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Trimethylsilyldiazomethane: A safe non-explosive, cost effective and less-toxic reagent for phenol derivatization in GC applications

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ABSTRACT

Diazomethane is a highly explosive and toxic gas routinely employed for the quantitative and clean derivatization of phenols. We investigated the commercially available trimethylsilyldiazomethane in the presence of diisopropylethylamine as a safe, non-explosive and less-toxic alternative using six phenolic polychlorinated biphenyls as model analytes and fluoro-tagged analogues as internal standards. We compared yields and derivatization times of each method employing a liver microsomal extract as a real matrix. Steric hindrance and electronic properties of the analytes were also investigated. The alternative method afforded equal to higher derivatization yields with increased reaction times, up to 100 min, while explosion and toxic exposure risks were minimized and cost efficiency was increased above 25%. These findings demonstrate that non-explosive trimethylsilyl diazomethane produces comparable derivatization results to the dangerous diazomethane under the conditions studied.

1. Introduction

The analysis of phenolic analytes is routine in a broad range of disciplines, *e.g.* environmental sciences, medicinal chemistry, pharmacology and toxicology (Hovander et al., 2002; Kraimer, 1995; Qiu et al., 2007; Halket and Zaikin, 2004). Before phenols can be analyzed by GC they need to be derivatized. Commonly a derivatization reaction by O-methylation using the explosive gas diazomethane (DM) is employed. The main advantages of DM are its quantitative and clean reactions (Hovander et al., 2002; Kraimer, 1995; Qiu et al., 2007; Halket and Zaikin, 2004). These advantages come with great risks including: spontaneous explosions and toxic reactions upon inhalation or by contact with skin and eyes (USDOL, 2000). There are numerous references to the explosive and toxic nature of DM (USDOL, 2000; Warr, 2002; NIOSH, 2008). DM is classified as carcinogenic in laboratory animals, and as an allergen in humans (USDOL, 2000). The OSHA Permissible Exposure Limit (PEL) for DM is (0.2 ppm or 0.4 mg/m^3) (USDOL, 2000), which is comparable to that of ozone (0.1 ppm or 0.2 mg/m^3) (NIOSH, 2005) and benzene (1 ppm or 3.19 mg/m^3) (NIOSH, 2005). However there were no statistical data on the number of accidents or fatalities to give a broader overview of the dangers of DM. Preparing and handling DM is not straight-forward, requiring skilled operators, special glassware (Sigma-Aldrich, AL-180), working behind blast shields at all times (USDOL, 2000), working in well ventilated hoods (USDOL, 2000) and the grounding and discharging of both operator and equipment (USDOL, 2000). As the benefits of using DM come at such high risk, our current study assesses trimethylsilyldiazomethane (TMS-DM) as a reliable, clean, safe, easier to handle, time-saving and cost-efficient alternative.

TMS-DM has been described as an O-methylation reagent in synthetic organic reactions, including the Arndt–Eistert homologation (Podlech, 1998), O-methylation of carboxylic acids (Podlech, 1998) and even phenols in pure solvents (Podlech, 1998; Aoyama et al., 1984). TMS-DM is frequently used as derivatization agent in the analysis of carboxylic acids (Kuehnel et al., 2007; Park et al., 2001). However, there has been little effort to investigate the factors influencing the effect of steric hindrance and acidity of the analytes in real matrices. This is surprising, since TMS-DM is commercially available (Podlech, 1998) and has in fact an unlimited shelf life, while DM needs to be freshly prepared and has very limited storage time even in a freezer. Presently, reactions involving TMS-DM and phenolic analytes have been published only for organic synthesis involving clean solvents (Podlech, 1998; Aoyama et al., 1984).

In the present study phenolic metabolites of polychlorinated biphenyls (OH-PCBs) were chosen as model compounds to investigate the potential of TMS-DM as O-methylation derivatization reagent for phenolic analytes; see Fig. 1. OH-PCB metabolites are perfect as model compounds due to the variety of structures available, allowing the investigation of steric and electronic effects on the O-methylation by

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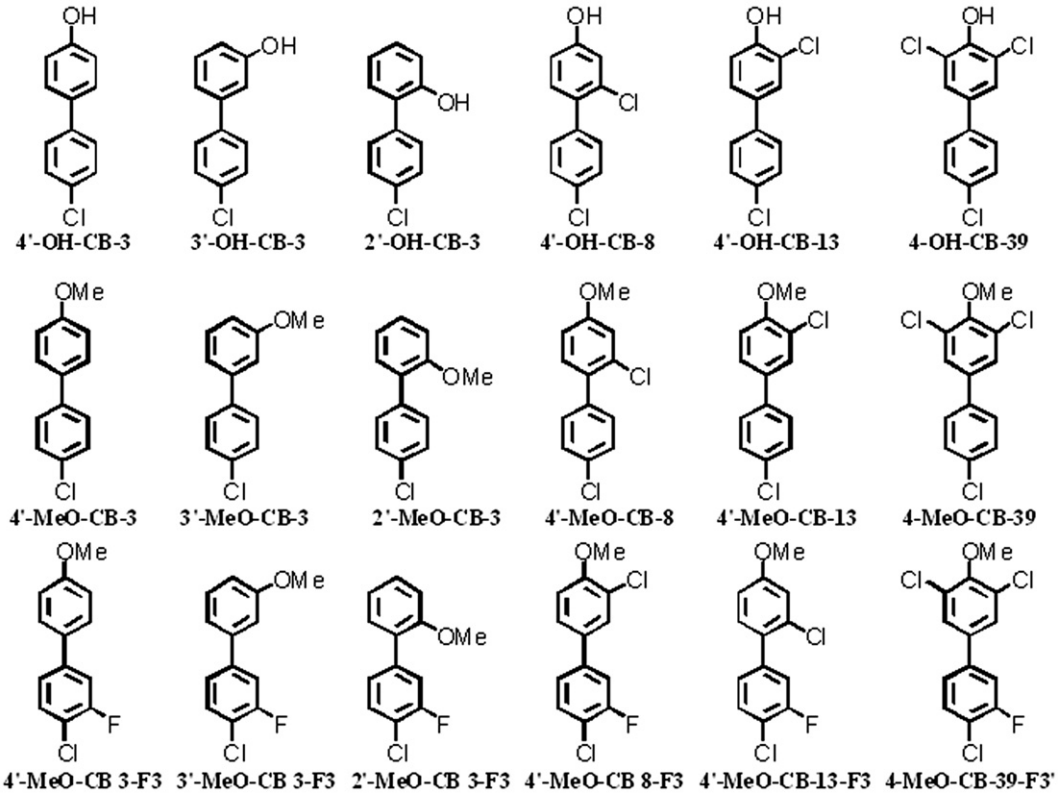

Fig. 1. [Top] Hydroxy-PCBs (OH-PCBs), [middle] methoxy-PCBs (MeO-PCBs) and [bottom] fluoro-substituted methoxy-PCBs (MeO-F-PCBs) used as model compounds. Nomenclature is according to Ballschmiter–Zell and Ballschmiter–Zell–Luthe systems.

ortho-chloro-substitution; see Fig. 1. In addition, extensive research is currently probing the biological effects of both parent PCBs and their metabolites (McLean et al., 1996; Lehmann et al., 2007; Espandiari et al., 2004).

Fluorine-tagged analogues (MeO-F-PCBs) of the expected methoxy PCB (MeO-PCBs) derivatives were used in this study as internal standards to monitor: discrimination effects, analyte losses and competing reactions. Fluoro-substituted or tagged analogues of aromatic analytes, *e.g.* PCBs, polybrominated diphenyl ethers and polycyclic aromatic hydrocarbons have shown their potential as internal standards in previous studies (Klösener et al., 2009). Fluorine-tagged analogues of aromatic compounds have the advantage that they resemble their parent compounds, but do not tend to scramble like deuterated standards and are easier to access compared with ^{13}C labeled analogues. In addition, they have advantages in their detection behavior, *e.g.* a mass difference of 18 *m/z* (Luthe et al., 2003).

In this study MeO-PCBs and MeO-F-PCBs were synthesized by an improved Suzuki-coupling (Luthe et al., 2009); the corresponding hydroxy-PCB (OH-PCBs) congeners were prepared by dealkylation of the corresponding MeO-PCB using borontribromide (Lehmler and Robertson, 2001). As a real matrix, a denatured extract of rat liver microsomes was used. Rat liver microsomal fraction is highly complex, consisting of lipophilic, hydrophilic and bipolar components with the tendency to form micelles (Hayes, 2001). Various components within the microsomal matrix are capable of reacting competitively with the derivatization regents including fatty acids and other phenols. We investigated the yields at a fixed time point with varying amounts of excess of TMS-DM and diisopropylethylamine (DIPEA) and optimized this to comparable levels as with DM. Kinetic studies were preformed under these optimized conditions. In addition, we conducted safety and cost analyses for both reagents.

2. Materials and methods

2.1. Chemicals

Trimethylsilyldiazomethane (TMS-DM) in hexanes (p.a.) and N,N-diisopropylethylamine (DIPEA) (99.5%) were purchased from Acros Organics (Morris Plains, NJ, USA). N-methyl-N-nitroso-p-toluenesulphonamide (diazald) (99%) was purchased as DM precursor from Sigma-Aldrich (St. Louis, MO, USA). CAUTION: *DM is an explosive gas, and should be prepared in a well ventilated hood. Operation of equipment should be carried out from a remote location behind safety glass. Exposure above the Occupational Exposure Limits (OEL = 0.2 ppm as TWA; (ACGIH 2004)) may result in death.* 4-Bromo-2-chlorophenol (99%) 4-bromo-3-chloroanisole (99%) and 4-chloro-3-fluorophenylboronic acid (99%), were purchased from Oakwood Products (Greenville, SC, USA), 2,6-dichlorophenol (99%) 4-bromo-2-fluoro-chlorobenzene (98+%), 4-chlorophenylboronic acid (97%), 2-methoxyphenylboronic acid (97+%), 3-methoxyphenylboronic acid (97+%), 4-methoxyphenylboronic acid (97+%), anhydrous magnesium sulfate (p.a.), anhydrous sodium sulfate (p.a.), hydrochloric acid (p.a.) (1 N) and $CDCl_3$ (99.8%) containing tetramethylsilane (0.03%) were purchased from Acros Organics (Morris Plains, NJ, USA). Methanol (HPLC grade), *n*-hexane (95%), acetonitrile (anhydrous), silica gel (60 Å C: C 40–63 μm) were purchased from Fisher Chemical (Pittsburgh, PA, USA). Tetrakis (triphenylphosphine) palladium (0) (99%) was purchased from Acros Organics (Morris Plains, NJ, USA).

The internal standards 4′-methoxy-3-fluoro-4-chlorobiphenyl (4′-MeO-CB-3-F3), 3′-methoxy-3-fluoro-4-chlorobiphenyl (3′ MeO-CB-3-F3), 2′-methoxy-3-fluoro-4-chlorobiphenyl (2′-MeO-CB-3-F3), 4′-methoxymethoxyl-3-fluoro-3,4′-dichlorobiphenyl (4′-MeO-CB-13-F3), 4′-methoxy-3-fluoro-2,4′-dichlorobiphenyl (4′-MeO-CB-8-F3) and 4-methoxy-3′-fluoro-3,4′,5-trichlorobiphenyl (4-MeO-CB-39-F3′),

reference compounds and precursors to demethylation 4′-methoxy-3′,4-dichlorobiphenyl (4′-MeO-CB-13), and 4-methoxy-3,4′,5-trichlorobiphenyl (4-MeO-CB-39), were synthesized by an improved method utilizing a palladium-catalyzed Suzuki-cross coupling between substituted boronic acids and bromobenzenes; please see Fig. 1 for structural formulas.

4′-Methoxy-4-chlorobiphenyl (4′-MeO-CB-3), 3′-methoxy-4-chlorobiphenyl (3′-MeO-CB-3), 2′-methoxy-4-chlorobiphenyl (2′-MeO-CB-3), 4′-methoxy-2,4′-dichlorobiphenyl (4′-MeO-CB-8), 4′-hydroxy-4-chlorobiphenyl (4′-OH-CB-3), 3′-hydroxy-4-chlorobiphenyl (3′-OH-CB-3), 2′-hydroxy-4-chlorobiphenyl (2′-OH-CB-3) and 4′-hydroxy-2,4′-dichlorobiphenyl (4′-OH-CB-8) were obtained at 99.5% purity.

4′-Hydroxy-3,4′-dichlorobiphenyl (4′-OH-CB-13) and 4-hydroxy-3,4′,5-trichlorobiphenyl (4-OH-CB-39) were synthesized by demethylation from the corresponding methoxy analogues with boron tribromide under stirring for 24 h in a protected atmosphere (argon). All compounds were purified using flash silica gel column chromatography followed by re-crystallization from methanol. The purity determined by GC-MS, was >99.5% ($n=5$).

2.2. Nomenclature

The nomenclature for the methoxy-PCBs (MeO-PCBs) and hydroxy-PCBs (OH-PCBs) is based on the Ballschmiter–Zell system (Luthe et al., 2009). The nomenclature for the monofluoro substituted methoxy PCBs (MeO-F-PCBs) is according the Ballschmiter–Zell–Luthe system (Luthe et al., 2009).

2.3. Synthesis

The series of MeO-PCBs (4′-MeO-CB-13, and 4-MeO-CB-39) and MeO-F-PCBs (4′-MeO-CB-3-F3, 3′-MeO-CB-3-F3, 2′-MeO-CB-3-F3, 4′-MeO-CB-13-F3, and 4′-MeO-CB-8-F3, 4-MeO-CB-39-F3′) were prepared using an improved Suzuki-coupling (Luthe et al., 2009), while OH-PCBs (4′-OH-CB-13 and 4-OH-CB-39) were prepared by demethylation (Lehmler and Robertson, 2006), and were synthesized for the first time. The purity of all model compounds was 99.5% (GC-MS). Yields for the Suzuki-coupling ranged between 16.1% (4′-MeO-CB-8 F 3) and 75.1% (2′-MeO-CB-3 F 3); see Supplementary Tables 1, 2 and 3. The demethylation yielded 45.5% (4′-OH-CB-13) and 68.1% (4-OH-CB-39). These values are in line with the literature (Lehmler and Robertson, 2006), and are good to excellent compared with other congeners (Luthe et al., 2009).

2.4. ^{1}H, ^{13}C and ^{19}F NMR characterization

All synthesized analytes, internal standards and reference compounds were characterized by means of proton (^{1}H), carbon (^{13}C), and where appropriate fluorine (^{19}F) nuclear magnetic resonance (NMR) spectroscopy. The ^{1}H and ^{13}C NMR spectra were recorded on 300 MHz NMR spectrometer (Bruker, Billerica, MA, USA), using $CDCl_3$ as solvent. Chemical shifts, δ, are given in ppm relative to TMS (0.03%), coupling constants, J, in Hz. ^{19}F NMR spectra were obtained with a 5 mm QNP probe, operating at 282.4 MHz. Chemical shifts were calibrated against hexafluorobenzene (C_6F_6) as standard. Supplementary Table 4 lists all NMR characterization spectra.

2.5. GC-MS analysis of synthesized compounds

Analysis of synthesized analytes, internal standards and reference compounds were carried out on a Thermo Trace 2000 GC-MS (Thermo Fisher, San Jose, CA, USA) coupled with a Thermo Voyager inert MS detection and auto sampler (Thermo AS 3000). 1 μL aliquots (1 mg/mL) were injected split less. The injection temperature was set at 225 °C. Separation was performed on an SLB-5ms capillary column (60 m × 0.25 mm I.D., 0.25 μm film thickness). Helium was used as the carrier gas at a flow of 1.2 mL/min. The split was opened after 2 min. The column temperature was programmed from 50 °C to 250 °C with 5 °C/min. The final temperature was held for 20 min. Detection was based on EI-MS mode in the total ion count mode (m/z 50–500) over the entire time range. Hydrogen was used as reagent gas at a flow of 3 mL/min. The ion source temperature was 200 °C. Supplementary Table 4 lists all GC-MS characterization spectra for the synthesized compounds.

2.6. Preparation of DM

DM was prepared using an Aldrich Mini Diazald® apparatus. According to the manufacturer's specifications; paraphrasing: the apparatus is filled with ethanol (95%, 10 mL), potassium hydroxide (5 g) and water (8 mL). Cool the receiver in dry ice/isopropanol bath. Place a separatory funnel over the reaction vessel and charge the funnel with Diazald® (5.0 g, 23 mmol) in ether (45 mL). Warm the apparatus to 65 °C. Add the Diazald® solution over 20 min. When finished add another 10 mL of ether and continue the distillation until the yellow color disappears. The ether will contain 700 mg to 900 mg (16.6 mmol to 21.4 mmol) of DM (Method according to Sigma-Aldrich bulletin A180). DM was stored at −80 °C in sealed 10 mL aliquots, and was used within a month of preparation.

2.7. Preparation of denatured microsomal extract

Pooled microsomes were prepared from the livers of 10 male Sprague–Dawley rats (120–170 g). The animals were euthanized, their livers excised and homogenized in 0.25 M sucrose solution containing 0.1 mM ethylenediaminetetraacetic acid (EDTA). The microsomal fraction was prepared by differential centrifugation, at 10,000 ×g for 20 min, and then at 100,000 ×g for 1 h. The microsomal pellet was resuspended in the sucrose solution and then denatured by adding isopropanol (37 mL), hydrochloric acid (100 mL, 1 mM), and sodium chloride (5 g) in nanopure water (100 mL); the mixture was extracted using diethyl ether (250 mL). The extract was homogenized by vortexing (5 min), the layers were separated, and the organic layer was dried using $MgSO_4$. Solvent was evaporated using nitrogen and replaced with 100 mL acetonitrile/methanol (9:1, v:v), aliquoted and the extract stored at −80 °C until use.

2.8. GC-MS analysis

Analysis of the derivatization mixtures was carried out on a Thermo Trace 2000 GC-MS (Thermo Fisher, San Jose, CA, USA) coupled with a Thermo Voyager inert MS detection and auto sampler (Thermo AS 3000). 5 μL aliquots were injected split less. The injection temperature was set at 225 °C. Separation was performed on an SLB-5ms capillary column (60 m × 0.25 mm I.D., 0.25 μm film thickness). Helium was used as the carrier gas at a flow of 1.2 mL/min. The split was opened after 2 min. The column temperature was programmed from 50 °C to 250 °C with 5 °C/min. The final temperature was held for 20 min. Detection was based on EI-MS in the single ion monitoring mode (SIM) selecting for six masses (218, 236, 255, 272, 289 and 306). Hydrogen was used as reagent gas at a flow of 3 mL/min. The ion source temperature was 200 °C. Supplementary Fig. 3 displays a typical GC chromatogram at an analyte concentration of 10 μmol.

2.9. Validation of the F-tagged MeO-PCBs analogues as internal standards

A mixture composed of MeO-F-PCBs (4′-MeO-CB-3-F3, 3′-MeO-CB-3-F3, 2′-MeO-CB-3-F3, 4′-MeO-CB-13-F3, 4′-MeO-CB-8-F3, and 4-MeO-CB-39-F3′) and MeO-PCBs (4′-MeO-CB-3, 3′-MeO-CB-3, 2′-MeO-CB-3, 4′-MeO-CB-8, 4′-MeO-CB-13, and 4-MeO-CB-39) (each analyte 10 μmol) was dissolved in ethyl acetate (1 mL) as stock

solution I. An aliquot (5 µL) of stock solution I was added to the microsomal extract (1 mL) (real matrix) and to acetonitrile/methanol (9:1 v:v) (1 mL) (reference matrix), homogenized (vortexed, 10 min) and kept at +4 °C in the dark until use. The analytical process for both matrices was followed according to the O-methylation with TMS-DM; see Fig. 2. The relative responses of the compounds were determined before and after extraction. These numbers were normalized and parent CB response was divided by internal standard response.

2.10. Stock solution for O-methylation of OH-PCBs with DM and TMS-DM

A mixture composed of MeO-F-PCBs (4′-MeO-CB-3-F3, 3′-MeO-CB-3-F3, 2′-MeO-CB-3-F3, 4′-MeO-CB-13-F3, 4′-MeO-CB-8-F3, and 4-MeO-CB-39-F3′) and OH-PCBs (4′-OH-CB-3, 3′-OH-CB-3, 2′-OH-CB-3, 4′-OH-CB-8, 4′-OH-CB-13, and 4-OH-CB-39) (each analyte 10 µmol) was dissolved in ethyl acetate (1 mL) as stock solution II. An aliquot (5 µL) of stock solution II was added to the microsomal extract (1 mL) (real matrix) and to acetonitrile/methanol (9:1 v:v) (1 mL) (reference matrix), homogenized (vortexed, 10 min) and kept at +4 °C in the dark until use; these solutions are further referred to as spiked matrices. Final concentration of each standard and analyte is 5 µM.

2.11. O-methylation with DM

1 mL aliquots of the spiked matrices were derivatized by DM in diethyl ether (0.5 mL, 0.07 M). The reaction mixture was kept at +4 °C for 3–4 h under stirring in the dark. Excess DM and diethyl ether was evaporated under a gentle stream of nitrogen; see Fig. 2. The total volume was corrected to 1 mL with *n*-hexane and transferred to GC vials for analysis. To investigate the time line of the reaction, an aliquot (20 mL) of spiked matrix was derivatized with DM (10 mL, 0.07 M). Aliquots (1 mL) were taken at 0, 1, 7.5, 15, 30, 60, 120, 240, and 1440 min; the procedure followed according to Fig. 2.

2.12. O-methylation with TMS-DM

1 mL aliquots of the spiked matrices, DIPEA (80 mg, 62.6 µL, 0.62 mmol) and TMS-DM solution (100 µL, 0.76 mmol, 2 M) in *n*-hexane were added with stirring at room temperature. The reaction mixture was stirred for 24 h at room temperature in the dark. Hydrochloric acid (1 mL, 1 M) was added to terminate the reaction, and diethyl ether (1 mL) was added for extraction. The organic layer was dried over $MgSO_4$. Please see Fig. 2. To investigate the time line of

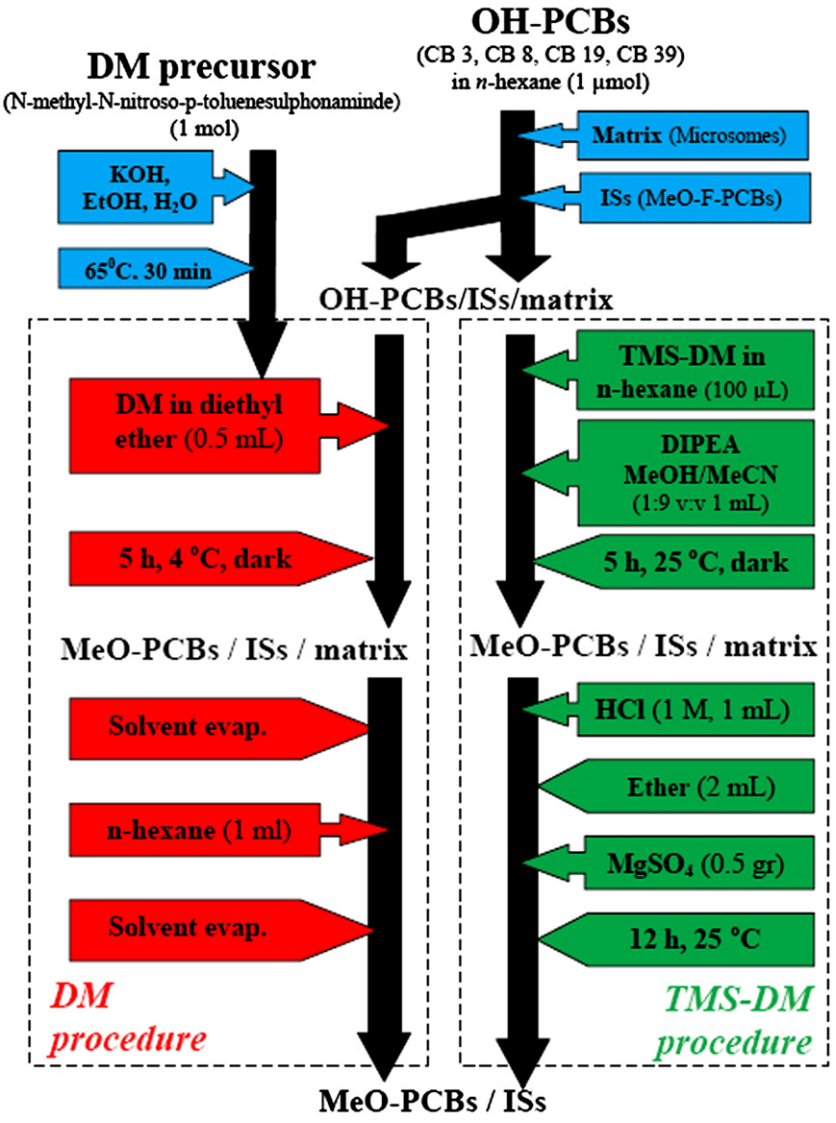

Fig. 2. General workflow chart showing the DM and TMS-DM derivatization procedures.

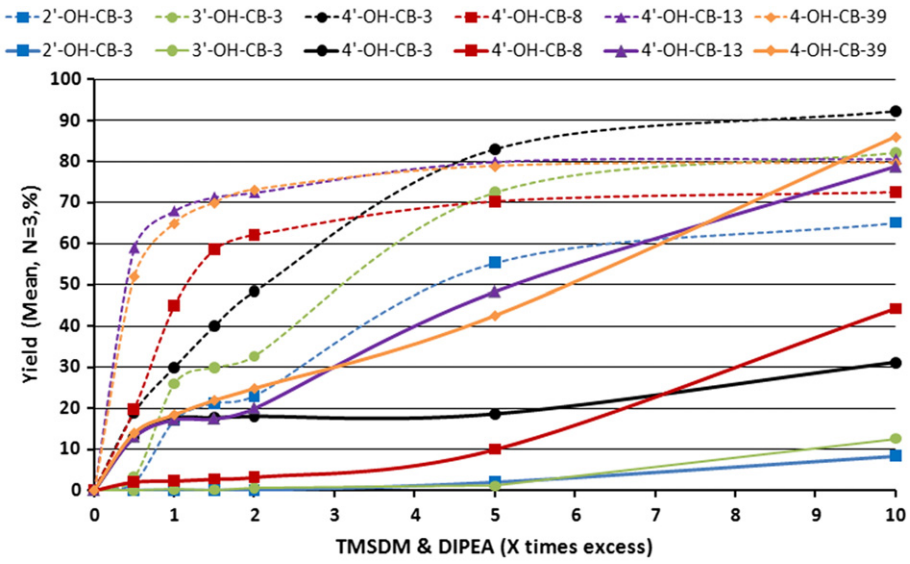

Fig. 3. Yields of the derivatization of OH-PCBs (2′-OH-CB-3, 3′-OH-CB-3, 4′-OH-CB-3, 4′-OH-CB-13, 4′-OH-CB-8, and 4-OH-CB-39) by TMS-DM at various conc. of TMS-DM and DIPEA in a real matrix (MeCN/MeOH (9:1) + denatured microsomal fraction extract) (*solid line*) compared to a reference matrix (MeCN/MeOH (9:1)) (*dotted line*) determined by GC-MS, utilizing MeO-F-PCBs analogues (4′-MeO-CB-3-F3, 3′-MeO-CB-3-F3, 2′-MeO-CB-3-F3, 4′-MeO-CB-13-F3, 4′-MeO-CB-8-F3, and 4-MeO-CB-39-F3′) as internal standards. The reaction time was a constant 24 h. Standard deviation range was between 0.002% and 0.2%.

the reaction, an aliquot (20 mL) of spiked matrix was derivatized with DIPEA (1.6 g, 12.4 mmol) and TMS-DM (2 mL, 15.2 mmol). Aliquots (2 × 1 mL) were taken at 0, 1, 7.5, 15, 30, 60, 120, 240, and 1440 min; the procedure followed according to Fig. 2.

2.13. Optimization of O-methylation with TMS-DM

1 mL aliquots of the spiked matrices excess TMS-DM and DIPEA were added. Please see Fig. 4.

2.14. Comparison of derivatization capability

1 mL aliquots of the spiked matrices, the DM and TMS-DM methods were applied under optimal conditions to compare both reactions on a statistical level. The statistical comparison was done using the Pearson correlation test.

2.15. Cost comparison

To compare the cost advantages of the TMS-DM and the DM methods, estimates were compiled based on the initial costs, *e.g.* stirrers, preparation apparatus, etc.; and on the cost per reaction, *e.g.* chemicals and person hours. Prices for chemicals were converted into their various units and the costs were calculated for a single and multiple reactions. The manufacturers' websites (http://www.acros.com and http://www.sigmaaldrich.com) were used as reference and no specific discounts for universities or large corporations were taken into account. Salary per hour was based on a "Postdoctoral fellow" at the University of Iowa in 2008.

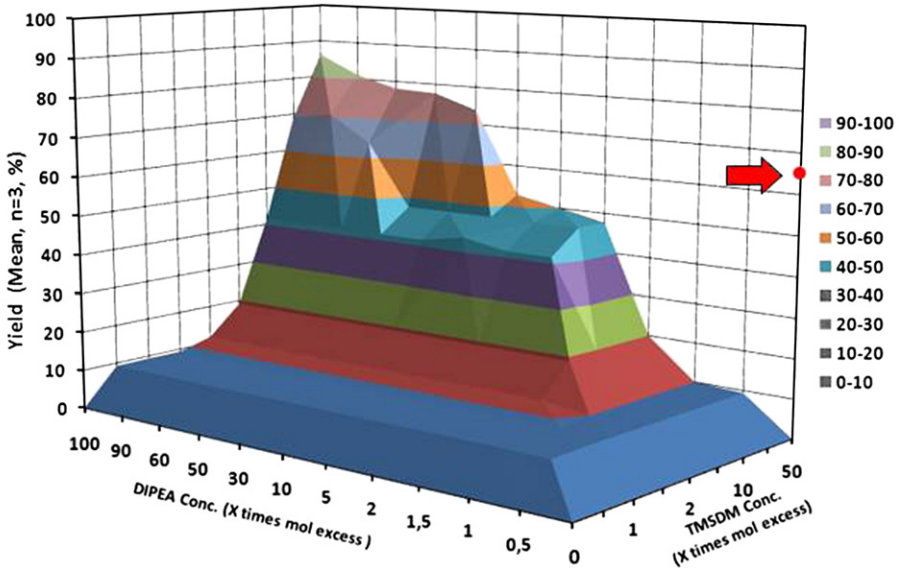

Fig. 4. Reaction optimization chart, with the DIPEA (base) mole excess plotted on the *X* axis and the TMS-DM (reagent) mole excess plotted on the *Y* axis. Yield represents mean of all six analytes measured 3 times individually. The red dot represents a derivatization of the same matrix with DM at 40 times excess and NO DIPEA. Both reactions were carried out in a real matrix (MeCN/MeOH (9:1) + denatured microsomal fraction extract). Standard deviation varies between 0.002 and 0.2.

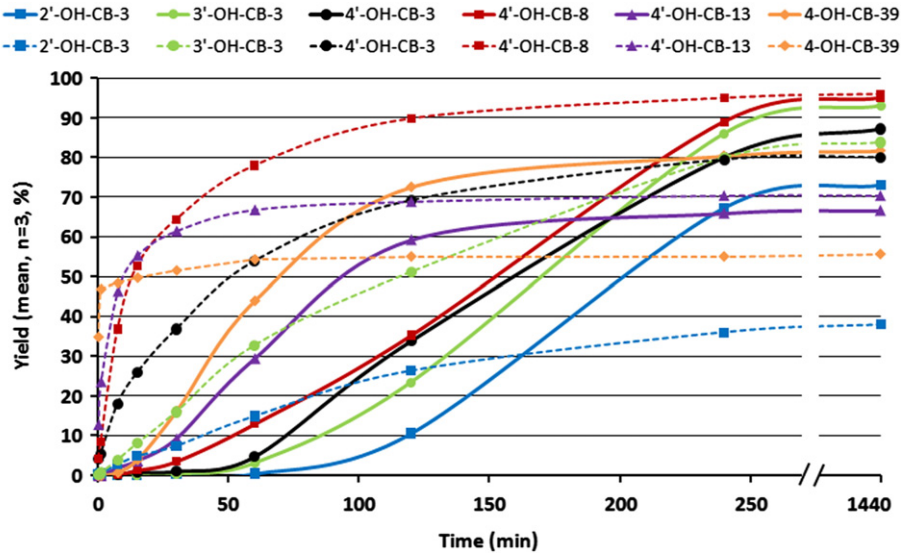

Fig. 5. Comparison of the derivatization reagent (TMS-DM) (*solid line*) with the conventional reagent (DM) (*dotted line*) over time, exhibiting the different kinetics and yields of both reagents with model compounds (2′-OH-CB-3, 3′-OH-CB-3, 4′-OH-CB-3, 4′-OH-CB-13, 4′-OH-CB-8, and 4-OH-CB-39). MeO-F-PCBs (4′-MeO-CB-3-F3, 3′-MeO-CB-3-F3, 2′-MeO-CB-3-F3, 4′-MeO-CB-13-F3, 4′-MeO-CB-8-F3, and 4-MeO-CB-39-F3′) were used as IS.

2.16. Hazard analysis

A hazard analysis was carried out for both DM and TMS-DM applying the Preliminary Hazard Analysis method as described by USDOD (2000) and Mohr (2002). We identified the three most prominent hazards encountered during the application of both reagents: 1) explosion of solution or vapors of the chemicals, 2) spill of the chemicals, and 3) exposure to the chemicals. For each hazard, we considered the potential hazard target including equipment damage, personal injury, downtime, and leaks into the environment. We estimated the risk level (severity and probability) for each potential hazard target. The severity was estimated on a scale from catastrophic to negligible, and the probability was estimated on a scale from frequent to improbable. Countermeasures recommended by OSHA were identified and the magnitudes of risk before and after these countermeasures were inventoried. The authors used official Material Safety Data Sheets, previous publications, work experience, and professional judgment in the identification of hazards and the classification of associated risks.

3. Results and discussion

3.1. Congener reaction profiles

The differences in reaction yields for the different analytes (2′-OH-CB-3, 3′-OH-CB-3, 4′-OH-CB-3, 4′-OH-CB-13, 4′-OH-CB-8, and 4-OH-CB-39) (for synthesis and characterization see Supplementary data) in the reference and real matrices with a 10 times excess of reagents are shown in Fig. 3. In this experiment, MeO-F-PCBs (for synthesis and characterization see Supplementary data) were used as internal standards. In general reaction yields in the real matrix were far lower than values found for the reference matrix at equal reaction conditions (time: 24 h, TMS-DM and DIPEA excess 0.5–10×). Lower chlorinated OH-PCBs (4′-OH-CB-3, 3′-OH-CB-3, and 2′-OH-CB-3) showed yields of around 10%, while higher chlorinated phenols (4-OH-CB-39 and 4′-OH-CB-13) reached comparable yields to the reference matrix (80%). The difference in yields and reaction times are due to higher pK_a or acidity of the phenol groups by negative inductive effect of the chlorines. The di-ortho-chloro-substituted 4-OH-CB-39, with the lowest pK_a value of our model compounds (calculated using software obtained from www.acdlabs.com/products/phys_chem_lab), showed the fastest reaction, and the highest reaction yield followed by the mono-ortho-chloro-substituted 4′-OH-CB-13. The effect of the meta-chloro-substituent in 4′-OH-CB-8 on the acidity of the hydroxy group was clearly lower, resulting in the lowest reaction turn-over of the three congeners. The difference between the OH-CB-3s is due to the position of the hydroxy group in relation to the chloro-phenyl substituent on the second phenyl ring, determining the acidity of the hydroxy group. Steric effects are secondary to acidity when looking at reaction kinetics.

3.2. Effects of excess DIPEA

Excess of DIPEA increased yields in the real matrix from 50% (5 times excess), up to 80% (30 times and above) determined on an average basis of all six analytes. As seen in Fig. 4, the optimum was reached with an 85% average yield of the six analytes in real matrix with a 50 times excess of TMS-DM and 100 times DIPEA; this is higher than reactions with DM (65%).

3.3. Validation of F-tagged PCBs as internal standards

The recovery factors after extraction with diethyl ether of the investigated MeO-PCBs (for synthesis and characterization see Supplementary data) were between 79% and 95% in the reference matrix and between 55% and 91% in the real matrix. Standard

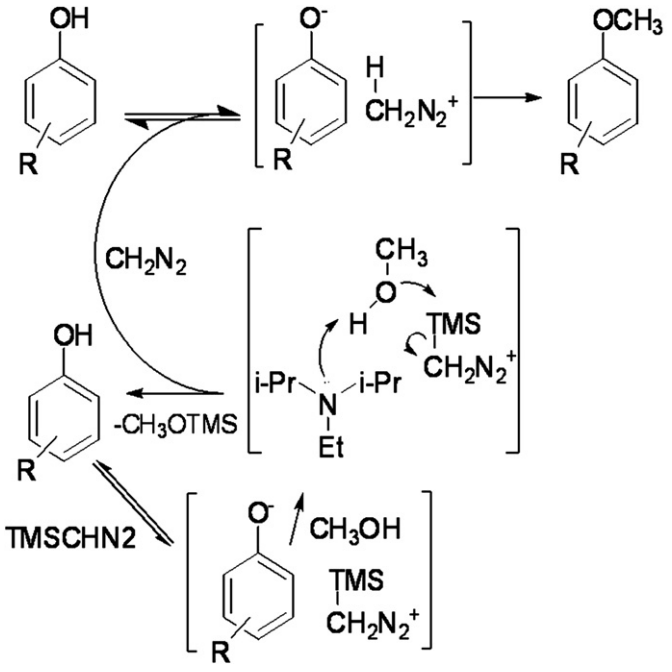

Fig. 6. Proposed reaction mechanism of the O-methylation with TMS-DM and DM as based on Kuehnel et al. (2007). The mechanism shows the abstraction of a hydrogen from the phenol with subsequent *in situ* generation of diazomethane which after a second hydrogen abstraction can react with the hydroxyl group of the analyte to form a methoxy derivate.

Table 1
Cost analysis for the DM and TMS-DM reagents, calculated per reaction and for 100, 1000 and 10,000 samples.

Initial costs ($)			Reaction costs ($)		
Equipment	Cost ($)	Item	Cost ($)/unit	Quantity	Cost ($)
Diazomethane (DM)					
Stirrer	726.60	Labor	30.00/h	0.15 h	4.50
Stirrer bar	3.54	KOH	0.04/g	0.05 g	0.01
Flask	42.90	EtOH	0.01/ml	0.09 ml	0.01
Funnel	120.00	Ether	0.01/ml	0.50 ml	0.01
Total	1186.54	Diazald	0.64/g	0.05 g	0.03
		Tube	1/reaction	1 tube	1.00
		Screwcap	1/reaction	1 cap	1.00
Labor (100 samples)	90.00	Isopropanol	0.05/ml	0.45 ml	0.02
Mini diazald	293.50	Dry ice	0.80 g	1 g	0.80
total	383.50	Nitrogen	160/2000 psi	2 psi	0.16
Total	1570.04	Total			7.54
Trimethylsilyldiazomethane (TMS-DM)					
Stirrer	726.60	Labor	30.00/h	0.20 h	6.00
Stirrer bar	3.54	DIPEA	0.55/g	0,08 g	0.04
Total	730.14	Ether	0.01/ml	2,00 ml	0.03
		$MgSO_4$	0.10/g	1,00 g	0.10
		MeCN	0.05/ml	0,90 ml	0.05
		MeOH	0.05/ml	0,10 ml	0.00
		Tube	1/reaction	1 tube	1.00
		Screwcap	1/reaction	1 cap	1.00
		TMS-DM	2.45/ml	0.10 ml	0.25
Total	730.14	Total			8.47

Cost ($) sample volume	TMS-DM	DM
100 samples	1577.14	2324.04
1000 samples	9200.14	12,561.54
10,000 samples	85,430.14	114,936.54

deviations were within 0.02 to 0.04% determined using MeO-F-PCBs as internal standards. Recovery factor determination of internal standards and derivatized analytes were determined in matrix between 0.998 and 1.002. Results are shown in Supplementary Fig. 2.

3.4. Congener kinetics

The general kinetic trend, seen in Fig. 5, shows that reactions with DM reach their maximum yield after 50 min, a yield reached with TMS-DM after 100 to 250 min depending on the substitution pattern. Taking into account the fact that derivatizations utilizing DM are, in most cases, carried out over night (8 to 15 h), the difference in reaction time between the methods is equalized. More interestingly, for all OH-PCBs investigated, reactions with TMS-DM demonstrated comparable or higher yields at an incubation time of 250 min and beyond. The difference in the reaction slopes is due to the different mechanisms followed by the reagents. While DM reacts spontaneously with the phenols, TMS-DM needs to be activated by the base DIPEA prior to reacting with the phenol, (see Fig. 6) resulting in a sigmoid reaction increase.

3.5. Cost analysis

To compare the cost of performing the derivatization reaction with either reagent, a detailed list of the initial costs for equipment and reagent preparation, and the individual running costs for each reagent was inventoried (Table 1). The initial costs were approximately $400 higher for DM compared to TMS-DM, due to the purchase of additional equipment to prepare fresh quantities of DM. While the costs for derivatizing a single sample were $0.90 lower for DM ($7.54) compared with TMS-DM ($8.47), this does not translate in lower costs for the overall method. For small lab scale experiment of approximately 100 samples, the costs are $600 lower when TMS-DM is used ($1577.14, DM: $2324.04). This trend continues when larger sample volumes are derivatized; $2300 for 1000 samples (DM: $12,561.54, TMS-DM: $9200.14) and $29,500 for 10,000 samples (DM: $114,936.54, TMS-DM: $85,430.14).

3.6. Hazard analysis

To evaluate the risks involved of using either TMS-DM or DM as a derivatizing reagent, a preliminary hazard analysis was performed, as shown in Table 2. The use of TMS-DM as reagent virtually eliminates the explosion risk associated with the use of DM. The explosion risk is present with TMS-DM only in extreme situations, such as additional heating. The TMS-DM reagent is sold commercially as a solution in hexanes. While the reagent TMS-DM itself adds no risk of explosion, the hexane is classified as highly flammable (NIOSH, 2005).

The risk level related to spills is high with the use of DM, as any DM spill involves the risk of explosion. The inhalation risk from airborne contaminants is considerable for both reagents. The OSHA Permissible Exposure Limit (PEL) for DM is 0.2 ppm. No PEL has been issued for TMS-DM. There is no OSHA PEL issued for DIPEA. With general laboratory countermeasures, hazardous exposures to DIPEA are minimized. Overall

Table 2
Hazard analysis of DM and TMS-DM reagents: risk categories were assessed before and after the application of counter measures defined by OSHA including: working in a hood, working behind explosion barriers or shields, low temperature work, using hood malfunction detectors, and using gloves, goggles, and aprons.

Hazard	Target effect	Risk category					
		DM method		TMS-DM method			
		Before counter measures	After counter measures	Before counter measures		After counter measures	
		DM	DM	TMS-DM	DIPEA	TMS-DM	DIPEA
Explosion	Equipment damage or destruction	Ib	Ic	IIIc	IVe	IIId	IVe
	Personal injury to operator or others	Ib	Ic	IIc	IVe	IIc	IVe
	Downtime of laboratory space	Ib	IIc	IIc	IVe	IId	IVe
	Leak of toxic compounds into environment	IIId	IIId	IIIc	IVe	IIId	IVe
Spill	Personal injury to operator or others	Ib	Ic	IIc	IIIc	IIc	IIIc
	Downtime of laboratory space	IIIb	IIIc	IIIb	IVb	IIIc	IVc
	Leak of toxic compounds into environment	IIId	IIId	IIIc	IIId	IIIc	IIId
Exposure	Personal injury to operator or others	IIb	IIc	IIb	IIIb	IIc	IIIc
	Leak of toxic compounds into environment	IIId	IIId	IIb	IIIb	IIIc	IIIc

Risk category coding:
Green low: operation permissible using customary laboratory safety measures.
Yellow medium: operation requires caution and strict adherence to counter measures.
Red high: operation requires suppressing risk to a lower level.
Severity classification:
I. Catastrophic. Death, loss exceeding $1M, or irreversible severe environmental damage that violates regulation.
II. Critical. Permanent partial disability, loss between $200K and $1M, or reversible environmental damage that violates regulation.
III. Marginal. Lost-time injury or illness, loss between $10K and $200K, or mitigatible environmental damage without violation of regulation.
IV. Negligible. Non-lost-time injury or illness, loss up to $10K, or minimal environmental damage not violating regulation.
Frequency classification:
a. Frequent.
b. Probable.
c. Occasional.
d. Remote.
e. Improbable.

even after countermeasures, the explosion hazard associated with DM remained in the high hazard zone, where it would be imperative to suppress the risk to a lower level.

4. Conclusions

As demonstrated in these experiments, derivatization with TMS-DM produces comparable results to the generally and routinely used DM for the derivatization of phenolic analytes. Derivatization using TMS-DM results in higher yields under optimized conditions. This occurs in clean samples as well as matrices (microsomal fraction from rat liver). Use of TMS-DM as a derivatization reagent is cost effective with an estimated reduction of up to 25% in material and labor costs. TMS-DM is also safer, based on the preliminary hazard analysis. The reduced risks of explosion and health effects favor TMS-DM as the reagent of choice on both low and high sample volume experiments and routine analysis. Since derivatization of phenols is carried out on a routine basis in a wide variety of disciplines, these results will be of major importance for several fields of research, including toxicology, pharmacy, analytical and medicinal chemistry, environmental and forensic sciences and public and occupational health.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envint.2010.02.011.

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