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Short communication

Suitability of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide as derivatization reagents for the determination of the estrogens estrone and 17α-ethinylestradiol by gas chromatography–mass spectrometry

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Abstract

This paper describes a previously unreported problem with the use of *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) to derivatise the natural hormone estrone (E1) and the synthetic estrogen 17α-ethinylestradiol (EE2). The resulting trimethylsilyl (TMS) and *t*-butyldimethylsilyl (TBS) derivatives of EE2 were partially converted to their respective E1 derivatives. Therefore, these reagents may not be suitable for simultaneous determination of estrogens in environmental samples, which raises questions about the reliability of results from some earlier studies.

Keywords: Derivatization, GC; Estrone; 17α-Ethinylestradiol

1. Introduction

Low concentrations (ng/l) of estrogens in environmental water samples are commonly determined by gas chromatography-mass spectrometry (GC–MS), following extraction and derivatization [1–15]. We would like to draw attention to a previously unreported problem with two of the more popular silylation methods, namely that 17α-ethinylestradiol (EE2) is partially or near 100% converted to estrone (E1) during the derivatization and chromatography. The structural formulae for the two estrogens are given in Fig. 1. Consequently these methods cannot reliably quantify mixtures of the two analytes in environmental samples under the conditions described here-conditions that are based on those adopted in previous studies [8,11,12,14].

Two popular reagents used to derivatise compounds bearing hydroxyl groups are *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA), which lead to the formation of TMS and TBS derivatives, respectively. Catalysts such as trimethylchlorosilane (TMCS) together with trimethylsilylimidazole (TMSI) or *t*-butyldimethylsilylchlorosilane (TBCS) are usually added to enhance derivatization. TBS derivatives are often preferred because they are more stable and sensitive than the conventional TMS derivatives [6,8,16–18].

Mol et al. [11] found that derivatization with MTBSTFA was complete under reaction conditions of 75 °C for 30 min for determination of phenolic estrogens including EE2. Kelly [14] determined E1 and EE2, among others, after derivatization with MTBSTFA containing 1% TBCS at ambient temperature. Promberger and Schmid [8] determined estrogens including E1 and EE2 after derivatization with BSTFA + 1%TMCS + 2%TMSI at 60 °C for 30 min. Helaleh et al. [6] determined phenolic estrogenic compounds after derivatization with BSTFA. From an evaluation of a number of similar reagents for detecting both natural and synthetic estrogens by GC–MS, Ding and Chiang [12] concluded that BSTFA with 1% TMCS at 70 °C for 30 min was the best derivatization reagent.

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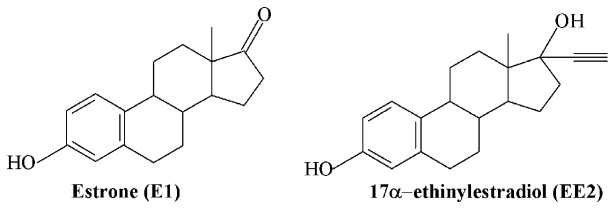

Fig. 1. Structures of estrone (E1) and 17α-ethinylestradiol (EE2).

2. Experimental

2.1. Chemicals

Estrogen standards (E1 and EE2) with a purity of 98% or higher, derivatization grade MTBSTFA, anhydrous methanol, ethyl acetate and deuterated bisphenol A (BPA-$_{d16}$) were supplied by Sigma–Aldrich (Melbourne, Australia). BSTFA containing 1% TMCS, and TMSI were supplied by Alltech (Melbourne, Australia).

2.2. Standard solutions

Individual standard solutions of E1 and EE2 were prepared at 100 mg/l in anhydrous methanol, from which appropriate dilutions were made up in methanol for determination of retention times and mass spectra for the analytes. A stock solution of internal standard, BPA-$_{d16}$, was made up at 100 mg/l in anhydrous methanol.

2.3. Derivatization

TBS derivatives of E1 and EE2 standards were prepared (as in [11]) by the addition of anhydrous ethyl acetate (100 μl) and MTBSTFA (100 μl) to a 2 ml amber reaction vial containing 100 μg of both standard and internal standard obtained by evaporating 1 ml of the appropriate standard solution to dryness under nitrogen. The vial was then capped, vortexed and heated in an oil bath at 75 °C for 30 min. The vial was immersed in the oil bath to the level of the fluid inside the vial. TMS derivatives were prepared in the same way as above (similar to the method described in [8]) using BSTFA + 1%TMCS + 2%TMSI instead of MTBSTFA and heating the reaction mixture at 60 °C for 30 min. After cooling, aliquots of the reaction mixtures were analysed directly by GC–MS employing SCAN mode.

2.4. GC–MS analysis

Derivatised samples were analysed on a Shimadzu GC-17A gas chromatograph equipped with a non-polar MDN-5S 30 m × 0.25 mm capillary column with a 0.25 μm film (Supelco, Australia). The injector with a split insert was set at 280 °C, and the oven temperature was programmed at 50 °C for 1 min, ramped at 20 °C/min to 120 °C, then ramped at 10 °C/min to 300 °C and maintained at this temperature for 10 min. The carrier gas was helium with a constant flow rate achieved by the column pressure programmed at 25.9 kPa for 1 min, increased at 3.9 kPa/min to 43.7 kPa, then increased at 2.5 kPa/min to 86.1 kPa and maintained at this pressure for 10 min. The GC was directly interfaced to a Shimadzu QP 5000 quadrapole mass spectrometer operated in the electron impact ionization mode at 70 eV with an interface temperature of 280 °C. Positive fragment ions were analysed over 50–700 *m/z* mass range in SCAN mode. All GC and MS

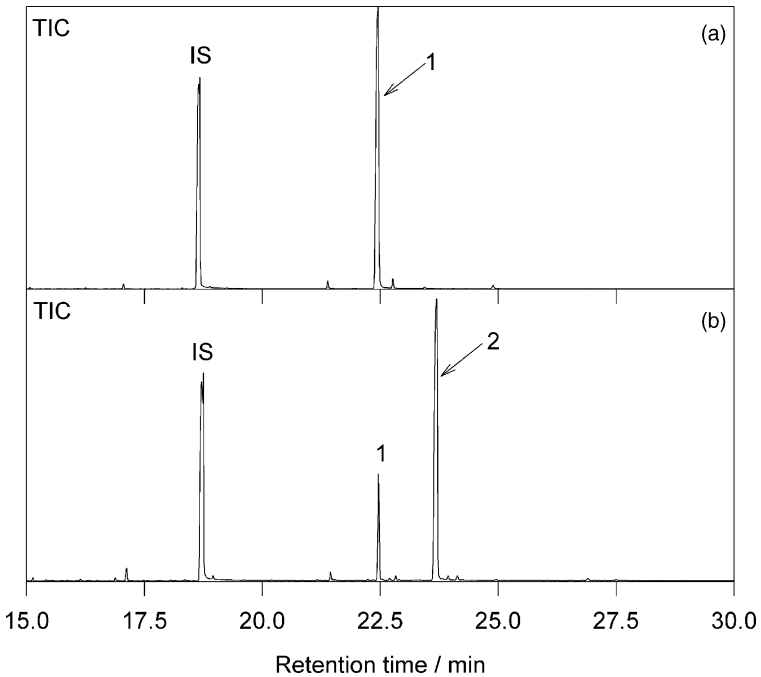

Fig. 2. GC–MS TICs of trimethylsilyl (TMS) estrone, E1 (a), and TMS ethinylestradiol, EE2 (b). The internal standard (IS) was deuterated bisphenol A.

parameters were implemented using Class-5K version 2.23 software.

3. Results and discussion

Typical total ion chromatograms (TICs) of the TMS derivatives of E1 and EE2 are given in Fig. 2, and the mass spectra from individual peaks are shown in Fig. 3. Peak 1 in the TIC of E1 (Fig. 2a) and peak 1 of EE2 (Fig. 2b) correspond to the same retention time of 22.5 min and have identical mass spectra as shown in Fig. 3a. The mass spectrum of peak 2 in the TIC of EE2 is shown in Fig. 3b.

The major ions for TMS-E1 (Fig. 3a) were the molecular ion 342 $[M]^+$ (base peak), 257 $[M-85]^+$, 327 $[M-15]^+$ due to the loss of a methyl group from the derivative, and an ion with *m/z* 218. EE2 (Fig. 3b) contained the molecular ion with *m/z* 440 $[M]^+$ resulting from reaction at both the 3-OH and 17-OH, an ion with *m/z* 425 $[M-15]^+$ due to the loss of a methyl group from the derivative, and an ion with *m/z* 285 $[M-155]^+$ due to the loss of $[(CH_3)_3–Si–O–C_3H_5]$ and ethynyl group from $[M]^+$ on the D ring [12].

Fig. 4 displays the TICs for the TBS derivatives of E1 and EE2. Peak 1 in the TIC of E1 (Fig. 4a) and peak 1 of EE2 (Fig. 4b) correspond to the same retention time of 25 min and have identical mass spectra as shown in Fig. 5a. The mass spectrum of peak 2 from TBS-EE2 is shown in Fig. 5b.

The diagnostic ions for TBS-E1(Fig. 5a) were the molecular ion with *m/z* 384 $[M]^+$, 327 $[M-57]^+$ (base peak) due to the loss of the *t*-butyl group from the derivative and an ion with *m/z* 163 which can be attributed to the fragment $[(CH_3)_2Si–O–C_6H_3–CH_2]^+$ due to the cleavage between C6 and C7 and C9 and C10 bonds [11]. The mass spectrum of peak 2 in Fig. 4b, shown in Fig. 5b contained the TBS-EE2 molecular ion *m/z* 410 $[M]^+$, 353 $[M-57]^+$ (base peak) due

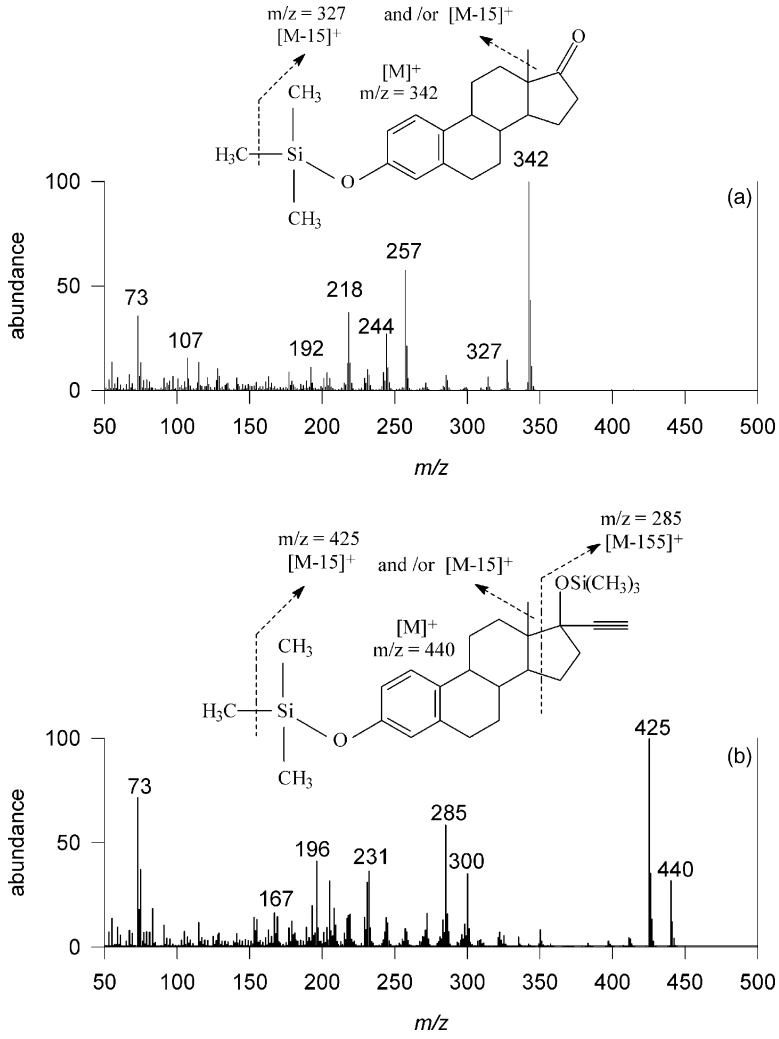

Fig. 3. Mass spectra of trimethylsilyl (TMS) estrone, E1 (a), and peak 2 of TMS ethinylestradiol, EE2 (b).The proposed fragmentation patterns of the TMS derivatives of E1 and EE2 are shown above the respective mass spectra.

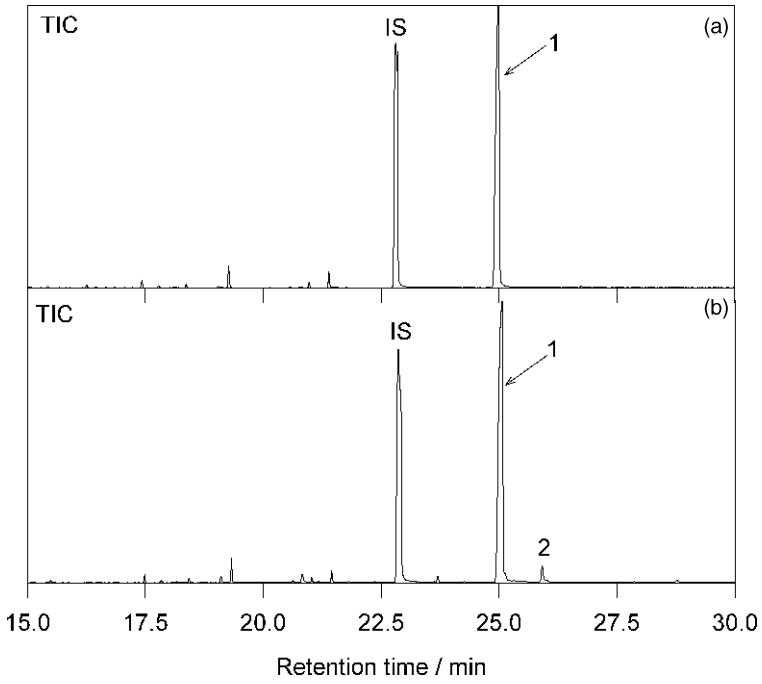

Fig. 4. GC–MS TICs of *t*-butyldimethylsilyl (TBS) estrone, E1 (a), and TBS ethinylestradiol, EE2 (b). The internal standard (IS) was deuterated bisphenol A.

to the loss of the *t*-butyl group from the derivative and an ion with *m*/*z* 327 $[M-83]^+$ due to the loss of C_3H_5OH and an ethinyl group from $[M]^+$ on the D ring [11,12,14]. The fragment with *m*/*z* 163 present in both mass spectra of E1 and EE2 suggest that silylation occurs at the 3-OH groups. Steric hindrance at the 17-OH group on EE2 may prevent derivatization [11].

The identical retention times and mass spectra, together with fragmentation patterns that match those expected for E1 and EE2, indicate that both the TMS and TBS derivatives of EE2 were broken down to form their respective E1 derivatives during the analysis.

Table 1 shows the relative peak areas (against the internal standard) observed for E1 and EE2 standards following reaction with each of BSTFA and MTBSTFA. From the relative peak areas for the E1 derivatives (Figs. 2a and 4a), and allowing for the slightly different molar concentrations of the standards, we estimate that about 42% of EE2 was converted to the E1 derivative after reaction with BSTFA, but from Fig. 4 it can be seen that close to 100% of EE2 was converted to the E1 derivative after reaction with MTBSTFA.

The sum of the relative peak areas for peaks 1 and 2 in the TICs for EE2 need not be the same as the relative peak area of peak 1 in the TIC for E1, because the molar concentrations of the two analytes are slightly different, and the response factors for the two derivatives need not be the same. The similarity in the case of the TBS derivatives (Table 1) is coincidential.

In the analysis of a mixture of E1 and EE2 only BSTFA derivatization would indicate the presence of EE2, but unless allowance were made for the partial conversion of EE2 to the E1 derivative the concentration of E1 would be overestimated, and quantitative estimation of EE2 would not be straightforward. MTBSTFA derivatization might give a good estimate of the combined concentrations of the two

Table 1
The relative peak areas (normalized against the internal standard) for E1 and EE2 after derivatization with BSTFA and MTBSTFA

Analyte	TMS derivatives		TBS derivatives	
	Peak no. (Fig. 2)	Relative peak area ± S.D. (*n*)	Peak no. (Fig. 4)	Relative peak area ± S.D. (*n*)
E1	1	1.01 ± 0.04 (5)	1	1.30 ± 0.04 (10)
EE2	1	0.39 ± 0.06 (9)	1	1.20 ± 0.04 (13)
	2	1.03 ± 0.04 (9)	2	0.11 ± 0.04 (13)

Peak 1 corresponds to the E1 derivative, and peak 2 to the EE2 derivative.

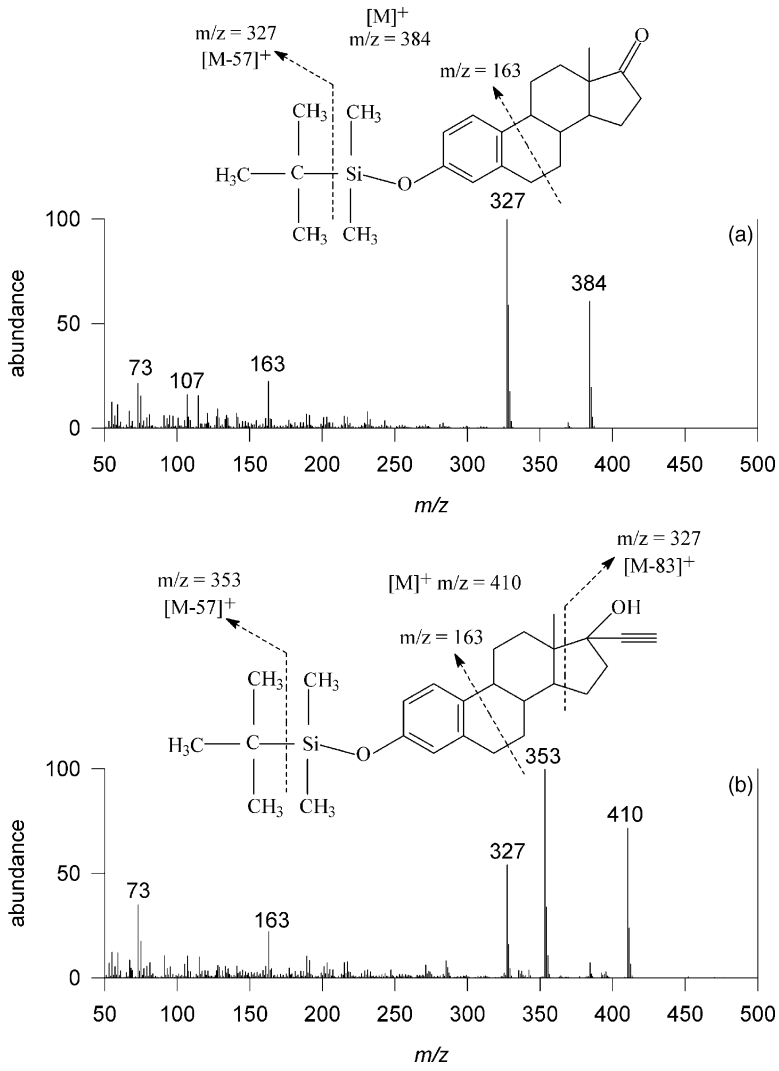

Fig. 5. Mass spectra of *t*-butyldimethylsilyl (TBS) estrone, E1 (a), and peak 2 of TBS ethinylestradiol, EE2 (b). The proposed fragmentation patterns of TBS derivatives of E1 and EE2 are shown above the respective mass spectra.

analytes, but would indicate nothing about their individual concentrations.

4. Conclusions

1. EE2 was broken down into E1 during the derivatization with MTBSTFA or BSTFA, or during chromatographic separation, or both. Therefore, the methods described here are not suitable for the simultaneous determination of these two estrogens in environmental samples. However, it may be possible to report the combined concentrations of E1 and EE2.
2. Results from previous studies [6,11,14] which used analytical methods similar to those described in this work may need to be re-evaluated. The earlier studies may have overestimated E1 and underestimated EE2 as a result of inter-conversion between them.

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