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Trace Analysis of Tributyltin Chloride in Fish Tissue by Electron Capture Gas Chromatography

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A method is described for sample preparation by gas chromatography in biological samples. TBT was extracted from samples as chloride under the acidic condition of HCl and was detected by GC-ECD system using an apolar capillary column doped with a dilute HBr-methanolic solution. Derivatization step has been eliminated from sample preparation procedure, but doping of the GC-ECD system with a dilute HBr-methanolic solution has been used to detect the TBT chloride and triphenyltin (TPhT) directly by halogen exchange from chloride to bromide. This combination made it possible to determine TBT as less than the ngg^{-1} level in fish tissue.

[**Keywords***:* Organotin; Fish tissue; Analysis; Derivatization; GC-ECD.]

Introduction

Organotin compounds (OTs) have been extensively used in recent decades in agriculture and industry as biocides. Among these, the most prominent are the tri-substituted forms, such as tributyltin (TBT) and triphenyltin (TPhT). Tributyltin has been used for several years in marine coatings[1-3]. TBT and TPhT can produce negative effects on the environment even at low concentration (sub ngl^{-1})[3-5]. It is recognized that low level concentration of especially, TBT in sea water can exert lethal and sub-lethal effects on a wide variety of marine organisms, particularly in the case of sensitive juvenile life forms[6,7]. Furthermore, the bioaccumulation of biocide and pesticide residues in fish, food stuffs and endocrine disrupting chemicals have been reported[8-11]. The danger includes the risk of toxicity to marine organisms and fishes after prolonged exposure and causes some severe environmental problems, such as neogastropod imposex, shell malformation of oysters and high accumulation by mussels[12,13].

A variety of analytical methods, such as atomic absorption (AA)[5,14,15], gas chromatography (GC)[14,16] and high-performance liquid chromatography (HPLC)[17,18], have been applied to determine organotin compounds. In order to reveal environmental contamination, by organotin compounds and their degradation and metabolism organotin, a rapid and sensitive method applicable to various organotin compounds is required. Several gas chromatography (GC) analytical techniques for the determination of TBT and TPhT have been reported. Many of them apply derivatization methods: alkylation by Grignard reagents and hydrogenation by sodium borohydride[1-3,14,16,18,19]. These methods are generally rather complicated and require many handling steps. Present procedure economizes costs. In addition, it is time consuming and affects the measurement results in respect of accuracy and precision. To monitor TBT and TPhT concentrations at less than ngg^{-1} level in the environment, highly sensitive analytical techniques are essential.

In the present study, GC-electron capture detection (ECD) has been used for the direct determination of trace TBT in fish tissue. TBT extracted from fish tissue as the chloride could be gas chromatographed directly by the capillary column doped with a dilute HBr-methanolic solution. During GC separation, halogen exchange occurred from the chloride to the bromide form. GC-ECD was used as a highly selective and sensitive method for the detection of organotin halides and this method showed superior sensitivity for TBT

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chlorides which were extracted from fish tissue under the acidic condition of HCl. The combination of GC-ECD using an apolar capillary column doped with HBr makes it possible to determine TBT at lower concentration than the ngg^{-1} level in fish tissue. This combination is enough sensitive for determination of halogens rather than GC with other detectors, such as (FPD), that have been applied and explained in other articles[20]. The proposed method presents a highly suitable analytical technique for ultra trace determination of TBT in fish tissue.

Materials and Methods

Tributyltin chloride [$(n\text{-}C_4H_9)_3SnCl$] and triphenyltin chloride [$(C_6H_5)_3SnCl$, as internal standard] were supplied by Merck Chemical Company. Working standard solutions were prepared by mixing the tributyltin chloride and ethanol, and diluting in the range from 2 to 200 ng/ml. TBT bromide was obtained from Aldrich (Milwaukee, WI, USA). TPhT bromide was prepared by treating the TPhTCl ethanol solution with a 10% HBr aqueous solution. HBr (25%)-acetic acid solution for doping was purchased from Wako (Osaka, Japan). HBr-methanolic solutions at the concentration of 1 m*M* and 0.5 m*M* were prepared by diluting the 25% HBr-acetic acid solution (3.3 *M*) with methanol. All solvents used were analytical grade. The standard solutions, the working standard solutions and HBr-methanolic solutions were stored at 4°C.

GC was performed on a Hewlett-Packard (Avondale, PA, USA 5890 series II) gas chromatograph equipped with an electron capture detector (ECD) with a 611.6 nm filter. The GC column used was a fused-silica capillary column (15m×0.25 mm I.D.) coated with DB-1 (0.1 µm film thickness) (J&W Scientific, Rancho Cordova, CA, USA). Injections were made in the splitless injection mode (1 min split time) with an inlet pressure of 70 KPa using helium as the carrier gas. A temperature program was employed in which the column temperature was initially held at 45°C for 1 min, then increased at 15°C/min to a final temperature of 255°C. The injector temperature was held constant at 260°C. The GC column was pretreated with HBr-methanolic solution to prevent the adsorption of TBT chloride within the column. After setting the capillary column to the gas chromatograph and holding the temperature at 45°C, pretreatment of the capillary column was performed by injecting three times 1 µl portions of 1 m*M* HBr-methanolic solution at 1.5 min intervals and programming the column temperature according to the GC conditions described above. The column end was connected to electron capture detector after the pretreatment with 1 m*M* HBr. One µl of 0.5 m*M* HBr-methanolic solution was doped at 45°C about 1 min prior to injection of a sample or standard solution in order to keep peaks sharp and sensitivity high[20].

Sample preparation for whole body of fish was carried out according to the flow chart of Fig. 1. The samples were homogenized in a commercial meat grinder.

TBT was extracted two times from 10 gr of the whole body of fish sample with 20 ml of hexane and ether (3:1, v/v) in the presence of HCl, ethanol-ethyl acetate (1:1, v/v). The combined extract was concentrated to about 5ml and then subjected to column chromatography using 2gr anhydrous sodium sulfate and 4gr silica gel. Preparation of the column was as follows: anhydrous sodium sulfate and silica gel was suspended in hexane and pack into a glass column (15m × 0.25 mm I.D.). When the concentrate was

Biological sample (ex. Fish tissue, 10g)
← HCl, 10 ml
← ethanol: ethyl acetate (1:1), 20ml
← hexane: ether (3:1), 40ml, x2
Organic layer
Silica gel column chromatography
(anhydrous sodium solfate, 2g + silica gel, 4g)
Wash ← hexane: ether (3:1), 40ml
← hexane: acetone (3:1), 40ml
Elute ← hexane: ether: acetic acid (75:25:1), 50.5ml
← sodium hydroxide: sodium chloride (1:1), 10ml
Eluate solution
Concentrate to 2ml
GC-ECD

Fig.1— Sample preparation procedure of TBT a biological sample.

poured into the column and the top of the solvent surface came to the anhydrous sodium sulfate layer, 40ml hexane-ether (3:1, v/v), then 40ml hexane-acetone (3:1, v/v) and 50.5ml hexane-ether-acetic acid (75:25:1, v/v/v) were added. The final acid portion was collected and washed with 10 ml sodium hydroxide-sodium chloride (1:1, v/v). Then this organic layer concentrated to 2ml. TPhT chloride was added as an internal standard to the final sample solution at the 10 ngg^{-1} level to control the precision of the manual injection. The injection volume of this standard was 2 µl. The solution obtained was subjected to GC-ECD.

Results and Discussion

An apolar fused-silica capillary column with a thin immobilized stationary phase of DB-1 (15m×0.25mm I.D., 0.1µm film thickness) was examined. When TBT chloride was injected into the gas chromatograph equipped with the capillary column not pretreated with HBr, the peaks of TBT and TPhT chlorides hardly appeared. It is evident that ordinary capillary columns are largely deactivated and have very few active sites compared to packed columns. Nevertheless, organotin halides readily react with OH groups and other reactive sites in the column. In addition, It is difficult to analyze TBT halide directly and precisely by capillary GC. Derivatization methods, such as those described above, are usually required. These attempts were made to use capillary GC for the analysis of halides of TBT and TPhT by applying a doping technique[20]. Previous experiments demonstrated that applying a DB-1 capillary column (15 m, 0.25 mm id, 0.1 mm film thickness) and a FID could not easily determine trace levels of TBT and TPhT halides (chloride and bromide) by direct injection and without any treatment or modification[20]. Since organotin halides are very apolar, most of them are adsorbed on the column and hence sufficient and quantitative peaks are not observed. Therefore, TBT appeared as relatively small peaks with degradation products by direct injection into GC (Fig. 2). To solve this problem, the HBr doped column was carried out. Doping was performed by injecting 1 µl of a dilute methanolic HBr solution into the gas chromatograph about 1 min before the injection of a standard solution. Different HBr concentrations were studied, such as 0.1, 0.3, 0.5, 1.0, 2.0 and 3.0 mM. All the TBTCl was converted to the TBTBr at 2.0 mM HBr doping concentration level and the TBT appeared as relatively sharp peak compared to no doping. In addition, the small degradation peak of TBTCl became a single large peak with increasing HBr concentration. The identity of each compound (TBTCl, TBTBr) was confirmed by GC retention time in preliminary studies. The mechanism of peak sharpening in GC with HBr doping was studied thoroughly by Mizuishi *et al*. (1998). Based on the present study and also previous works, the chromatograph, detector and column did not suffer any damage due to HBr doping and the shape peak has remained fairly constant through the study[20].

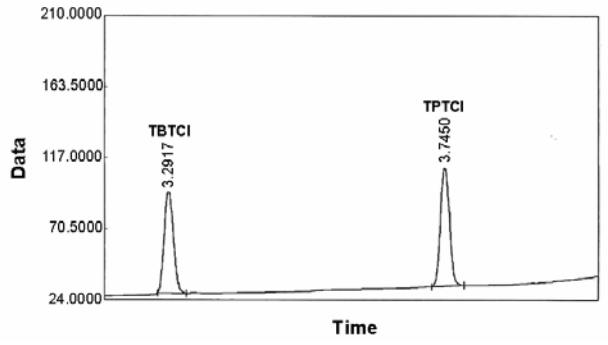

Fig. 2 — GC-ECD chromatogram of TBT chloride and TPhT chloride (I.S.) with DB-1 capillary column under HBr doping.

Fig.2 shows GC-ECD chromatogram when 10 ngg-1 TBT chloride and TPhT chloride (I.S.) was injected into the gas chromatography equipped with the capillary column pretreated by injecting three times 1 µl of 1 m*M* HBr solution.

The pretreated column gave sufficiently sharp peaks of TBT and TPhT chlorides. Furthermore, 1µl doping of 0.5m*M* HBr solution prior to the injection of sample solution was successful in keeping the peaks sharp and sensitivity high. Doping of a 0.5m*M* HBr solution had a persistent effect of sharpening peaks of TBT and TPhT chlorides.

For the preparation of the calibration curve and the development of the assay, standard solutions concentrations ranging from 2-200 ng/ml in ethanol, were prepared. An internal standard containing TPhTCl at concentration of 100 ng/ml was prepared. Tributyltin chloride and triphenyltin chloride were not detected in the fish which were used for the preparation of the calibration samples. Before analysis, 0.5 ml of I.S. solution was added to the calibration samples. Calibration curves were constructed by plotting the relative peak area to TPhT chloride as I.S.; the detection limit was defined as the signal equal to three times the standard deviation (3σ) of the baseline noise.

The calibration curve parameters listed in Table 1 were obtained under the optimized conditions. Linearity of calibration curves were examined in the range of 0.1-50 ngg^{-1} for the analyses. Correlation

coefficient was 0.9992. Limit of detection (LOD) was calculated as three times of the baseline noise (S/N = 3) after 5 successive extractions of blank sample. According to the ICH (International conference on harmonization of technical requirements for analytical methods) criteria for analytical method validation, limit of quantification (LOQ) for the analyte was determined as the lowest concentration on the calibration curve with a precision of less than 20% of coefficient of variation (CV) and an accuracy of 80 to 120%[21]. For target analyte, LOD and LOQ were determined 0.05 and 0.1 ngg^{-1}, respectively, which indicate sensitivity of the method. The precision of the method was evaluated in terms of repeatability (or intraday precision) by calculation the analyte concentration in quality control samples, which prepared at three levels (each six replicates) on three consecutive days. Intraday precision values were always <15%. Expression of the intraday precision are the coefficients of variation (CV%) of determined responses of five replicates of quality control (QC) samples, which were prepared at three levels and reported in Table 2. The estimated recoveries at three different concentration levels were also shown in Table 2. Recovery studies were performed by spiking the analyte standard in a blank sample at 0.1 and 5 ngg^{-1} levels (n = 6). Internal standard was added just before sample treatment and the ratio of the peak area count of the analyte to that of internal standard was calculated. These values were compared with the ratios obtained for reference QC extracts, which were prepared by adding the same amount of the analyte standard to the final blank sample extract. All these results indicate the validity and reliability of the developed method for determining the TBT in fish tissue samples.

The proposed method was applied to studies of bioaccumulation of tributyltin chloride (Bu_3SnCl) by fish (*Scatophagus argus*). This experiment was carried out by exposing fish with food contaminated to 0.00264 – 0.264 μgml^{-1} for 7- 21 days. As a case in point, the results of bioaccumulation of TBT chloride in *Scatophagus argus* are shown in table 3 after 7th, 14th and 21st days exposure of fishes to food with two different concentrations of biocide[22]. Fig. 3 shows typical gas chromatogram of tributyltin chloride in fish tissue after 14 days.

Table 1—Calibration lines parameters of the developed method for determination of TBT in fish samples.

Compound	Linear range (ngg^{-1})	LOQ (ngg^{-1})	Calibration line equation	Square of the correlation coefficient (r^2)
TBT	0.1 - 50	0.1	y = 0.1011x	0.9992

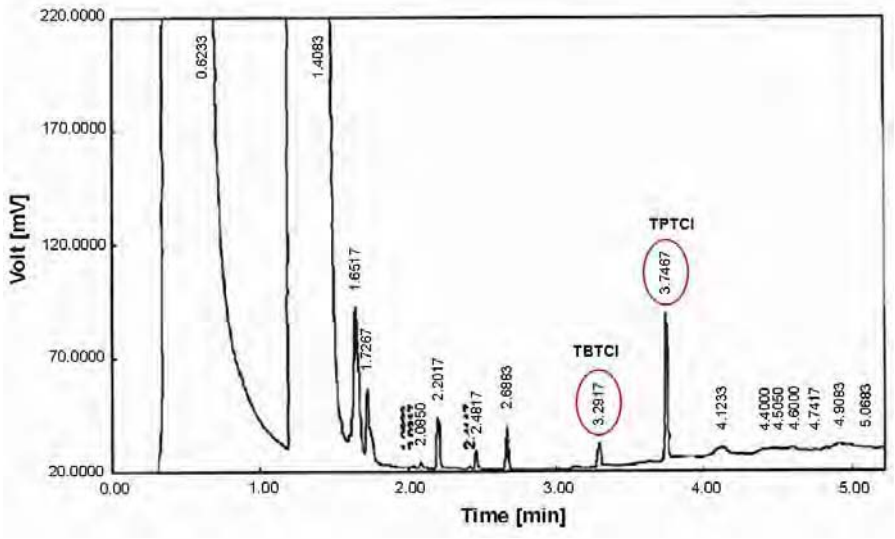

Fig. 3— GC-ECD chromatogram of TBT chloride in fish tissue after 14 days.

Table 2—Estimated recoveries and method precision for TBT at different concentrations (n = 6) in QC samples.

Compound	Sample	Nominal Conc. (ngg^{-1})	Mean of Calc. Conc.[a] (ngg^{-1})	CV% of Calc. Conc.	RE % of Calc. Conc.[b]	Estimated Recoveries %	CV% of Calc. Recovery
TBT	QC_1	0.1	0.09	10.4	-10	84	12.2
	QC_2	5	4.6	8.5	-8	86	10.6

[a] Calculated concentration
[b] Relative error = [(Calculated Conc./Nominal Conc.)-1]×100

Table 3—TBT chloride concentrations in fish (*Scatophagus argus*) samples at 7th, 14th and 21st day.

Test No.	Concentration in food (μgml^{-1})	Time (day)	Concentration in fish (ngg^{-1})	Standard Deviation
		7	0.375	0.0195
3	0.0528	14	0.410	0.0540
		21	0.920	0.0200
		7	0.940	0.0100
4	0.2640	14	1.724	0.0155
		21	1.700	0.0291

Conclusion

The combination of GC-ECD using a pretreated column with HBr-methanolic solution, as a reagent gas, gave enough sensitivity to determine trace levels of TBT compounds in biological samples for investigation of bioconcentration and metabolism in aquatic animals. The pretreatment of column with HBr produced good results in that TBT chloride eluted smoothly without adsorption. Consequently TBT chloride could be determined directly without derivation. From GC-ECD spectra, it was found that TBT chloride converted to and eluted as the respective bromide by the reaction with HBr remaining in the column.

The results showed that this sample preparation method and the proposed method of pretreatment of a column with HBr-methanolic solution and GC-ECD measurement, presents a highly suitable analytical technique for the trace measurement at less than the ngg^{-1} level of TBT in fish tissue Table 3. Hence the technique was significantly selective and sensitive, amounts of sample were comparatively small, and subsequently the interference of sample matrix and the amounts of reagents used for extraction analysis could be minimized, and might be also applicable for trace level concentration of TBT in biological samples, sea water and sediment.

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