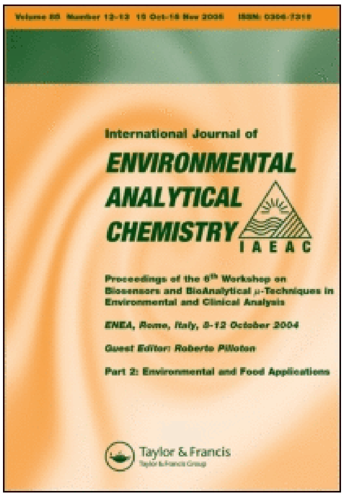

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# Evaluation of *N*-methyl-*N*-tert-butyldimethylsilyl trifluoroacetamide for environmental analysis under both EIMS and electron capture NICIMS conditions and comparison to trimethylsilyl reagents under EIMS

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# Evaluation of *N*-methyl-*N*-tert-butyldimethylsilyl trifluoroacetamide for environmental analysis under both EIMS and electron capture NICIMS conditions and comparison to trimethylsilyl reagents under EIMS

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*N*-Methyl-*N*-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) is a silylating agent with a range of applicability in clinical and environmental analysis, particularly when substrates possess at least moderate acidity. In this paper, we demonstrate its applicability and limitations as a reagent for environmental analysis by comparing and contrasting two different target analyte problems in a sewage effluent matrix. In one case, electron ionization was used for the determination of three potential endocrine disrupting compounds: 17$\beta$-oestradiol, ethynyl oestradiol, and oestrone where the phenolic functionality was silylated with MTBSTFA and compared with results using *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) as the reagent. In this instance, a large volume of effluent was subjected to either solid-phase extraction followed by cleanup using high-performance gel permeation chromatography (AppI) or liquid/liquid extraction followed by SPE fractionation and HPLC fractionation (AppII). The method using BSTFA rather than MTBSTFA was demonstrated to work down to low and sub-ppt levels where the target compounds were found. In a parallel and contrasting study, sewage effluent was analysed for 3,5,6-trichloropyridinol (TCP) by extracting one liter of water using liquid–liquid extraction and determined by GC/MS operated in the negative ion chemical ionization (electron capture) mode after derivatization with MTBSTFA. TCP is the major metabolite of the commonly used insecticide, chlorpyrifos, and herbicide trichlorpyr. The recoveries using dichloromethane as the extractant were 59%, with a relative standard deviation of 2%. This method was used to investigate levels of TCP in sewage effluent. During this analysis, a tentatively identified additional isomer of TCP (X-TCP) was found. The 3,5,6-TCP, the common chlorpyrifos metabolite and the synthesized isomer, 3,4,5-TCP were compared with X-TCP. All three isomers have significantly different retention times. The average level of 3,5,6-TCP was 3.4 $\mathrm{ng\,L^{-1}}$, while the level of X-TCP was 39.8 $\mathrm{ng\,L^{-1}}$. The two approaches are compared and contrasted with respect to artefact formation and matrix component effects. The reagent MTBSTFA is found to be suitable for quantitative analysis of environmental samples for relatively acidic substrates (e.g. phenols and carboxylic acids). More powerful silylating agents such as *N*-methyl-*N*-trimethylacetamide or BSTFA are required for sterols and similar substrates. The stability of the two silylating reagents appears

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to be similar and practical for accurate quantitative analysis. Differences in EI spectra with respect to fragmentation may also dictate which reagent is preferred.

*Keywords:* BSTFA; MTBSTFA; Oestradiol; Effluent; Trichloropyridinol; GC/MS; Cleanup; Porous graphitic carbon HPLC

## 1. Introduction

*N*-Methyl-*N*-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) is a typical silylating reagent that is suitable for derivatizing various oxygen functionality in the target molecules. This chemistry may be rationalized to some extent by the exceptional strength of the silicon–oxygen bond at 108 kcal mol$^{-1}$ [1]. Thus, alcohols, phenols, and acids are its primary potential substrates. The reactivity of MTBSTFA is expected to be similar to the more familiar trimethylsilyl reagents such as *N*,*O*-trimethylsilyltrifluoroacetamide (BSTFA), but its greater bulkiness may be a factor that enhances the stability of the derivative relative to hydrolysis [2]. This feature also creates the possibility of steric hindrance to the reaction in some substrates. One of the chief concerns in applications of silylating reagents is the possibility of hydrolysis of the resulting derivative. Another limiting concern in environmental and other analysis is the production of derivatization artefacts as well as derivatization of matrix components [3] that could interfere with specificity. These processes impart a greater burden on the separation technique to eliminate coelutions of extraneous compounds with target analytes or may dictate a need for more selectivity in the detection phase of the analysis.

An emerging area of environmental interest is the exposure and effects presented by the persistent infusion of pharmaceuticals and personal care products (PPCPs) into ecosystems [4]. Among the many potential analytes and classes of analytes belonging to PPCPs are those also classified as endocrine-disrupting compounds (EDCs) [5]. Within this group, oestrone (EST), 17$\beta$-oestradiol (ESD), and ethynyl oestradiol (EED) have been of much analytical interest [2]. In the analysis of waste effluent streams, various solid phase extraction, cleanup, and derivatization approaches have been used [6–8] where GC/MS has been the most widely used final separation/detection approach. Detection limits in the 0.1–10 ng L$^{-1}$ concentration range are usually appropriate for these analytes [2] in waste streams.

Another group of compounds that may act as EDCs or exhibit other deleterious effects is that of pesticides. A common concern is the possible harmful effect of pesticides experienced by nontarget organisms. Pesticides are monitored either as the native compound or as a metabolite that can serve, in some instances, as a biomarker of exposure. The compound, 3,5,6-trichloropyridinol (TCP), has been used in several studies as a biomarker for chlorpyrifos exposure in humans [9], catfish [10], trout [11], and rats [12]. Chlorpyrifos degrades to TCP through hydrolysis and photolysis, making TCP appropriate as a environmental indicator as well [13]. Due to its versatility, chlorpyrifos is the most widely applied organophosphate insecticide. Currently, chlorpyrifos is used as an insecticide on almost every major crop grown in the USA. It has also been used for domestic protection against cockroaches, ticks, fleas, termites, spiders, and fire ants. Residential chlorpyrifos products were phased out in December 2001, and all retail sales have stopped. However, it will remain available for nonresidential uses such as golf courses, ornamental nurseries, and crop protection (except tomatoes) [14].

Several methods have been developed to measure chlorpyrifos directly [15–17]. Most of the published methods used to measure TCP levels are specific to clinical samples, including urine [10, 18, 19], blood [10, 20] and bile [10]. An immunoassay was developed to measure both chlorpyrifos and TCP [21]. It should also be noted that TCP is also the major metabolite of chlorpyrifos-methyl and the herbicide trichlorpyr [22].

In this paper, we compare the use of MTBSTFA in an EIMS method for EST, ESD, and EED to the use of BSTFA or similar reagents using two approaches termed AppI and AppII; we then contrast EIMS work with MTBSTFA to its use in an ECNIMS method for TCP in the analysis of large volume samples of effluent from a tertiary sewage treatment plant. The two studies (EIMS and ECNIMS) together provide a direct quantitative comparison of the relative ecological exposure to the two compound categories of pesticide metabolites and PPCPs. The reactivity of MTBSTFA is also compared with BSTFA for derivatization of alcohol/sterol functionality. The EI and ECNIMS spectra of compounds derivatized with MTBSTFA and BSTFA are discussed with respect to fragmentation using additional substrates as examples.

## 2. Experimental

### 2.1 *Reagents and chemicals*

3,5,6-Trichloro-2-pyridinol was obtained in neat form (Sigma-Aldrich, Milwaukee, WI). Standards were made up in methanol and serially diluted. The derivatizing agent used was *N*-methyl-*N*-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) (Sigma-Aldrich). The first of the two internal standards used was 7-hydroxy-4-methylcoumarin (Sigma-Aldrich), and the second internal standard, octachlorobiphenyl, PCB (#204) was part of a mixture of polychlorinated biphenyls (PCBs) (Chem Service Inc., West Chester, PA), including 2,2′,6-trichlorobiphenyl, 2,2′,6,6′-tetrachlorobiphenyl, 2,2′,4,6,6′-pentachlorobiphenyl, 2,2′,4,4′,6,6′-hexachlorobiphenyl, 2,2′,3,4,4′,6,6′-heptachlorobiphenyl, 2,2′,3,4,4′,5,6,6′-octachlorobiphenyl. The concentrated HCl was 36.5–38.0% (J.T. Baker, Phillipsburg, NJ). The dichloromethane (DCM) was HPLC-GCMS grade (Fisher, Pittsburg, PA). The toluene was reagent-grade (J.T. Baker). The filtered (0.2 µm pore size) deionized (DI) water came from a Barnstead Nanopure ultrapurification system (Barnstead, Dubuque, IA).

EST (99%), ESD (98%), and EED (98%) were obtained from Sigma Chemical Co. (St. Louis, MO) as neat solids. 17$\beta$-Oestradiol-2,4,16,l6 $d_4$ (>98% deuterated) was obtained from CDN Isotopes (Pointe Claire, Quebec, Canada) as a neat solid. Stock solutions of neat oestrogen standards were prepared by measuring out approximately 0.0010 g of each oestrogen and dissolving them in separate 10 mL amber glass volumetric vials with acetonitrile. Serial dilutions in acetonitrile were prepared by transferring aliquots of stock solutions, using Rainin electronic digital pipettes, to 2 mL glass vials with Teflon-lined caps. MTBSTFA (99+%), BSTFA (99+%), and chlorotrimethylsilane (CTMS) (99+%) were obtained from Aldrich. BSTFA with 10% CAMS was obtained from Pierce. Anhydrous sodium sulphate was from J.T. Baker (Phillipsburg, NJ). CPI Technologies (Santa Rosa, CA) solid-phase extraction disks were Nu.Phase C18 material, 47 mm and 90 mm diameters. Varian solid-phase extraction disks were styrenedivinylbenzene material, 47 mm in diameter. Acetonitrile, ethyl acetate, dichloromethane, and methanol were chromatography-grade solvents from J.T. Baker and Burdick and Jackson (Muskegon, MI). Deionized water (17.4 MΩ resistivity) was supplied by a Barnstaed NANOPURE system.

### 2.2 *Liquid/liquid extraction of TCP*

The Bartels and Kastl [18] urine analysis method was used as a basis for developing the method for TCP. A major difference between the two methods is that Bartels and Kastl concentrated the urine fivefold, while the present method concentrates the sample 1000-fold.

One litre of water was taken as the sample volume. The pH of the sample was adjusted to 1.0 using concentrated HCl to facilitate extraction of TCP into organic solvent (DCM). These conditions may liberate the TCP from conjugates including the major transformation product, the glucuronide of TCP [10], although the conditions are not as vigorous as the clinical method (10% HCl and 80°C for 1 h). 3,5,6-Trichloro Pyridinol has a log $K_{ow}$ of 3.21 [23], suggesting that DCM should be a favourable extractant. Approximately 30 mL of DCM was added to the water sample in a separatory funnel; the sample was then shaken vigorously; the extraction procedure was repeated two additional times using about 20 mL of DCM each time. The final extract volume containing TCP was about 50 mL. Initial concentration used a refluxing condenser with heating manifold to reach about 4 mL volume. Final concentration used a gentle nitrogen stream to evaporate the sample to dryness for the derivatization step.

For derivatization, solutions of MTBSTFA (100 μL), Cou (25 μL), and PCB 204 (50 μL) were added to the dried extract and brought to 1.0 mL with toluene. The derivatization mixture was then heated at 60°C for 1 h in a tightly capped (Teflon seal) glass vial.

### 2.3 *Approach I (AppI)*

**2.3.1 Disk extraction of ESD, EED, and EST.** Comparison of solid-phase extraction media was carried out to assess the extraction efficiency of the nonpolar C 18 and slightly polar styrenedivinylbenzene (SDB) solid phases to isolate the oestrogens from aqueous matrices. For these experiments, only 47 mm disks were used. Duplicate samples were prepared for each phase and extracted using the CPI manifold. For each sample, 0.5 L of deionized water (17.4 MΩ) was spiked with 10 ng each of EST, ESD, and EED. Samples were acidified to pH 3 using HCl, and 2.5 mL of methanol was added (5 mL $L^{-1}$) to the sample prepared for C18 extraction.

Washing and conditioning steps for the disks were performed as prescribed by each manufacturer. For the SDB disks, pre-washing was done by soaking the disks in acetone for 45 min, draining, and soaking again in fresh acetone for an additional 45 min. Ethyl acetate was used in pre-washing steps for C18 disks. The disks were then placed in the CPI extraction manifold for washing and conditioning steps. The SDB disks were washed with acetone, followed by ethyl acetate. C 18 disks were washed only with ethyl acetate. For both phases, washing entailed adding 20 mL of solvent to the reservoir, allowing the disk to soak for 3 min, then draining the wash to waste and drying the disks for 1 min under vacuum (15″ Hg). Conditioning steps were identical for both phases and entailed adding 10 mL of methanol to each reservoir, allowing the disks to soak for 3 min, then draining the methanol until within 1 mm of the disks' surface. A final rinse of 20 mL of deionized water (17.4 MΩ), acidified to pH 3, was then added to each disk and drained to within 2 mm of the disks' surface.

Spiked samples were added to each reservoir and extracted, diverting flow to waste. Extraction rates were maintained at 100 mL min$^{-1}$ or less by adjusting the valve opening. Vacuum was not necessary to maintain flow of the samples through the disks, since no particulate matter was present in the spiked waters.

Once extracted, excess water was removed from disks by applying vacuum (15″ Hg) for 30 min. Disks were eluted with aliquots of solvent previously used to rinse down the walls of the flasks used to hold the respective samples. Rinses were transferred to the sample reservoirs by pipette, washing the walls of the reservoirs as well. Samples extracted over SOB material were eluted first with aliquots of 10 mL of ethyl acetate, which were allowed to soak the disk for 3 min, then drained to the respective collection tube. Elution steps were repeated twice again with 5 mL aliquots of ethyl acetate. C18 disks were extracted in the same manner, but with ethyl acetate/dichloromethane (1 : 1).

To compensate for the larger disk size and subsequent greater amount of material, solvent volumes were increased from 10 to 20 mL for washing and elution steps, and from 5 to 10 mL for the second and third elutions. The final disk eluent (40 mL) of ethyl acetate/dichloromethane (1 : 1) was concentrated and dried.

After GPC cleanup, the eluates were taken to dryness and reconstituted in 200 μL of ethyl acetate, 25 μL of MTBSTFA, and 10 ng of the internal standard 17$\beta$-oestradiol-d$_4$, capped, and heated at 60°C for 30 min.

Recoveries of EST, ESD, and EED from the disks (C15 and SDB) disk were 83% and 80%; 83% and 74%; 46%, and 26%, respectively.

**2.3.2 SPE with 90 mm C18 disks.** Analysis of environmental samples and treated waste effluent for human derived oestrogens was expected to entail extracting up to 4 L samples with SPE disks. When suspended particulates are present in samples, extraction could become time-consuming, if not impossible, as pore spaces in the disk would become clogged with particulate material. Ninety-millimetre disks designed to extract large samples containing suspended particulates, which would otherwise impede flow of the sample through the smaller 47 mm disks, were chosen for this reason.

Analysis of oestrogen-spiked water was performed to verify that oestrogens could be extracted from several litres of sample using 90 mm C18 disks, and recovered from the disk using the solvent conditions described in section 2.3.1. A 4 L amber glass bottle was filled with 2 L of tap water. The spiking solution of 1 ng L$^{-1}$ of each of EST, ESD, and EED was prepared in acetonitrile. Twenty microlitres of this solution was transferred to the water sample using a Rainin electronic digital pipette (100 μL maximum volume) to give a sample with 10 ng L$^{-1}$ of each oestrogen spiked. The sample was acidified to pH 2 with 12 N Rd (Seastar). Washing and conditioning of the disk were performed as described before. To compensate for the larger disk size and subsequent greater amount of material, solvent volumes were increased from 10 to 20 mL for washing and elution steps, and from 5 to 10 mL for the second and third elutions. The final disk eluent (40 mL) of ethyl acetate/dichloromethane (1 : 1) was concentrated and dried. Recoveries from 90 mm disks for EST, ESD, and EED were 70, 96, and 41%, respectively.

**2.3.3 Cleanup following disk extraction using HPGPC.** Gel-permeation chromatography was performed with a high-pressure liquid chromatograph arranged for preparative work. The system consisted of a Waters 515 HPLC pump, a Waters 717 plus antosampler (2500 μL syringe and 5000 μL sample loop), a Phenomenex Phenogel 10 μm linear/mixed pre-column (7.8 × 50 mm), followed by two Waters Envirogel styrenedivinylbenzene columns (19 × 150 mm and 19 × 300 mm). A Waters 2487 dual-wavelength absorbance detector was used to monitor extracts (214 nm and 281 nm). A Waters Fraction Collector II was used to collect various time-based fractions of column eluent following injection of samples. Dichloromethane mobile phase was helium-degassed, and the flow rate was set at 5 mL min$^{-1}$. Fractions were collected and combined from 14.12 to 17.47 min.

## 2.4 *Approach II (AppII)*

**2.4.1 Liquid/liquid extraction of ESD, EED, and EST.** Three extractions of the effluent as received were performed with methylene chloride (100 mL, 50 mL, 50 mL) in a 2000 mL separatory funnel. Emulsions were broken up with silylated glass wool. The pH was not adjusted (pH 7.0) because the oestrogens would not be ionized at this pH but carboxylic acids would be. The extract was dried over sodium sulphate and then concentrated in a round-bottom flask with a three-stage Snyder column under refluxing conditions using Teflon boiling chips. The concentrated extract was further concentrated by a gentle nitrogen stream and evaporated to dryness in a 60 mL glass tube. The dry film was redissolved with 1 mL of hexane for application to the SPE cartridge cleanup using silica.

**2.4.2 SPE Si cleanup following liquid/liquid extraction.** A 3 mL cartridge of LC-Si (Supelco, Bellafonte, PA) was washed with 6 mL of hexanes with a small portion retained above the frit. The 1 mL sample extract was added and pulled through along with 2 mL of hexanes, always retaining a small liquid level above the frit. Next, a solvent gradient was applied in the following sequence: 2 mL (v/v, 50/50) of hexanes/methylene chloride, 2 mL of methylene chloride, 3.5 mL of acetone. The oestrogens were recovered in the acetone fraction and concentrated to dryness under a gentle nitrogen stream in a 13 mL test tube. A 1 mL aliquot of hexanes was added and used to wash down the walls. The hexanes solution was then filtered prior to injection on the HPLC porous graphitic carbon (PGC) column.

**2.4.3 HPLC fractionation on PGC following SPE Si.** A 1 mL hexanes solution of the sample was loaded on a Valco 10-port injector (VICI, Houston, TX) for injection on a 5 μm PGC HyperCarb (Hypersil, Keystone, Bellefonte, PA) column 4.6 mm ID × 100 mm. A flow rate of 2.5 mL/min of mobile phase was used. The gradient consisted of: 100% hexane for 2 min, from 2 to 5 min developing 18.7% toluene; from 5 to 10 min developing 50% toluene, 20% acetonitrile; from 10 to 16 min developing 100% acetonitrile; from 16 to 18 min maintaining 100% acetonitrile; from 18 to 26 min developing 100% toluene; from 26 to 30 min developing 100% hexane; from 30 to 36 min developing 100% hexane to equilibrate before the next run. Fractions were collected by a Foxy Junior Collector (ISCO, Lincoln, NE) with 1 min/13 mL test tube. Tube 9 contained EED and 10 contained EST and ESD. The overall recovery of spiked analytes in DI water using AppII averaged 52%.

Table 1. Selected ion monitoring (SIM) ions.

| Compound | Retention time (min) | Quantification ion | Qualifying ions |
|---|---|---|---|
| Cou | 11.47 | 290.10 | 175.10 |
| 3,5,6-TCP | 7.66 | 160.90 | 162.90,<br>164.90,<br>161.90 |
| 3,4,5-TCP | 8.17 | 160.90 | 162.90,<br>164.90 |
| PCB 204 | 12.78 | 429.80 | 431.80 |

## 2.5 *MS conditions for TCP*

The gas chromatograph was an Agilent 6890 GC with 5973 Mass Selective Detection. The column was an HP-5MS (Agilent, Palo Alto, CA). The injection parameters were 2 μL pulsed splitless injection, 250°C injection temperature, and a helium carrier gas flow rate of $1\,\mathrm{mL\,min^{-1}}$. The GC programme initially at 100°C was held for 1.0 min, and then it increased to 300°C at a rate of $15^\circ\mathrm{C\,min^{-1}}$ and held for 8.0 min. The transfer line was set at 280°C. For TCP analysis, the MS was set to the negative-ion chemical ionization (NCI) mode with the following conditions for electron capture: ion-source temperature 150°C, electron energy 70 eV, the methane reagent gas flow rate set at $0.8\,\mathrm{mL\,min^{-1}}$. After running a sample of derivatized TCP with the internal standards in scan mode, ions were chosen for use in the selected ion monitoring (SIM) mode (see table 1).

## 2.6 *AppI GC/MS conditions*

A Hewlett Packard 5890 A gas chromatograph with an autosampler (5 μL syringe) was used for final chromatographic separation. A Supelco fused-silica intermediate polarity capillary column (0.5 m × 0.53 mm ID) was used as a retention gap to allow 3 μL injection volumes. The retention gap also served to protect the analytical column from sample impurities and aggressive derivatizing reagents. A J&W Scientific DB-5MS capillary column (30 m × 0.25 mm ID) with a 0.25 μm film thickness was used for gas chromatography (GC). Mass spectral (MS) analysis was performed with a VG-70SE mass spectrometer. Electron ionization (EI) was performed, and positive ions were monitored by a full scan or select ion recording (SIR). The VG-70SE was operated in low-resolution mode. GC parameters were as follows: initial temperature of 80°C held for 1 min following injection, constant ramp of $15^\circ\mathrm{C\,min^{-1}}$ to 300°C final temperature, held for 4 min. In the SIR mode, molecular ions of the MTBSTFA derivatives of EST (384.2486 *m*/*z*), ESD (386.2641 *m*/*z*), and EED (410.2641 *m*/*z*) were monitored.

TMS derivatives were also studied, and in this case, acetonitrile was used as the make-up solvent for the derivatization reaction to provide a polar, aprotic environment for exchange of the protons from the hydroxyl groups of the oestrogens with TMS groups from BSTFA. BSTFA/CTMS (100 : 1) was added at one-tenth the total sample volume. Samples were heated at 60°C for 60 min. In this case, ions monitored included the mono-TMS derivative of EED (368 *m*/*z*), di-TMS ESD (416 *m*/*z*) and mono-TMS ESD (342 *m*/*z*), and EST (*m*/*z* 342).

## 2.7 *AppII GC/MS conditions*

This work was performed on an Agilent 5973 GC/MSD with a 6890 gas chromatograph. A 40 m (0.18 mm ID with 0.18 µm film) DB5 ms (J&W, Folsom, CA) was used with a He flow of 0.5 mL min$^{-1}$. The injection parameters were 1 µL pulsed splitless injection (40 psig pulse pressure), 280°C injection temperature, and transfer line temperature of 280°C. The GC initial temperature at 105°C was held for 1.0 min, and then it increased to 300°C at a rate of 20°C min$^{-1}$ and was held for 20.0 min. The transfer line was set at 280°C.

The following ions (*m*/*z*) were monitored by compound: 480.8 for tetrabromocresol (second internal standard found unnecessary for this study); 504.4 for oestriol (3-TMS derivative) as internal standard; 342.2, 257.1, 218.1, 327.2 for EST (1-TMS derivative); 416.3, 285.2, 326.2, 232.1 for ESD (2-TMS derivative); 368.2, 285.2, 301.2, 232.1 for EED (1-TMS derivative); 425.3, 440.3, 301.2, 285.2 for EED (2-TMS derivative).

## 2.8 *Reagent blanks*

Reagent blanks taken through the respective procedures gave no response for the monitored ions of the target analytes at their retention times.

## 3. Results and discussion

## 3.1 *EIMS of ESD, EST, and EED*

The mass spectra of the derivatives of the target compounds with either derivatizing reagent exhibit relatively abundant molecular ions and characteristic fragment ions. As the concentration of the standard decreases, the relative importance of artefacts and other background increases but seems to be about the same for each reagent. Artefacts become a more important concern when a sample matrix is encountered, and derivatization of matrix components adds to the burden of separation. The chromatography of the derivatives is illustrated in figure 1 where it is obvious that the MTBSTFA derivative of ESD tails significantly. This results from the inability of

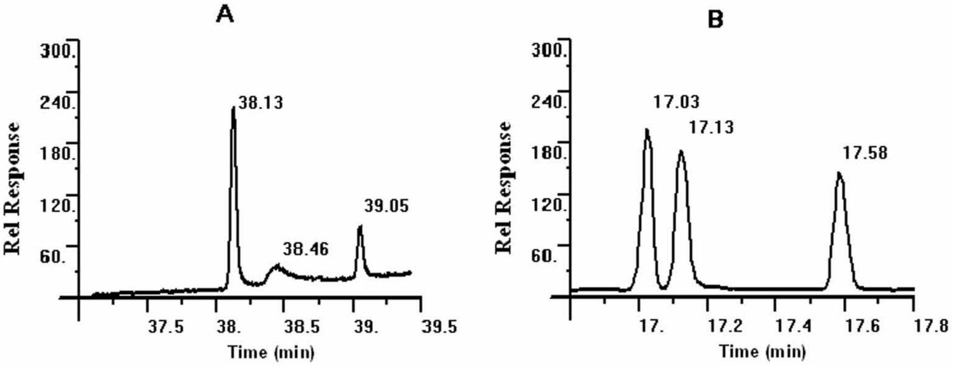

Figure 1. GC/MS total ion chromatogram of EST, ESD, and EED derivatized by (A) MTBSTFA (38.13 min, 38.46 min, and 39.05 min, respectively) and (B) BSTFA (17.03 min, 17.13 min, and 17.58 min, respectively); conditions are given in section 2.

MTBSTFA to derivatize the alcohol functionality of either ESD or EED (di-TMS derivatives are formed with BSTFA). The chromatography suffers less in the case of EED. Such a situation would have a deleterious effect on the detection limit and would possibly increase problems with coextractives that could tail into the target peak. This lack of reactivity of MTBSTFA seems to be a general feature as we have observed it with common sterols such as cholesterol as well. Thus, some care must be taken in approaching any problem where the use of MTBSTFA is anticipated and steric hindrance or an alcohol/sterol group is at issue.

### 3.2 *ESD, EST, and EED found in effluent*

The application of AppI to tertiary treated sewage effluent resulted in the detection of oestrone at 0.66 ng $L^{-1}$. ESD and EED could not be detected at the low-ppt levels required due to coextractive inteferences. Thus, HPGPC was not an adequate cleanup for low ng $L^{-1}$ detection limits of all three analytes in sewage effluent.

Use of AppII with both SPE and HPLC fractionation afforded two fractions, one containing EED and one containing EST/ESD. The results of analyses yielded values of 0.53 ng $L^{-1}$, 1.07 ng $L^{-1}$, and 6.31 ng $L^{-1}$ for EST, ESD, and EED, respectively. The agreement between the two methods on the value for EST is good. Concentrations of oestrone have been reported for treated waste water ranging from 4.5 to 27 ng $L^{-1}$ [8, 24]. Figure 2 presents the chromatographic results for standard and sample selecting the *m*/*z* 416 ion of ESD for illustrative purposes. The cleanup was highly effective in removing coextractives, and the two responses visible are due to ESD and an additional ion from the internal standard. Figure 3 compares the background subtracted mass spectrum afforded by a standard and from the sample using ESD as an example. The agreement between sample and standard is excellent.

In our work with BSTFA using AppII, the derivatizations of ESD and EED were complete (the bis trimethylsilyl ethers were formed in both cases) in 1.5 h at 65°C using toluene as the solvent. This may represent a solvent effect relative to the use of acetonitrile in AppI. where a mixture of the singly and double derivatized ESD was obtained and only the singly derivatized EED.

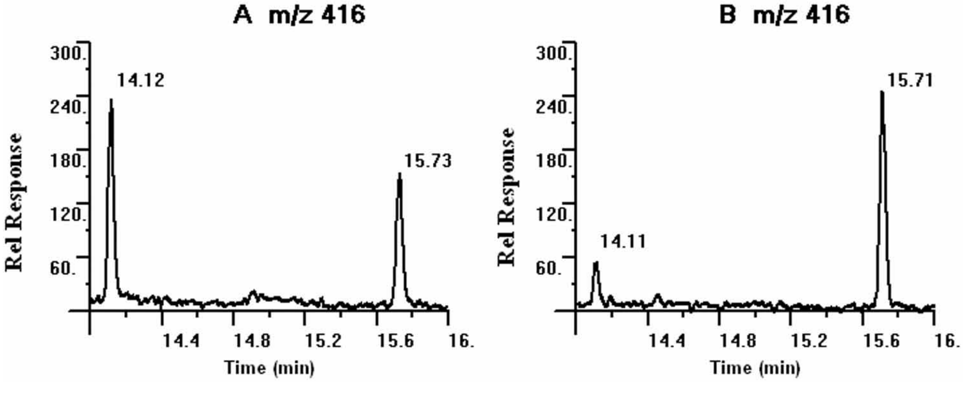

Figure 2. Ion chromatogram for *m*/*z* 416 of ESD (2-TMS derivative) for (A) a standard (5 pg) and (B) a sample (extract of effluent) where the response at 14.11 min is due to ESD, and that at 15.71 min is the oestriol (3-TMS derivative) internal standard; conditions given in section 2.

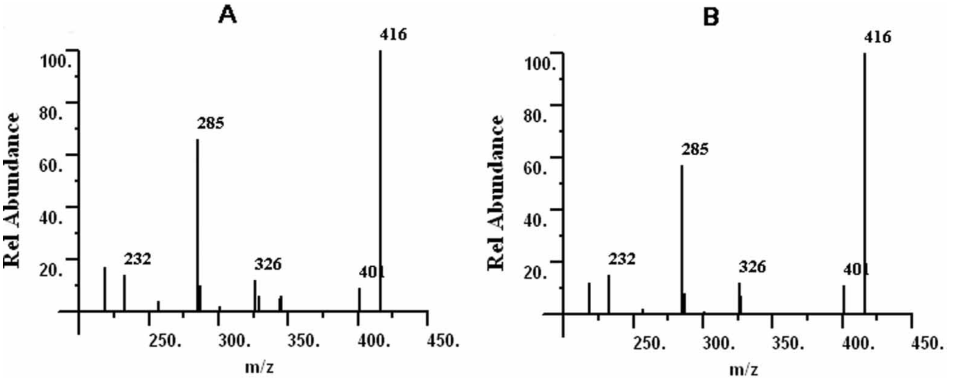

Figure 3. Background-subtracted mass spectra for (A) standard and (B) sample of ESD (2-TMS derivative) for the responses shown in figure 2.

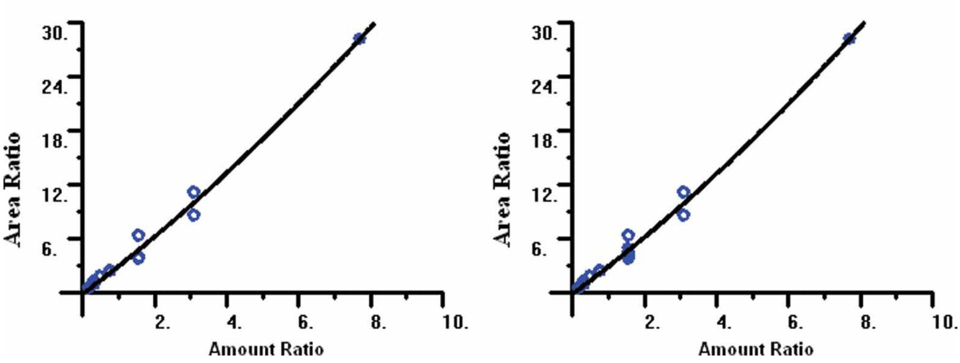

Figure 4. Original calibration curve for ESD (2-TMS derivative) (left) and the same curve after plotting additional points at the 5 pg µL$^{-1}$ level over a 3-week period injected from the same GC vial (right).

The use of oestriol as the internal standard in AppII requires some comment in view of the possible occurrence of oestriol in effluent samples. In our work, the internal standard was spiked at approximately 100 times the level expected in effluent. Moreover, the oestriol was not collected in the fractions containing the analytes because it elutes at 11.28 min from the HPLC column, and this would correspond to collection Tube 12.

### 3.3 *Stability of derivatives*

The use of MTBSTFA is sometimes promoted on the basis that its derivatives may have a greater stability than more common reagents. We looked at this issue in the context of our analysis using BSTFA from a very practical standpoint. Figure 4 shows the original calibration using freshly derivatized analytes/internal standard and the results of continuing calibrations which are plotted near the 1.6 ratio value on the $x$-axis. These values were obtained over a 3-week period from the original calibration standard solution left in the punctured GC vial at room temperature in the autosampler. Clearly, the trimethylsilyl ether derivatives are reasonably stable, and we found no apparent difference in stability between the two reagents. One can have confidence

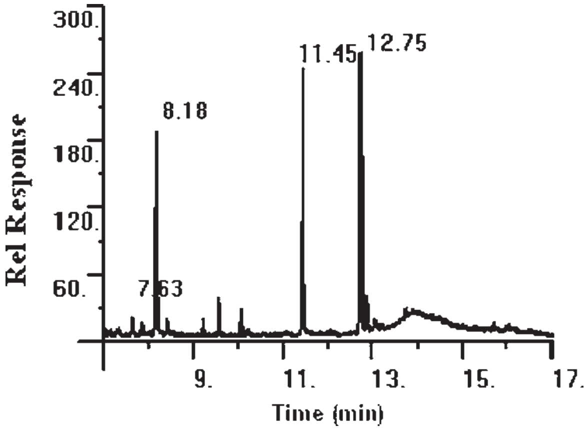

Figure 5. Total ion chromatogram (nine ions) for effluent extract with 3,5,6-TCP at 7.7 min; unknown TCP isomer at 8.18 min; 7-hydroxy-4-methylcoumarin internal standard at 11.45 min; and PCB internal standard at 12.75 min. Conditions: 30 m DB5 column (0.25 mm ID) 100–300 @ 15°C $\mathrm{min}^{-1}$; injector 250°C; source 150°C; 0.8 mL of methane $\mathrm{min}^{-1}$ negative ion mode.

Table 2. Southwestern city/county tertiary sewage-treatment effluent analysis.

| Sample date | 3,5,6-TCP (ng/L) | Environmental TCP isomer (ng/L) |
|---|---|---|
| 1/5/01 ($n=1$) | 3.2 | 31.1 |
| 3/14/01 ($n=2$) | 4.2 (0.3)[a] | 52.6 (40.4)[a] |
| 3/23/01 ($n=3$) | 2.8 (1.8)[b] | 35.8 (24.7)[b] |
| Average | 3.4 | 39.8 |

[a] (% difference).
[b] Standard deviation.

that the quantitative values generated from use of BSTFA are representative of the actual amount.

### 3.4 *EC NICIMS of TCP*

The change in ionization conditions has a profound effect on the analysis of effluent and on the mass spectra of silyl ether derivatives. Essentially no artefacts are present beyond the very early stages of the chromatograph when MTBSTFA is used with TCP. This results in immediate reduction of the burden on the separation system compared with EIMS conditions. Under EC conditions, the derivative apparently loses the silyl group together with one chlorine, presumably by rearrangement, to produce a two-chlorine cluster beginning at $m/z$ 161 characterizing the pyridinol moiety. The selectivity of the detection is such that no cleanup is needed to detect TCP at low-ng $\mathrm{L}^{-1}$ levels, in sharp contrast with the situation with regard to the oestrogens. Figure 5 illustrates a 9-ion chromatogram showing the responses from TCP, an unknown ostensibly similar to TCP, and the two internal standards. Quantitative results are given in table 2. In this example, 3,5,6-TCP was determined at 3.2 ng $\mathrm{L}^{-1}$ while the unknown was estimated at 31.1 ng $\mathrm{L}^{-1}$ (see table 2) based on the calibration response for 3,5,6-TCP. It is apparent

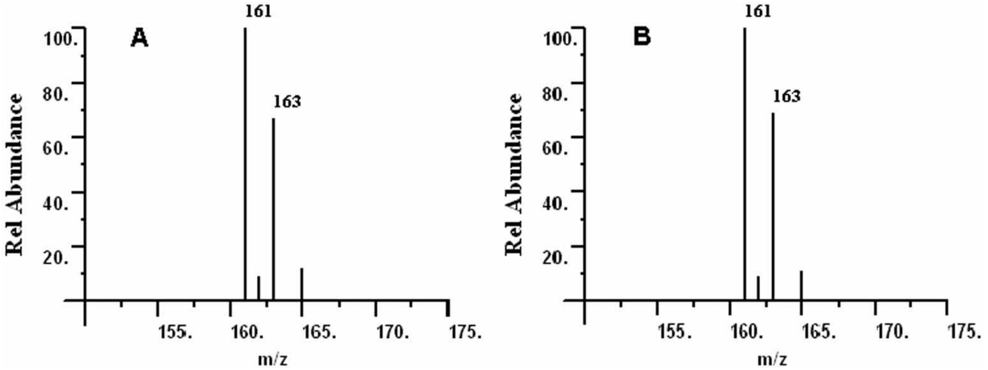

Figure 6. Background subtracted mass spectrum for (A) standard of 3,5,6-TCP and (B) sample of effluent extract.

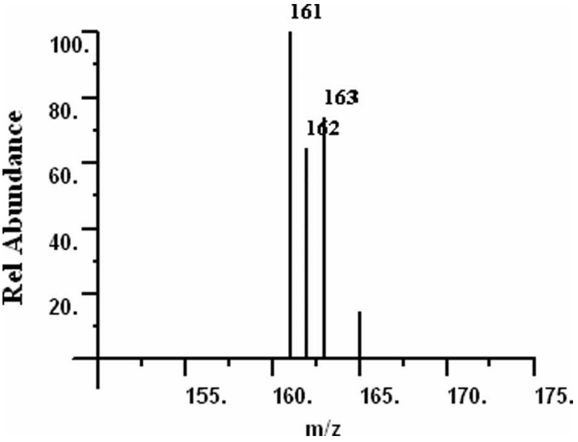

Figure 7. Ion-monitoring mass spectrum for 3,4,5-TCP isomer; conditions as in figure 5.

that the electron-capture negative-ion mode provides sufficient sensitivity and selectivity to allow determinations down to the low-ng $L^{-1}$ levels in effluent.

The spectrum of TCP standard and that obtained from the sample extract is given in figure 6. In addition to 3,5,6-TCP, the response for an unknown compound at retention time 8.2 min gave the mass spectrum shown in figure 7. Its structure has not been determined, but it cannot be the 3,4,5-trichloropyridinol isomer, since this afforded a mass spectrum identical to that of 3,5,6-TCP and had a retention time of 7.9 min. There are at least nine isomers possible that still retain the trichloropyridinol structure as well other structural variations, and we do not have definitive evidence on the structure of the unknown.

The fragmentation under EC conditions is usually limited and is also structurally dependent. For analytes such as TCP and pentachlorophenol, the main observed ion corresponds to loss of the tert-butyldimethylsilyl unit and a chlorine atom (presumably by a rearrangement) resulting in the appearance of a radical anion reflecting this loss. In the case of 4-nitrophenol, for example, where no ortho effect is possible, the molecular anion is the observed moiety with the tert-butyldimethylsilyl moiety still attached to the molecule. Thus, the chemistry of each target analyte must be investigated in order to choose the appropriate ions to monitor.

### 3.5 *Recoveries of TCP*

The clinical methodology for TCP was modified to handle the sample size of 1 L rather than 1 mL. A total of three recovery analyses were performed with a mean recovery of 59% and a relative standard deviation of 2%. The calibration curve used in this analysis was linear in the range of 0.8–17.5 ng $mL^{-1}$ corresponding to quantitative levels of 0.8–17.5 ng $L^{-1}$. Linear regression resulted in an $R^2$ value of 0.991. Thus, the modified approach appears applicable to environmental analysis and revealed the presence of at least one unanticipated compound of apparently similar structure.

## 4. Conclusions

The application of the derivatizing agents MTBSTFA and BSTFA was compared using EIMS conditions for oestrogens and evaluated under ECNIMS for TCP in waste-treatment effluent. The MTBSTFA reagent is suitable for trace-level quantitative environmental analysis with substrates of sufficient acidity but is more selective than common reagents such as BSTFA. The relative stability of MTBSTFA derivatives *versus* TMS derivatives does not appear to be greater within the context of punctured GC vials in an autosampler over a period of 21 days. Oestrogens were present at low- or sub-ng $L^{-1}$ levels, and during the study, novel use was made of HPLC cleanup with porous graphitic carbon packing. The average level of TCP was 3.4 ng $L^{-1}$ in the effluent, whereas the level of TCP in human urine ranges from 4 to 400 ng $mL^{-1}$ [18]. For comparative purposes, pharmaceuticals and personal care products (PPCPs) are often found at the same levels or greater than those reported here for TCP [25, 26]. Thus, within the scope of this study, PPCPs occur at levels comparable to or even greater than those of pesticide metabolites.

Sources of TCP include the many environmental uses of chlorpyrifos in addition to the metabolic processes resulting from human exposure and the treatment process itself. TCP is the breakdown product of several biological and environmental processes, and these contributions to the sewage effluent are most likely much higher than the levels found in humans. The unknown compound, assumed to be an isomer of TCP, does not appear to have been detected in the clinical analysis [10, 18–20] or the aquatic environmental analysis [21]. Further research needs to be done to determine the origin and structure of the isomer.

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