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Determination of Butyltin Compounds in Mussel Samples: A Comparative Study of Analytical Procedures

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Three different sample pretreatment methods (methanolic HCl with or without tropolone and enzymic extraction) and two final determination procedures (GC–MS and GC–QFAAS) have been applied independently to the determination of butyltin compounds in two mussel samples polluted at different levels. The results obtained validate the sample storage and transport conditions as well as the analytical procedures.

Keywords: Butyltin compounds; extraction; mussels; analytical procedures

INTRODUCTION

Heavy use of tributyltin (TBT)-based antifouling paints for use in boats, for fish-farming installations or in industry, despite regulations promulgated in several countries, still creates widespread environmental problems in freshwater or marine coastal environments.

TBT toxicity in marine environments is more easily demonstrated with molluscs such as oysters[1] or dogwhelks[2] and many laboratory studies have been devoted to the evaluation of toxicity thresholds for organisms such as molluscs, crustaceans, fish and worms.[3] Mollusc larvae appear extremely sensitive to TBT pollution, but adults seem to be more resistant and able to accumulate this lipophilic pollutant; this accumulation is greater as decontamination rates (by excretion or degradation) are slow.

Some of the marine molluscs prone to TBT contamination (e.g. oyster, mussel, scallop) are commercially produced for human consumption as also are fish reared in fish farms (e.g. salmon, sea perch, sea bream). The toxicity of TBT for mammals is such that the WHO (World Health Organization) and Japanese Ministry of Health and Welfare have defined limits to daily human consumption of respectively 1.3 and 0.6 μg of TBT (as Sn) per kg body wt.[4] It is therefore important to develop and test analytical procedures able to determine TBT and its degradation products dibutyltin (DBT) and monobutyltin (MBT) in sea food.[5,6] These procedures will allow the evaluation of the extent of butyltin pollution in the environment, the possible intake of TBT by man through consumption of sea food and any eventual threats to human health.

The determination of TBT in water and sediments has been the objective of very detailed studies in the last 10–15 years, and sediment reference materials are available (NRCC PACS 1 at high TBT levels (1.27 ± 0.22 μg g^{-1}) and CEC-BCR CRM 462 for low TBT concentrations (70 ± 14 ng g^{-1})). In contrast, analysis of biological matrices has been much less studied, probably owing to difficulties encountered in defining quantitative solubilization processes for tin speciation.

Two completely different procedures for the determination of butyltin compounds in mollusc samples have been recently and independently developed: hydride generation–cold trapping–gas chromatography–quartz furnace atomic adsorption spectrometry (HG–GC–QFAAS) after acidic extraction or enzymic hydrolysis (Method I)[7] and GC–MS following Grignard derivatization after acidic extraction in the presence of tropolone (Method II).[8,9]

This paper presents the results of an intercomparison study on two mussel samples, one taken in a highly polluted environment (La Spezia), the other in a quite pristine area (Oristano), both on Italian coasts. All organotin concentration values in this paper are reported as mass of tin per unit mass or volume.

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MATERIALS AND METHODS

Sample collection

The mussel samples were collected in La Spezia harbour (Liguria, Northern Italy) and in S'ena Arrubia Lagoon (Sardinia). La Spezia harbour is located in the internal part of the Gulf of La Spezia and is protected by an artificial dyke (2200 m in length). There exist two accesses into the harbour, the Passo di Levante (200 m wide) and the Passo di Ponente (400 m wide), which is the main way for the maritime traffic through a canal 14 m deep. The main depth of the basin is 13 m and the surface is 12 km^2. In the Gulf of La Spezia there is also a military harbour near the Arsenal in the Western part, joined to the other part by two canals (120 and 50 m wide).

La Spezia harbour is characterized by high levels of maritime traffic and dockyard, industrial and urban activities and is a heavily polluted area. One of the most important Italian mussel farms is located inside the harbour and the mussels used in this study were directly furnished by the farm managers. Further details are reported elsewhere.[10]

S'ena Arrubia lagoon is located a few kilometres south-east of Oristano on the west coast of the island of Sardinia. The lagoon is a protected natural area of international importance (it is considered in the Ramsar Convention). It is characterized by a low salinity with a mean depth of about 50 cm and is connected to the sea by a number of small canals closed by sluice-gates; the main activities in the area are those associated with the fisheries. The area is characterized by a low pollution level as the only anthropogenic source is irrigation water coming from agricultural activities.

Reagents

Method I

Standard solutions (1 g l^{-1}) of butyltin compounds were prepared in methanol (Prolabo Normapur). TBT chloride (96%) and DBT chloride (98%) were Merck products. MBT chloride (95%) was purchased from Aldrich. Intermediate standard solutions were prepared weekly (10 mg l^{-1}) or daily (0.1 mg l^{-1}) by dilution in MilliQ Milli Ro water acidified with 10% nitric acid (Merck Suprapur).

The 10% (w/v) sodium tetrahydroborate (Fluka) solution was stabilized with 1%(w/v) sodium hydroxide (Merck Suprapur). Acetic acid and ammonium dihydrogenophosphate were Prolabo products, hydrochloric acid and ammonia were Merck products and lipase type VII and protease type XIV were purchased from Sigma.

Method II

Organic solvents are 'RS-per determinazione pesticidi' from Carlo Erba. Hydrochloric acid (Aristar), sulphuric acid (Spectrosol), silica gel (Davison 923) activated overnight at 180 °C and TBT chloride (laboratory reagent) were BDH products. Tropolone was purchased from Lancaster Synthesis, and tripropyltin chloride (98%) from Merck. Anhydrous sodium sulphate RPE-ACS from Carlo Erba was treated at 550 °C for 6 h before use. *N*-Pentylmagnesium bromide, DBT chloride (97%) and MBT chloride (95%) were Aldrich products. They were used as received and were checked for the presence of degradation products by GC–MS after Grignard derivatization.

The organotin stock solutions were prepared gravimetrically in methanol at about 1 mg ml^{-1} (as Sn) concentration and diluted 1000-fold to give the working standard solutions. When stored refrigerated in the dark, stock solutions are stable for at least three months and the working solutions at least for one month, but the latter were renewed weekly.

Instrumentation

Method I

Two different automated HG–GC–QFAAS systems were used in this study, both furnished with a Perkin–Elmer MHS1 furnace mounted in a Il 151 or Varian AA10 spectrometer coupled to a Varian 2070 or Shimadzu CRA4 integrator.[3,7]

Method II

GC–MS analyses were performed on a Hewlett–Packard HP 5890 GC/HP 5970B MSD system with the following conditions: electron impact ionization mode (70 eV); carrier gas helium (65 kPa head pressure); column HP-5 (methyl-5% phenylsilicone, 0.20 mm i.d., 0.11 mm film thickness, 25 m length; Hewlett–Packard); temperature programme 80 °C × 2 min then 10 °C min^{-1} to 280 °C; injector splitless, 240 °C; transfer line temperature 280 °C; SIM (selected ion monitoring) operation method. The peak identification was based on the matching of retention times and isotopic mass ratios. 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.[8,9]

Analytical procedures

Method I

Extraction procedures Two different extraction procedures have been tested after optimization.

Acidic extraction. It has been previously demonstrated that lyophilized mussel samples should be pre-wetted before attack. Sample (0.5 g) was mixed with 5 ml of a 1:1 methanol/water solution during 4 h.[11] Then 12.5 ml of 0.1 mol l^{-1} HCl was added and the mixture submitted to sonication during 1 h. The solution volume was then adjusted to 25 ml with water; 0.2-ml subsamples of this solution were submitted to analysis.

Enzymatic hydrolysis. A mixture of 0.1 g of sample together with 10 mg each of lipase and protease was incubated at 37 °C with 10 ml of buffer solution in a haemolysis tube during 4 h, then 0.2-ml homogenized subsamples were submitted to analysis.[12]

Analysis Sample extracts (0.2 ml in this study) were introduced into the hydride generation reactor with 100 ml water and 1 ml acetic acid. After purging with helium the sodium tetrahydroborate solution was introduced by a peristaltic pump. Stannanes produced were purged by hydrogen evolving from the solution and carried to the cold trap (in liquid nitrogen) by helium flux. After the end of this collection step the cold trap was gently electrically heated; sequentially volatilized stannanes were then carried by the helium flux to the quartz cell (T=950 °C), where a H_2/O_2 flame improved atomization yield. Detection was at 286.3 nm.

This procedure had already been described in more detail elsewhere.[7,13]

Method II

The freeze-dried sample was homogenized and tripropyltin chloride was added, to aliquots of 100–500 mg, before extraction. Extraction was performed twice with 15 ml of 0.05% tropolone in methanol and 1 ml of concentrated HCl under sonication for 15 min. The liquid phases were collected after centrifugation at 3000 rpm for 10 min and liquid–liquid partitioning which was then performed twice with 30 ml of methylene chloride (CH_2Cl_2). The methylene chloride phases were collected through anhydrous sodium sulphate and, after solvent evaporation to almost dryness, solvent exchange (methylene chloride to iso-octane) was operated by evaporating methylene chloride after addition of iso-octane by a gentle stream of nitrogen. Derivatization with pentylmagnesium bromide in a 2 M ethereal solution was performed in a reaction vial for at least 15 min. Pentylmagnesium bromide (and other common Grignard reagents) often contain TBT impurities, so a careful blank evaluation was required. The excess reagent was destroyed by carefully adding water and then 1 M sulphuric acid. Derivatized organotins were extracted twice with 2–3 ml of hexane, purified on a silica-gel column and eluted with a mixture of hexane–benzene (1:1). Finally, the volume was concentrated under a moderate flow of nitrogen to *ca.* 0.5 ml, and 1 µl was injected for GC–MS determination. No losses of pentylated organotins were observed during volume concentration. Further details are reported elsewhere.[8,9]

Performance of the various methods

Analytical performance

Table 1 presents the analytical performance of the two methods. For Method I, calibration curves were perfectly linear for all butyltin compounds, at least in the range 0–10 ng (Sn) introduced in the reactor. Detection limits were calculated following IUPAC rules with k=3 (i.e. a 99.86% confidence level). Sensitivities were somewhat different, depending on the analyte; these may be explained by a systematic variation of atomization conditions during one analysis.

Table 1 Analytical performance of the methods

Method	Extraction	TBT DL[a]	TBT RSD[a]	DBT DL	DBT RSD	MBT DL	MBT RSD
Ia[b]	MeOH/HCl	5	9	2.7	9	2.2	8
Ib[c]	Enzymic	1.7	5	1.3	6	1.1	4
II[d]	MeOH/HCl/ tropolone	25	2	25	3	20	2

[a] DL, detection limit [µg(Sn) g^{-1}]; RSD, percentage relative standard deviation of 10 different analyses of an extract solution of the same freeze-dried mussel sample.
[b] Sample size 500 mg; final volume 25 ml; volume injected 0.2 ml.
[c] Sample size 100 mg; final volume 10 ml; volume injected 0.2 ml.
[d] Sample size 100 mg; final volume 0.5 ml; volume injected 1 µl.

Detection limits in the analysis of enzymic extracts were lower by a factor of 2–3. It did not appear necessary to reduce further these detection limits for the purposes of this study, but serious improvements could be obtained simply by increasing the volume of the subsamples analysed. Similar improvements of detection limits (DLs) using the acidic extraction procedure are less likely, due to the formation of foam during the hydridization reaction which may prevent a noticeable increase in the volume of the subsample introduced into the reactor.

For Method II, calibration curves were linear (as peak areas) in the range 5 pg–2 ng, 5 pg–1.6 ng and 4 pg–1.2 ng (as Sn) injected, for TBT, DBT and MBT, respectively. These correspond, under the usual analytical conditions for mussel samples (100 mg of sample, 0.5 ml final volume of the extract, 1 μl injected) to the DLs reported in Table 1. DLs can easily be lowered to a few nanograms per gram by increasing the size of samples to 1–2 g. Higher amounts of samples are not advisable as they require very long clean-up procedures, during which losses of analytes could occur.

Relative response factors were calculated daily (by injecting standard mixtures) before starting the analytical runs, because relatively small variations occurred in tuning conditions.

Recovery tests

Recovery tests on biological material are difficult to perform because reference materials are not yet commercially available (except the NIES (Japanese National Institute for Environmental Sciences) fish tissue, certified for TBT).

Method I The efficiency of the two extraction processes described was evaluated by analysing (in five replicates) 100–500 mg of freeze-dried spiked samples prepared several months previously as follows. Shells and flesh of 'clean' mussels were separated by hand after freezing. A large amount of flesh was then crushed and separated in four large samples, three of which were separately spiked with a known mass of one butyltin compound each and freeze-dried. After homogenization these samples (about 60 g each) were kept frozen for long-term conservation.

Analysis of unspiked mussel flesh revealed negligible concentrations of butyltin compounds.

Method II Freeze-dried mussel samples were analysed before and after spiking and the recoveries were calculated with respect to the sum of the concentrations of the incurred compounds and the spikes.

Samples (five replicates) were wetted before spiking and left to equilibrate for 24 h after spiking. Recoveries of the tri- and di-substituted species were around 90% while lower recoveries were obtained for the monosubstituted ones (ranging from 80 to 85%). Extraction yields obtained by the two methods are presented in Table 2

Table 2 Extraction recoveries[a] from spiked samples

Method	Extraction	TBT	DBT	MBT
Ia	MeOH/HCl	98±3	97±5	98±4
Ib	Enzymic	101±1	99±3	100±2
II	MeOH/Hcl/tropolone	91±7	89±11	85±11

[a] Recoveries and standard deviations are expressed as percentages.

RESULTS AND DISCUSSION

Methods I and II have been applied to the analysis of two lyophilized mussel samples highly (La Spezia) or very slightly (Oristano) contaminated by butyltin compounds.

Samples from La Spezia harbour

Analytical data obtained are compared in Table 3. Data presented are mean values calculated from six independent experiments for Method I and five for Method II; results are not corrected for the residual water content (humidity). It is worth stressing that the water content of samples increased slightly, from about 3% to about 6%, during the shipping of samples from Rome to Pau.

These results are in quite satisfying agree-

Table 3 Comparison of Methods I and II for the analysis of a highly polluted lyophilized mussel sample[a]

Method	TBT	DBT	MBT	ΣBu
Ia[b]	1050±90	820±120	630±40	2500±160
Ib[b]	1020±40	760±20	710±20	2490±50
II	920±30	860±30	850±30	2630±50

[a] Concentrations and standard deviations are in ng(Sn) g^{-1}.
[b] Ia, acidic extraction; Ib, enzymic extraction.

ment: there are no significant differences between the values of the total concentration of butyltin compounds (ΣBu=TBT+DBT+MBT), TBT or DBT obtained by Methods I and II. Slight differences appeared in MBT determinations. Comparison of the methanolic HCl extraction procedures of Method Ia and Method II may suggest a significant role for tropolone, a well-known ligand of MBT and DBT to improve MBT extraction from polluted mussel flesh.[14,15] The extraction of MBT spiked mussel flesh by Method I (without tropolone) was found to be quantitative (98%). There appears to be a noticeable difference of extractability of MBT in polluted samples and spiked samples, even when spiking was done several months previously. An explanation could be that MBT ingested by living mussels is metabolized and more strongly retained than MBT spikes added to dead organisms.

The slight differences in the whole set of data obtained by Methods I and II (Table 3) could also be interpreted by a slight degradation of TBT to DBT and of DBT to MBT during sample pretreatment in Method II. However, this hypothesis was not confirmed by complementary studies.[8,9]

Enzymic extraction leads to an intermediate MBT value.

Samples from S'ena Arrubia Lagoon

Analytical data presented in Table 4 are mean values calculated from six independent experiments for Method I and five for Method II.

Residual water content in samples analysed by Method I was measured as 10.3%; this value is higher than that measured in samples analysed by Method II before shipping (2.8%); it may be suspected that water vapour altered the material during transport or storage. For Method I, data presented are corrected for residual water content while for Method II they are not corrected.

A very satisfying agreement for determinations at low concentrations appears in Table 4. The only exception is the low DBT value obtained with Method Ia, for which no explanation could be found. The formation of abundant foam in the hydridization reactor with this sample necessitated a reduction in the volume of subsample analysed, inducing therefore an increase in the concentration detection limit. In these conditions the DBT and MBT concentrations measured were close to the quantitation limits.

Previous work has shown that methanolic HCl extraction of butyltin compounds from biological samples could induce a degradation of analytes by loss of butyltin radicals.[11,16] It is apparent from the data presented here that these phenomena were avoided in the procedures used, with HCl concentrations lower than 1 M and rather short extraction times.

Table 4 Comparison of Methods I and II for the analysis of a slightly polluted lyophilized mussel sample[a]

Method	TBT	DBT	MBT	ΣBu
Ia[b,c]	134±9	27±5	26±3	187±11
Ib[b]	140±4	55±8	32±5	227±10
II	130±10	66±5	35±7	231±13

[a] Concentrations and standard deviations are in ng(Sn) g^{-1}.
[b] Ia, acidic extraction; Ib, enzymic extraction.
[c] Sample volume reduced to prevent excessive foaming in the hydridization reactor.

CONCLUSIONS

Three analytical methods using three different extraction procedures and two different speciation methods have been applied in two different laboratories to two mussel samples, one highly and one slightly polluted by butyltin compounds. The good general agreement obtained, besides validating these methods, indicates that the sample storage and transport conditions used do not alter the speciation of tin.

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