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Formation of multiple trimethylsilyl derivatives in the derivatization of 17α-ethinylestradiol with BSTFA or MSTFA followed by gas chromatography-mass spectrometry determination

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Abstract

N,O-bis(trimethylsily)trifluoroacetamide (BSTFA) and N-methyl-N(trimethylsily) trifluoroacetamide (MSTFA) are common derivatization reagents used in the GC-MS analysis of estrogen steroids such as estrone (E1) and 17α-ethinylestradiol (EE2). In this study, three trimethylsilyl (TMS) steroid derivatives, mono- and di-trimethylsilyl EE2 and mono-trimethylsilyl E1, were observed during the derivatization of EE2 with BSTFA or MSTFA and/or GC separation. Factors influencing the production of multiple TMS derivatives and their relative abundance were examined. It was found that both methanol and bisphenol A competed with estrogenic esteroids when reacting with silylation reagents, and thus affected the formation of TMS derivatives and their relative abundance in the derivatization products. Methanol was found to be more reactive than bisphenol A with the BSTFA reagent. None of the three solvents tested in this study could prevent the generation of multiple TMS derivatives during the derivatization of EE2 with BSTFA, followed by GC analysis. A similar result was observed using MSTFA as the derivative reagent followed by GC analysis. Thus, the suitability of BSTFA or MSTFA as the derivatization reagent for the determination of E1 and EE2 by GC-MS, under the conditions reported here, is questionable. This problem can be solved by adding trimethylsilylimidaz (TMSI) in the BSTFA reagent as recommended, and the performance of the method has been proved in this study.

Key words: estrogens; water sample; GC/MS determination; trimethylsilyl steroid derivatives

Introduction

Steroid estrogens of natural and anthropogenic origin have been identified as the major contributors to endocrine-disrupting activities in both sewage effluent and surface water (Lai *et al*., 2000; Gomes *et al*., 2004; Zuo *et al*., 2004; Zhang and Zuo, 2005). Therefore it is very important to determine a low concentration (ng/L) of estrogens in environmental water. Considering the low volatility of steroid estrogens, LC/MS (Hu and Cheng, 2003; Hu *et al*., 2005) and LC/MS/MS methods have recently been developed for the determination of these estrogenic compounds. However, at present, the application of LC/MS and LC/MS/MS in environmental analyses of estrogenic steroids appears to be limited because of their capital costs. Traditionally, low concentrations (ng/L) of estrogenic steroids in environmental water samples are determined by gas chromatography-mass spectrometry (GC-MS), following extraction and derivatization (Montserrat and Damià, 1997; Mol *et al*., 2000; Jones *et al*., 2000; Lee Ferguson *et al*., 2001; Xiao *et al*., 2001; Helaleh *et al*., 2001; Orwa *et al*., 2002; Kelly, 2002; Jeannot *et al*., 2002; Brossa *et al*., 2002; Regan *et al*., 2002; Rodríguez *et al*., 2003; Miao and Metcalfe, 2003; Shareef *et al*., 2004; Quintana *et al*., 2004; Zuo *et al*., 2004, 2005; Zhang and Zuo, 2005). N,O-bis(trimethylsily)trifluoroacetamide (BATFA) and N-methyl-N(trimethylsily) trifluoroacetamide (MSTFA) have been widely used in the derivatization of estrogenic steroids by reason of their facile and low cost, which leads to the formation of the trimethylsilyl (TMS) derivative. Catalysts such as trimethylchlorosilane (TMCS), trimethyksilyimidazole (TMSI) or *t*-butyldimethylsilylchlorosilane are usually added to enhance derivatization efficiency (Shareef *et al*., 2004). It has been recently reported that di-trimethylsilyl (TMS) derivative of 17α-ethinylestradiol (EE2) was formed by the silylation reaction at both 3-OH and 17-OH, and partially converted to its respective estrone (E1) derivative when N, O-bis (trimethylsily)trifluoroacetamide (BSTFA) was used to derivatize the synthetic estrogen EE2. Quintana *et al.* (2004). observed the BSTFA reagent was able to react with the aromatic hydroxyl group, but not with the aliphatic hydroxyl group of EE2. Thus, a controversy arose about

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the feasibility of the simultaneous determination of the estrogens E1 and EE2 by BSTFA prior to column derivatization and GC-MS. Recently, Zuo *et al*. (2004), reported that using pyridine as a solvent can obtain di-trimethylsilyl EE2 derivative as a sole trimethylsilyl derivatized product, for the silylation of EE2 with the BSTFA reagent (Zuo *et al*., 2004, 2005; Zhang and Zuo, 2005). However, in the previous studies, various solvents and matrices were involved in the analytical derivatization of EE2 and other steroid compounds with MSTFA followed by GC-MS measurement. In this study, a systemic investigation was performed to examine the effects of solvent and matrices on the derivatization of EE2 with BSTFA and MSTFA reagents, including the formation and relative ratio of TMS derivatives of EE2 and E1, which could be used in the evaluation of the analytical results of EE2 and E1 obtained in the previous studies, and to avoid potential problems in the GC-MS analysis of estrogenic steroids. This research attested that adding TMSI properly could produce the di-trimethylsilyl EE2 derivative as a sole trimethylsilyl derivatized product, for the silylation of EE2 with the BSTFA or MSTFA reagent, using hexane as the solvent. This finding provides an efficient analytical tool for the future study of estrogenic steroids in the environment.

1 Experimental

1.1 Chemicals

Estrone (E1), deuterated bisphenol A, TMSI, MSTFA, and EE2 standards were purchased from Aldrich Chemistry Corporation (Milwaukee, WI). Anhydrous methanol, acetone, ethylacetate, and hexane were supplied by Dima (Richmond Hill, ON., Canada). BSTFA + TMCA (99:1, v/v) was obtained from Supelco (Supelco Park, PA).

1.2 Standard solution

An individual standard solution of E1 and EE2 was prepared at 5.0 × 10 mg/L and 1.00 × 10^2 mg/L in anhydrous methanol, from which appropriate dilutions were made according to need. A stock solution of internal standard, deuterated bisphenol A, was made at 2.00 × 10^2 mg/L in anhydrous methanol.

TMS derivatives of E1 and EE2 standards were respectively prepared by the addition of anhydrous ethyl acetate (100 μl) and BSTFA + 1% TMCA (100 μl) to a 2-ml amber reaction vial containing 50.0 μg of standard, obtained by evaporating 1.0 ml of the standard solution to dryness under a low nitrogen flow. Then the vials were capped and heated in an air bath at 65°C for 30 min. After cooling, the products of derivative were analyzed directly by GC-MS employing the SCAN mode.

1.3 GC-MS analysis

Derivatized samples were analyzed using an Agilent GC-6890 Gas Chromatograph and 5973 Quadrapole Mass Spectrometer equipped with a nonpolar HP-5MS 30 m × 0.25 mm capillary column with 0.25 μm film (Agilent, USA). The injector was set at 300°C, and the oven temperature was programmed at 80°C for 1 min, ramped at 20°C/min to 200°C, and 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 of 1 ml/min. The mass spectrometer was operated in the electron impact ionization mode at 70 eV with an interface temperature of 280°C and a source temperature of 230°C. Positive fragment ions were analyzed over 50–500 *m/z* mass range in the SCAN mode. All control of GC and MS parameters and analysis of data were performed by the MSD Productivity Chemstation Software Rev. D.00.00.

2 Results and discussion

2.1 TMS derivatives of E1 and EE2 resulted from silylation with BSTFA + TMCA

Total ion chromatograms (TICs) of the TMS derivatives of E1 and EE2 are given in Fig.1, and the mass spectra from individual peaks are displayed in Fig.2. Peak 1 in the TIC of E1 (Fig.1b) and Peak 1 of EE2 (Fig.1a) correspond to the same retention time of 12.25 min and have identical mass spectra as shown in Fig.2a. The mass spectra of peaks 2 and 3 in the TIC of EE2 are shown in Figs.2b and 2c.

The major ions for TMS E1 (Fig.2a) were the molecular

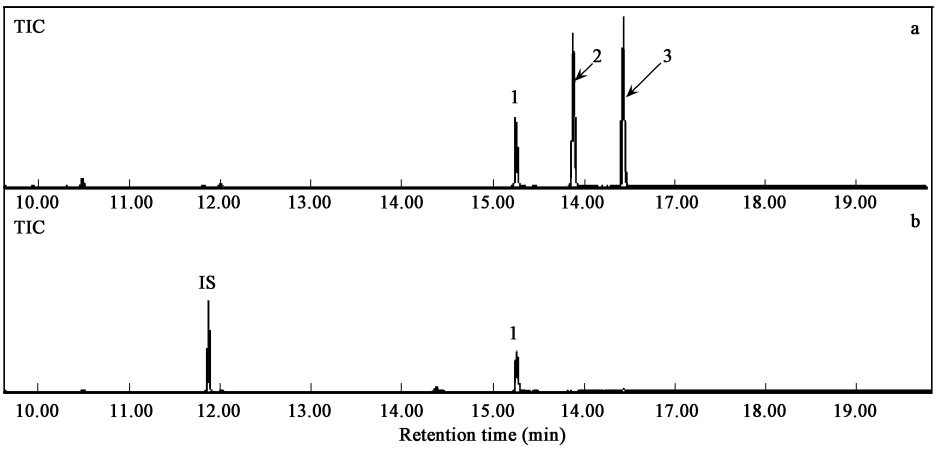

Fig. 1 GC-MS TICs of the derivatization products of (a) 17α-ethinylestradiol (EE2) without internal standard, (b) TMS estrone (E1) and the internal standard (IS), deuterated bisphenol A.

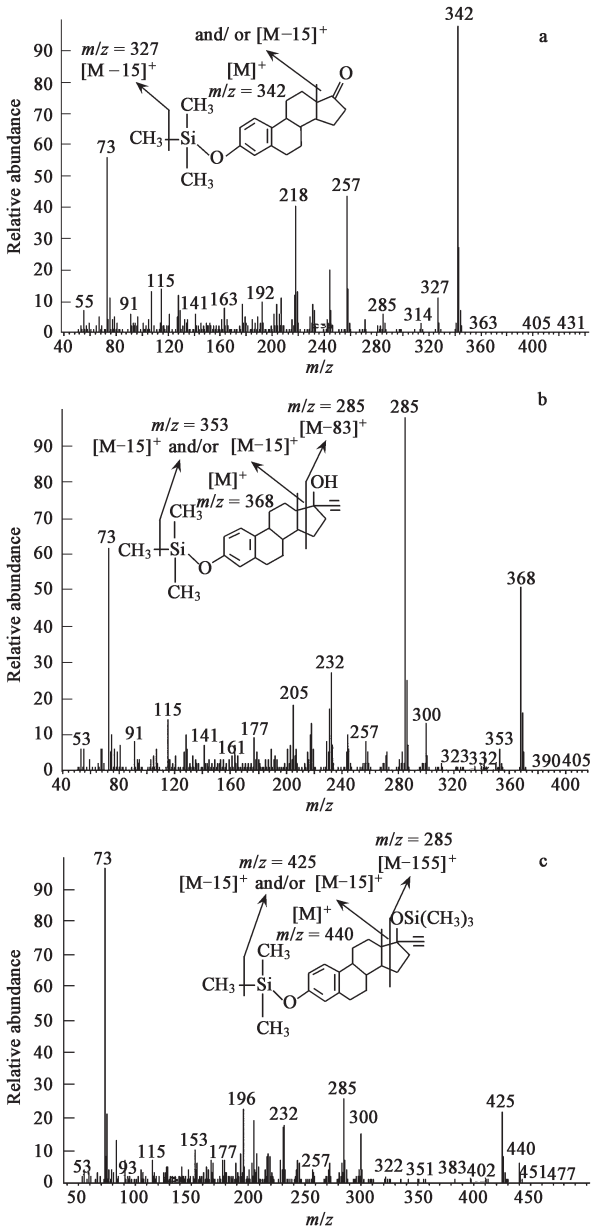

Fig. 2 Mass spectra. (a) mono-trimethylsilyl (TMS) estrone (E1), (b) mono-TMS 17α-ethinylestradiol (EE2), and (c) di-TMS 17α-ethinylestradiol (EE2).

ion at *m/z* 342 $[M]^+$ (base peak), 257 $[M\text{-}85]^+$, and 327, because of the loss of a methyl group from the derivative, and an ion with *m/z* 218. Peak 2 in Fig.1a resulted from a mono-trimethylsilyl derivative of EE2, which was formed via silylation with the 3-OH group of EE2. The mass spectrum of the mono-trimethylsilyl derivative of EE2 contained the molecular ion with *m/z* 368 $[M]^+$, and an ion with *m/z* 285 $[M–83]^+$, because of the loss of $[C_3H_5\text{-}OH]$ and ethynyl group from $[M]^+$ on the D ring. A compound of TMS reaction with both 3-OH and 17-OH of EE2 (Fig.2c), contained the molecular ion with *m/z* 440 $[M]^+$, and an ion with *m/z* 425 $[M–15]^+$ because of the loss of a methyl group from the derivative, and an ion with *m/z* 285 $[M–155]^+$, because of the loss of $[C_3H_5–O–Si–(CH_3)_3]$ and ethynyl group from $[M]^+$ on the D ring (Helaleh *et al*., 2001; Shareef *et al*., 2004).

The identical retention times and mass spectra, together with fragmentation patterns that match those expected for E1 and EE2, indicate that TMS derivatives of EE2 include two peaks, which are products of the TMS reaction, with only 3-OH (Peak 2) and with both 3-OH and 17-OH (Peak 3) of EE2. EE2 derivatives are also partially broken down into E1 derivative (Peak 1), during the derivatization with BSTFA, or during chromatographic separation, or both (Helaleh *et al*., 2001).

Previously, Quintana *et al*. (2004) observed that the BSTFA reagent derivatized only the aromatic hydroxyl group of EE2. Neither the di-TMS derivative of EE2 (Shareef *et al*., 2004) nor the derivative of E1 converted from the EE2 derivative (Quintana *et al*., 2004), was reported by them.

2.2 Effect of active hydroxyl on the silylation of EE2

Compounds containing active hydroxyl groups, such as, methanol and bisphenol A, are frequently involved in the determination of estrogens E1 and EE2 by BATFA pre-column derivatization and GC-MS. To test the effect of the active hydroxyl group in methanol and bisphenol A, on silylation of EE2, 100 μg of EE2 standard, in the presence of 200 μg deuterated bisphenol A or 20 μl methanol was derivatized as described in Section 1.1. The influence of active hydroxyl on the stability of TMS derivatives of EE2 was also examined, by adding 200 μg deuterated bisphenol A or 20 μl methanol into the reaction vial after the derivatization of EE2, with BSTFA + 1% TMCS reagent. As shown in Fig.3a, the addition of deuterated bisphenol A, after the derivatization of EE2, did not change the TMS derivatives formed or their relative peak areas, when the chromatographic results were compared with those obtained in the absence of deuterated bisphenol A or methanol (Fig.1a). Three TMS derivatives, mono-TMS E1, mono- and di-TMS EE2, were generated. However, when EE2 was derivatized in the presence of deuterated bisphenol A, only two derivatives, mono-TMS E1 and mono-TMS EE2, were observed (Fig.3b). The stability of these two TMS derivatives is shown in Fig.4. The ratio of peak areas of mono-TMS E1 to internal standard increases with the reaction time, but the ratio of peak areas between mono-TMS EE2 and internal standard decreases with reaction time. The relative abundance of mono-TMS EE2 to mono-TMS E1 decreases rapidly. In a previous study, Shareef *et al*. (2004) reported that mono-TMS E1 and di-TMS EE2 were the derivatization products because less amount of bisphenol A was used in their experiment.

No TMS derivative of EE2 was found when the BSTFA + 1% TMCS reagent was added into the mixture of EE2 and methanol, because of the reaction of methanol with the silylation reagent. Even though methanol was added after the derivatization of EE2, the relative ratio of mono-TMS E1, and mono- and di-TMS EE2 derivatives was altered. This indicated that different types of hydroxyl groups had different types of reactivity with the BSTFA + 1% TMCS silylation reagent and affected the silylation of estrogenic steroids differently.

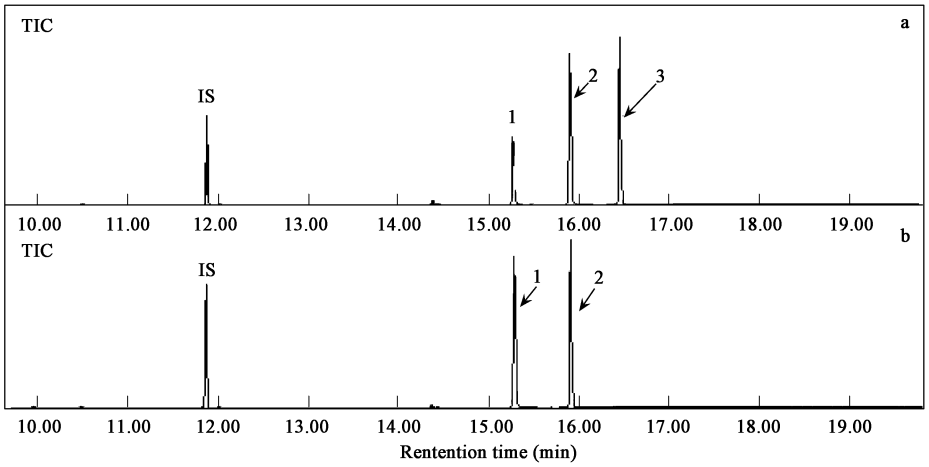

Fig. 3 GC-MS TICs of trimethylsilyl derivative products of 17α-ethinylestradiol (EE2). (a) deuterated bisphenol A was added after TMS reaction with EE2, and (b) deuterated bisphenol A was added at the same time with EE2.

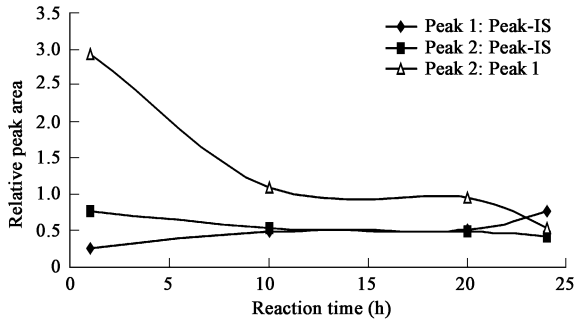

Fig. 4 Stability of the TMS derivative products of EE2 in ethyl acetate solvent.

2.3 Effect of solvent on conversion of EE2 to the E1 derivative

Table 1 shows the relative peak areas (against the internal standard) of mono-TMS E1 and mono-TMS EE2 in three solvents. Peak areas of mono-TMS EE2 versus those of mono-TMS E1 are 0.23:0.16, 0.18:0.20, 0.30:0.18 in hexane, acetone, and ethyl acetate, respectively. The tendency of conversion of the mono-TMS derivative of EE2 to the respective derivatives of E1 in the solvents studied follows the order: acetone > hexane > ethyl acetate.

2.4 Solution for formation of multiple trimethylsilyl derivatives

Taking MSTFA as the derivative reagent, a result similar to the above is observed. To solve the problem formation of multiple trimethylsilyl derivatives, addition of several kinds of reagents to the derivative reagent, such as triethylamine and TMSI, has been tried. The result shows that the reaction of triethylamine with BSTFA+1% TMCS or MSTFA produces a white sediment. It can be seen from Table 2, however, that adding 0.5% TMSI to BSTFA+1% TMCS or MSTFA reagent produces di-trimethylsilyl EE2 derivative as a sole trimethylsilyl derivatized product for the silylation of EE2, with the BSTFA reagent. The silylation reaction is viewed as a nucleophilic attack upon the silicon atom of the silyl donor, producing a bimolecular transition state (Knapp, 1979). As a weak base, TMSI can activate the hydroxyl groups and also serve as an acid scavenger by removing the acidic product resulting from the TMS derivatives, which make the derivatization reaction of EE2 complete. Comparison of the sensitivity of E1 and EE2, silylated by 0.5% TMSI in BSTFA + 1% TMCS and MSTFA in Table 3, demonstrates that it is a good choice to take BSTFA + 1% TMCS + 0.5% TMSI as the derivative reagent. Ratio of the peak area of the derivative product of E1 with BSTFA + 1% TMCS +

Table 1 Relative peak areas (normalized against the internal standard) for E1 and EE2 after derivatization with BSTFA in different solvents

Analyte	Hexane solvent		Acetone solvent		Ethyl acetate solvent	
	Peak No. in Fig.1	Relative peak area ±SD (*n*)	Peak No. in Fig.1	Relative peak area ±SD (*n*)	Peak No. in Fig.1	Relative peak area±SD (*n*)
E1	1	0.46±0.03 (4)	1	0.48±0.02 (4)	1	0.53±0.02 (10)
EE2	1	0.16±0.00 (4)		0.20±0.02 (4)		0.18±0.01 (5)
	2	0.23±0.01 (4)		0.18±0.03 (4)		0.30±0.01 (5)

Table 2 Percentage of mono-trimethylsilyl E1, mono-trimethylsilyl EE2, and di-trimethylsilyl EE2 using different derivatization reagents

Derivative reagent	mono-Trimethylsilyl E1 (%)	mono-Trimethylsilyl EE2 (%)	di-Trimethylsilyl EE2 (%)
MSTFA	54.0	22.1	23.9
MSTFA + 0.5%TMSI	0.0	0.0	100.0
BSTFA + 1%TMCS	24.0	60.0	16.0
BSTFA + 1%TMC + 0.5%TMSI	0.0	0.0	100.0

Table 3 Comparison of the sensitivity for E1 and EE2 silylated by different derivatization reagents (hexane solvent)

Derivative reagent	E1	EE2
BSTFA + 1%TMCS + 0.5%TMSI	1.23[a]	1.20
MSTFA + 0.5%TMSI	1	1

[a] Ratio of derivative product of specific compound with BSTFA + 1%TMCS + 0.5%TMSI and MSTFA + 0.5%TMSI.

Table 4 Calibration curve for E1 and EE2 using BSTFA + 1%TMCS + 0.5%TMSI as derivative reagent (hexane solvent)

Compound	Calibration curve[a]	R^2	IDL[b] (μg/L)
E1	$Y = 1885.7X$[c]	0.9979	0.10
EE2	$Y = 5226.8X$	0.9991	0.02

[a] All data are mean of five in dependent assays; [b] IDL is instrumental detection limit; [c] X is the concentration of specific compound.

0.5%TMSI, and MSTFA + 0.5%TMSI is 1.23, and that for EE2 with BSTFA + 1% TMCS + 0.5%TMSI, and MSTFA + 0.5%TMSI is 1.20. When taking BSTFA + 1% TMCS + 0.5%TMSI as the derivative reagent, it can be seen from Table 4 that a good linearity ($R^2 > 0.99$) is achieved in the hexane solvent for the E1 and EE2 mixture standard. Derivative reaction was performed under 65°C for 30 min. Addition of TMSI in the derivative reagent prevents the effective formation of multiple trimethylsilyl derivatives and ensures availability of analysis result of EE2, by using the similar pre-column derivative tandem GC-MS method. Furthermore, the results in Table 4 have proved that the approved method can be applied to simultaneous determination of E1 and EE2.

3 Conclusions

Three trimethylsilyl steroid derivatives, mono-TMS E1 and mono- and di-TMS EE2, were generated in the derivatization with BSTFA or/and subsequent GC separation. Both compounds containing active hydroxyl groups, such as methanol and bisphenol A, and derivatization solvents, affect the formation of multiple TMS steroid derivatives and their relative abundance in the silylation of EE2 with the BSTFA reagent. Methanol is much more reactive with BSTFA than bisphenol A. With each of the three solvents tested in this study, more than one TMS steroid derivative was produced in the derivatization of EE2; mono-TMS E1 derivative was observed in every case studied. Taking MSTFA as the derivative reagent, a similar result was observed.

In the present study, addition of 0.5% TMSI effectively prevents the formation of multiple trimethylsilyl derivatives in the derivatization of EE2 with BSTFA or MSTFA, followed by GC-MS determination. Developed methods have testified that it can be applied to simultaneous determination of E1 and EE2. The linearity relativity coefficients of the calibration curve for E1 and EE2 are 0.9979 and 0.9991 respectively. The result of this research provides an efficient analytical tool for a further study of estrogenic steroids in the environment.

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