AN IMPROVED DERIVATIZATION METHOD WITH *N,O*-BIS(TRIMETHYLSILYL)TRIFLUOROACETAMIDE (BSTFA) FOR SIMULTANEOUS DETERMINATION OF STEROID ESTROGENS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract
An improved derivatization method with *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) for simultaneous determination of four natural and synthetic steroid estrogens, such as estrone (E1), 17β-estradiol (E2), estriol (E3) and 17α-ethynylestradoil (EE2) by gas chromatography-mass spectrometry (GC-MS) is described. Compared with conventional derivatization method (typically, derivatization with BSTFA+1% trimethylchlorosilane (TMCS) in pyridine at 70 °C for 30 min), the improved method didn't need heating and catalyst, better derivatization results were obtained when the steroid estrogens were derivatized with BSTFA in pyridine at room temperature (20 °C) for 15 min. The RRFs (Relative Response Factor: peak areas of an analyte against that of an internal standard) of steroid derivatives generated at room temperature (20 °C) were superior to that at any other temperature between 30-80 °C, so heating was unnecessary. For the derivatization at room temperature (20 °C), 15 min was the optimized reaction time. A good linearity of calibration curve in the concentration range from 1.0 or 10 to 250 pg/μL of steroids with the square of the correlation coefficient (R^2) 0.99462-0.99942 was achieved under the improved derivatization method.

Introduction
Steroid estrogens, particularly the synthetic contraceptive steroid 17α-ethynylestradiol (EE2) and natural steroids estrone (E1), 17β-estradiol (E2) and estriol (E3), which show stronger estrogenic activity than other well known chemical pollutants such as nonylphenols and PCBs, have been suggested as the major contributors to endocrine disrupting activities in both sewage effluent and surface water[1-7]. For the determination of steroid estrogens, GC-MS has been a preferred approach because of its superior separation and identification capabilities, following derivatization to increase the volatility and thermal stability of the analytes and thus improve the chromatographic separation and sensitivity[8-9]. As one of the most commonly used derivatization reagents, *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) has been employed for the derivatization of the hydroxyl groups contained in the steroids, which lead to the formation of trimethylsilyl (TMS) derivatives[8-22]. Most previously described derivatization procedures for the GC-MS analysis of steroids adopted a time-consuming conventional heating process of 45-80 °C (typically 70 °C) in an oven or water bath for 25-60 min, usually in combination with a small proportion of catalysts such as trimethylchlorosilane (TMCS) or trimethylsilyl-imidazole (TMSI) to enhance derivatization efficiency[8-22].

Materials and Methods
Chemicals
Steroid estrogen standards (E1, E2, E3 and EE2) with a purity of 97% or higher and internal standard 5α-androstane (purity>97%) were supplied by Sigma-Aldrich (USA). Derivatization grade BSTFA, TMCS; and GC grade anhydrous methanol and pyridine were obtained from Fluka (USA).

Solutions
Individual standard stock solutions of E1, E2, E3, EE2 and 5α-androstane were prepared at 1 μg/μL in anhydrous methanol, from which appropriate dilutions were made in methanol according to need. A mixture of working standards containing each steroid compound at 100 pg/μL was prepared weekly by diluting the stock solution in

methanol. Calibration curves under derivatization condition were performed using the stock solution to prepare six standard solutions with different concentrations of steroids at 1, 5, 10, 25, 50, 100, 150, 200, and 250 pg/μL, respectively, and the inject concentration of the internal standard was 50 pg/μL. All the solutions should be stored in a freezer at -15 °C and protected from light.

Derivatization

100 μL of a prepared standard mixture solution (100 pg/μL) was pipeted into a 2 mL amber vial and evaporated to dryness under a gentle nitrogen stream at room temperature (20 °C). TMS derivatives of analytes were prepared by the addition of 30 μL BSTFA and 60 μL pyridine to the vial, and then capped, vortexed and placed at room temperature (20 °C) for 15 min. After adding 10μL internal standard with the inject concentration of 100 pg/μL, the mixtures were analyzed by GC-MS employing selected ion monitoring (SIM) mode. For conventional method, derivatization was conducted with 30 μL BSTFA+1%TMCS and 60 μL pyridine at 70 °C in a dry block heater for 30 min. Chemical structures of E1, E2, EE2, E3 and their derivatives are given in Fig. 1.

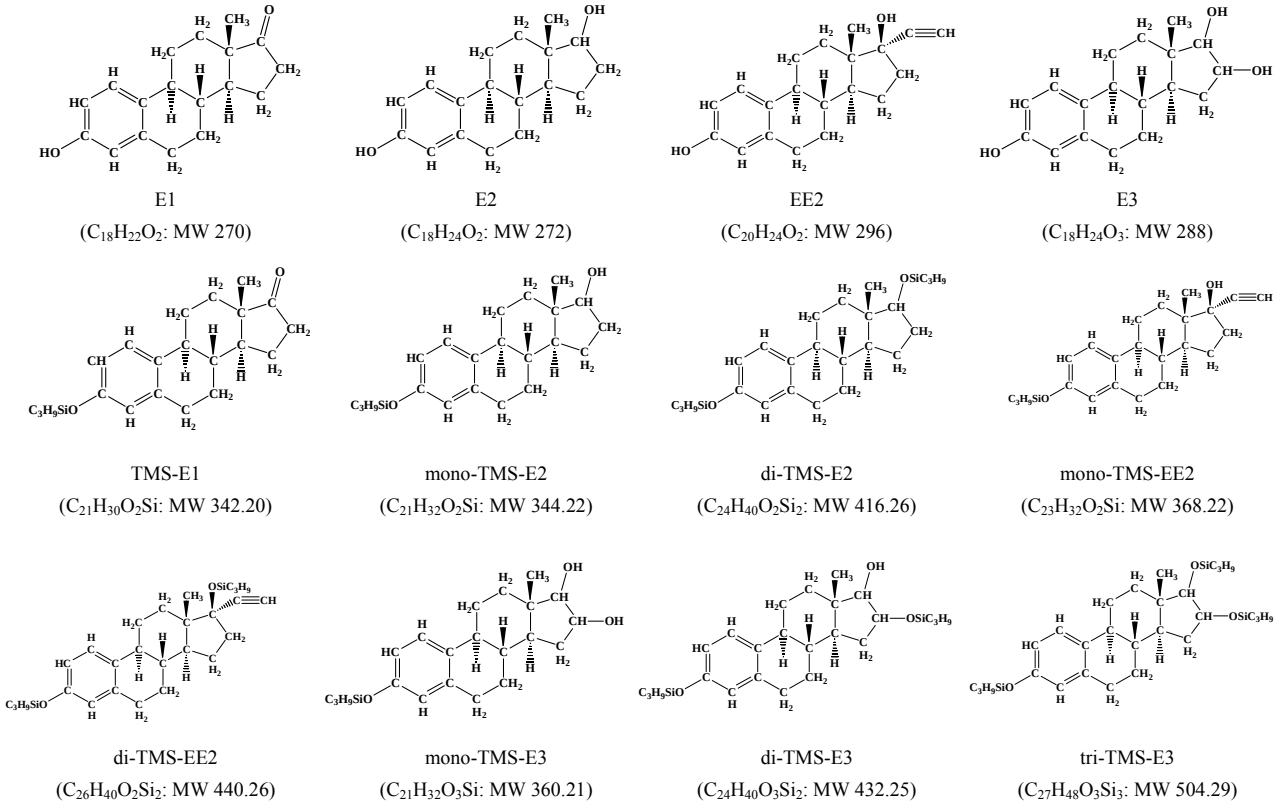

Fig. 1. Chemical structures of E1, E2, EE2, E3 and their derivatives

GC-MS analysis

The GC-MS analysis was performed using a Trace DSQ quardrupole mass spectrometer coupled to a Trace GC (Thermo Finnigan, USA) fitted with a DB-5MS (30 m×0.25 mm×0.25 μm) column (J&W Scientific, USA) and an autosampler Triplus AS. The injector with a splitless insert (1 μL) was set at 280 °C, and the oven temperature was programmed at 50 °C for 2 min, ramped at 12 °C /min to 260 °C and maintained at this temperature for 8 min, then ramped at 3 °C /min to 280 °C and held for 5 min. The carrier gas was helium with a constant flow rate of 1 mL/min.

The GC-MS interface and the ion trap temperature were set at 280 and 250 °C, respectively. Mass spectra were obtained in full scan mode from 50-600 *m/z* mass range for qualitative analysis or selected ion monitoring (SIM) mode for quantitative analysis, using electron impact ionization mode at 70 eV. All GC and MS parameters were implemented using Xcalibur version 1.4 software.

Results and discussion

Comparison of the derivatization under conventional and improved method

In conventional method for simultaneous derivatization of steroid estrogens, heating is an absolutely necessary, usually adding a small proportion of catalyst to enhance derivatization efficiency. For the purpose of comparison with the following improved method, the typical conventional method, reaction with BSTFA+1%TMCS in pyridine at 70 °C in a dry block heater for 30 min, was selected in this study. The SIM chromatogram of TMS derivatization of the four steroids and internal standard is given in Fig. 2A. The result shows that E1, E2, EE2 and E3 are derivatized to TMS-E1 (RRF, 1.544), di-TMS-E2 (RRF, 1.691), di-TMS-EE2 (RRF, 0.710) and tri-TMS-E3 (RRF, 0.774), respectively, and there are no peaks of E1, E2, EE2 and E3, so the hydroxyl groups contained in the four steroids have been derivatized completely. The derivatization results (Fig. 2B) obtained under the improved method (reaction with BSTFA at room temperature for 15 min, without catalyst) are a little better than those generated by conventional method. The derivatizations of E1, E2, EE2 and E3 are also completely, with the RRF 1.551 for TMS-E1, 1.794 for di-TMS-E2, 0.780 for di-TMS-EE2 and 0.828 for tri-TMS-E3.

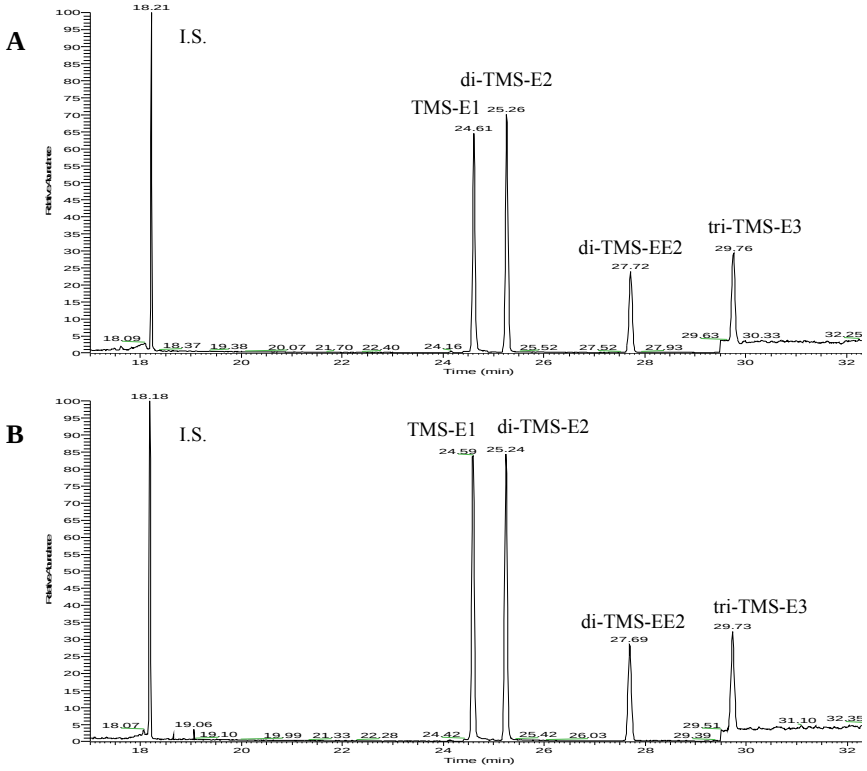

Fig. 2. SIM chromatogram of steroid derivatives and internal standard at 100 pg/μL.
(A) Derivatization performed under conventional method
(60 μL pyridine and 30 μL BSTFA+1%TMCS, reaction at 70 °C for 30 min)
(B) Derivatization performed under the improved method
(60 μL pyridine and 30 μL BSTFA, reaction at room temperature (20 °C) for 15 min)

Effect of reaction temperature and time

To investigate the effect of reaction temperature on the derivatization of steroid estrogens, derivatization was conducted with BSTFA in pyridine at different temperature (room temperature 20, 30, 40, 50, 60, 70, 80 °C) for 15 min. The results are shown in Fig. 3. E1, E2, EE2 and E3 have been derivatized completely to their TMS derivatives at various temperatures. Although the RRFs of steroid derivatives generated at 70 °C are superior to those at other temperatures between 30-80 °C, the difference is not significant. These results indicate that the derivatization can be conducted in the wide temperature range examined in this study, which explains why most previously studies used heating of 45-80 °C for the analysis of steroids, especially 70 °C. However, compared with the results at 70 °C, the RRFs generated at room temperature (20 °C) are better, so heating is unnecessary.

The effect of reaction time on the derivatization of the steroids was performed with BSTFA in pyridine at room temperature for 0 (analyzed directly by GC-MS after vortexed), 5, 10, 15, 20, 25, 30 min. The results are shown in Fig. 4. The RRFs of di-TMS-E2, di-TMS-EE2 and tri-TMS-E3 are increased rapidly with increase of the reaction time from 0 to 5 min, but RRF of TMS-E1 was decreased and mono-TMS-EE2 disappeared. This is because three products are formed for EE2 at 0 min, TMS-E1, mono-TMS-EE2 and di-TMS-EE2, while at 5 min, only di-TMS-EE2 is formed. The RRFs of TMS-E1 and di-TMS-E2 reach the maximum when the reaction time increases to 15 min, while the time is 20 min for di-TMS-EE2 and tri-TMS-E3. By comprehensive consideration, 15 min is the optimized reaction time in this study.

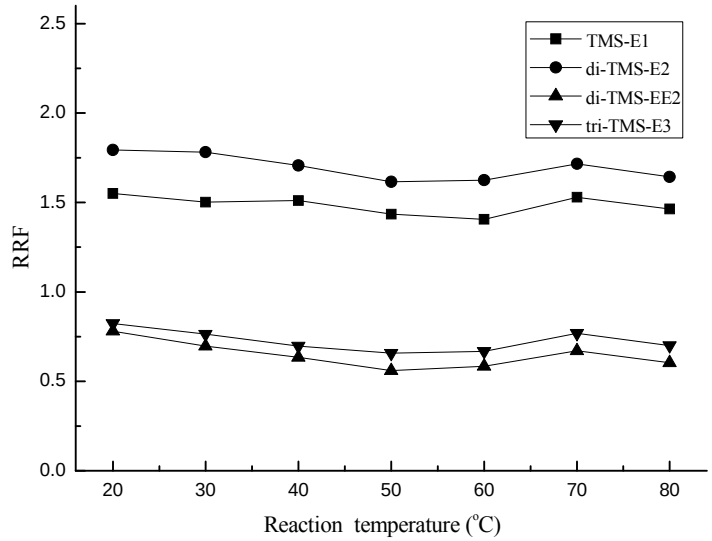

Fig. 3. Effect of reaction temperature on the derivatization of steroid estrogens at 100 pg/μL.
(30 μL BSTFA and 60 μL pyridine, reaction at room temperature, 30, 40, 50, 60, 70, 80 °C for 15min)

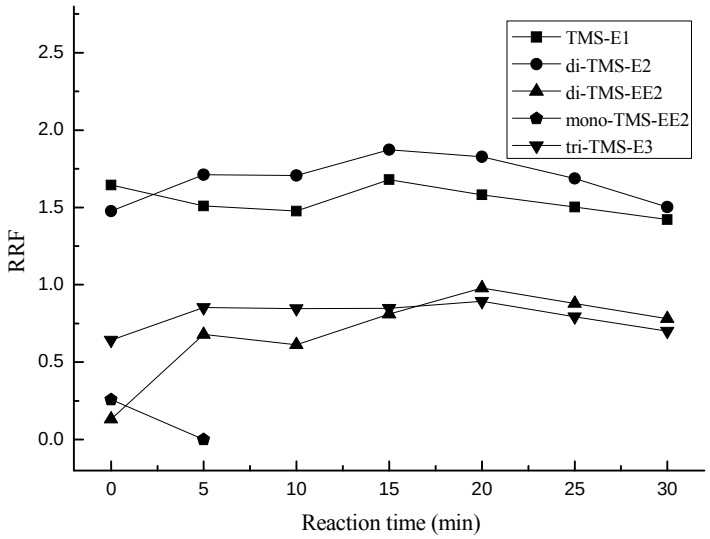

Fig. 4. Effect of reaction time on the derivatization of steroid estrogens at 100 pg/μL.
(30 μL BSTFA and 60 μL pyridine, reaction at room temperature for 0, 5, 10, 15, 20, 25, 30 min)

Calibration curve

The calibration equations for each steroid estrogen generated under the improved method (room temperature, without catalyst, reaction for 15 min) are shown in Table 1. Calibration curves of TMS-E1 and di-TMS-E2, or di-TMS-EE2 and tri-TMS-E3 are linear between the concentration range of 1.0-250 pg/μL and 10-250 pg/μL, respectively. The square of the correlation coefficient (R^2) varies from 0.99462 to 0.99942. The instrumental quantification limit (IQL), determined as a signal-to-noise ratio 3:1, using SIM detection mode for TMS-E1 and di-TMS-E2 is 0.3 pg/μL, while that is 5.0 pg/μL for di-TMS-EE2 and tri-TMS-E3. The results further support that the improved method can be applied to simultaneous determination of steroid estrogens.

Table 1 Calibration curve of steroid derivatives generated under the improved method
(30 μL BSTFA and 60 μL pyridine, reaction at room temperature for 15 min)

Steroid derivatives	Equation of linear regression	R^2	linear range (pg/μL)	IQL[b] (pg/μL)
TMS-E1	Y = 0.01434X - 0.06594[a]	0.99807	1-250	0.3
di-TMS-E2	Y = 0.01471X - 0.05199	0.99942	1-250	0.3
di-TMS-EE2	Y = 0.00380X + 0.00987	0.99462	10-250	5.0
tri-TMS-E3	Y = 0.00551X + 0.01192	0.99778	10-250	5.0

[a] X is the concentration of analyzed steroid estrogen; Y is the peak area ratio of analyzed estrogen and internal standard.
[b] IQL is instrumental quantification limit.

Acknowledgments

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