrepancies between both methods (7.26 ± 0.40 μg g^{-1} for enzymic hydrolysis; 5.91 ± 0.44 μg g^{-1} for TMAH solubilization) were found. It should be noted, however, that the reference value reported for TPT (6.3 μg g^{-1}) is only indicative, as during the certification campaign analytical problems related to extraction, measurement and clean-up occurred.[25] This confirms the difficulty of the accurate determination of unstable compounds such as TPT and may be the explanation for the differences in detected TPT concentration by both methods. A chromatogram for the analysis of a sample of reference tissue after enzymic hydrolysis is shown in Fig. 2(b).

Comparison with other methods

Table 3 summarizes the operating conditions for a selection of sample preparation methods used for organotin speciation analysis in biological samples. Procedures involving Grignard derivatization require a complexation reaction, often making the procedure tedious and time-consuming because of the large number of handling steps. Methods based on hydride generation are easier to perform but the hydridization reaction is said to be subject to interferences whenever materials with high organic material content are analysed.[11,26]

The methods presented in this work reduce the number of handling steps to a minimum, as *in situ* derivatization in the hydrolysate requires no copmplexing agents or a separation of the analytes from the sample matrix. At the same time it

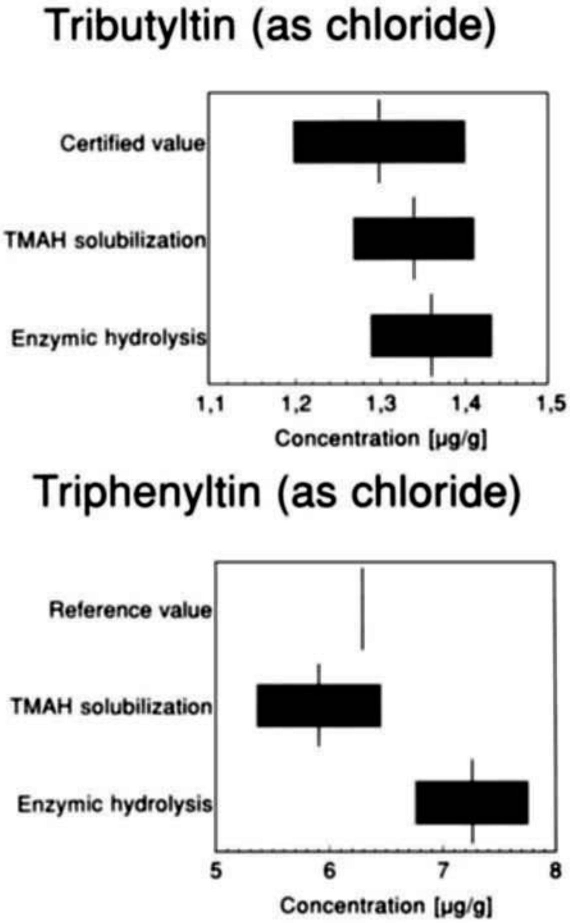

Figure 4 Results of the analysis of fish reference tissue (NIES No. 11) (mean value of five replicates ± 95% confidence interval).

Table 3 Selection of analytical procedures used for organotin speciation in biological samples

Analytes	Sample	Sample preparation	Extraction and derivatization	Separation and detection	Detection limit (ng g^{-1} as Sn)	Reference
$Me_nSn^{(4-n)+}$ $Bu_nSn^{(4-n)+}$ Sn^{IV}	Oyster	MeOH/HCl (7 M), 1 h ultrasound, 60 °C	$NaBH_4$	Thermal desorption QF AA	11–25	10
$Bu_nSn^{(4-n)+}$	Oyster	HCl (2 M), 12 h leaching	$NaBH_4$	GC–QF AA	0.5–3.5 (absolute)	9
$Bu_nSn^{(4-n)+}$	Mussel algae	Conc. HOAc; overnight stirring/30 min ultrasound	$NaBH_4$	GC-QFAAS	1.0–1.8	11
$Bu_nSn^{(4-n)+}$ $Ph_nSn^{(4-n)+}$	Mussel Fish	HCl(pH 2)	Diethyl ether/0.25% tropolone; EtMgCl	GC–FPD	9–23	6
$Bu_nSn^{(4-n)+}$	Fish	Conc. HCl, 4 h shaking	Pentane/0.05% tropolone; PeMgBr	GC–FPD	40	7
$Bu_nSn^{(4-n)+}$ Ph_3Sn^+	Mussel Fish	4 h enzymic hydrolysis or 4 h TMAH solubilization	Hexane; $NaBEt_4$	GC–MIP AE	2	This work
$Bu_2Sn^{(4-n)+}$ $Me_nSn^{(4-n)+}$ R_4Sn	Mussel Oyster Fish	24 h enzymic hydrolysis (lipase, protease)	Dichloromethane/ hexane/0.05% dithizone; MeMgCl or BuMgCl	GC-QFAAS	0.2–0.8	14

offers a detection limit which is comparable with the more sensitive methods hitherto reported. Further, the use of enzymes or TMAH does not impose a problem for altering the chemical structure of the analytes, in contrast to methods based on acids where care must be taken to avoid degradation.[23]

Application

The developed methods were applied to the analysis of mussels soaked in vinegar purchased in a supermarket and originating from Zeeland (The Netherlands). They were homogenized with a meat grinder and stored at −20 °C until analysis.

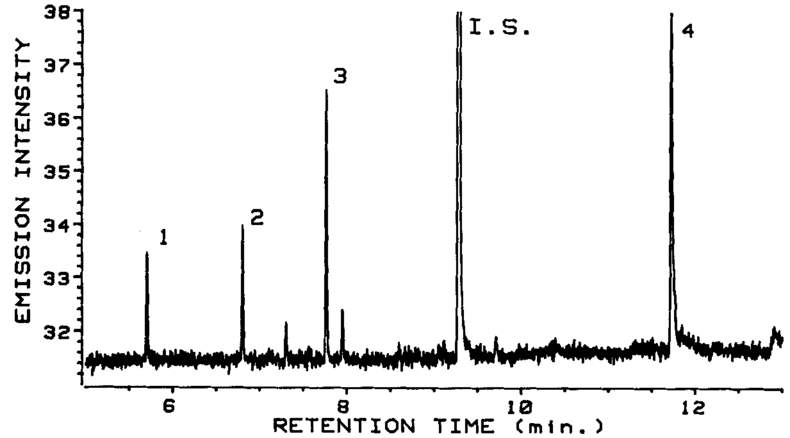

Figure 5 Chromatogram for a sample of mussel tissue: 1, monobutyltin; 2, dibutyltin; 3, tributyltin; I.S., internal standard (Pe_3SnEt); 4, triphenyltin.

Table 4 Results of the determination of organotin in mussel tissue

	Concentration ± 95% confidence limit (ng g^{-1} as compound)			
	$BuSn^{3+}$	Bu_2Sn^{2+}	Bu_3Sn^+	Ph_3Sn^+
Enzymic hydrolysis	5.4 ± 0.8	7.6 ± 1.0	14.2 ± 1.5	18.6 ± 3.4
TMAH solubilization	3.3 ± 0.5	9.1 ± 0.9	13.1 ± 1.0	14.2 ± 2.5

As the detected concentrations were low, 0.5 g of the tissue (wet weight) was used for analysis and 5 μl of the extract was injected after extensive clean-up. It should be noted that whenever larger volumes (>1 μl) are introduced into the system, the solvent has to be vented from the injection liner prior to the release of the analytes onto the chromatographic column. The technique of PTV injection has been described in detail elsewhere.[20] This venting of the solvent is responsible for the 1 min shift in retention time between the chromatograms in Figs 5 and 2. The mean concentration of four replicates is presented in Table 4. Figure 5 show a chromatogram for the analysis of the mussel tissue and clearly shows the presence of TBT, DBT and MBT, while TPT was also detected. The detected concentrations are at a level (<20 ng g^{-1}) which does not impose a potential hazard, as the acceptable daily intake established by the World Health Organization (WHO) is set at 3.2 μg and 0.5 μg per kg of body mass for TBT[27] and TPT[28] respectively.

CONCLUSIONS

Two simplified methods for speciation analysis of organotin in marine biomaterials were developed and successfully applied to the analysis of a fish reference material (NIES No. 11). The effect of sample clean-up was demonstrated by measurements of the carbon emission of tissue extracts, showing a correlation between the baseline and peak shapes on the tin channel and the amount of eluting high-boiling carbon compounds. The methods allow the accurate determination of butyl- and triphenyl-tin compounds in biological matrices down to the level of 2 ng g^{-1} (as tin). The use of *in situ* derivatization allows the procedures to be kept simple as no separation of the analytes prior to the ethylation, nor off-line evaporation, is necessary. Application of the methods to mussels for nutritive consumption showed the presence of butyl- and triphenyl-tin compounds at a low level (<20 ng g^{-1}).

Acknowledgement A research grant by the N.F.W.O., Belgium, to one of the authors (M.C.) is gratefully acknowledged.

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