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Study on the Derivatization Process Using *N*-*O*-bis-(trimethylsilyl)-trifluoroacetamide, *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide, Trimethylsilydiazomethane for the Determination of Fecal Sterols by Gas Chromatography-Mass Spectrometry

Jingming Wu, Ruikang Hu, Junqi Yue, Zhaoguang Yang, and Lifeng Zhang

***Abstract*—**Fecal sterol has been proposed as a chemical indicator of human fecal pollution even when fecal coliform populations have diminished due to water chlorination or toxic effects of industrial effluents. This paper describes an improved derivatization procedure for simultaneous determination of four fecal sterols including coprostanol, epicholestanol, cholesterol and cholestanol using gas chromatography-mass spectrometry (GC-MS), via optimization study on silylation procedures using N-O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA), and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), which lead to the formation of trimethylsilyl (TMS) and tert-butyldimethylsilyl (TBS) derivatives, respectively. Two derivatization processes of injection-port derivatization and water bath derivatization (60 ^{o}C, 1h) were inspected and compared. Furthermore, the methylation procedure at 25 ^{o}C for 2h with trimethylsilydiazomethane (TMSD) for fecal sterols analysis was also studied. It was found that most of TMS derivatives demonstrated the highest sensitivities, followed by methylated derivatives. For BSTFA or MTBSTFA derivatization processes, the simple injection-port derivatization process could achieve the same efficiency as that in the tedious water bath derivatization procedure.

***Keywords*—**Fecal Sterols, Methylation, Silylation, BSTFA, MTBSTFA, TMSD, GC-MS.

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I. Introduction

TO protect public health, it is important to monitor drinking water and recreational water bodies to ensure the pathogens are not present. Recently, various chemical compounds have been used for identifying human sewage contamination in water bodies [1-3]. Coprostanol, formed during catabolism of cholesterol by indigenous bacteria present in the gut of humans or animals, is the primary fecal sterol detected in the domestic wastewater or sediment samples [1-3]. It has been proposed as a chemical indicator of human fecal pollution even when fecal coliform populations have diminished due to water chlorination or toxic effects of industrial effluents [4]. The other metabolites of cholesterol are epicoprostanol, epicholestanol and cholestanol, which are stereoisomers. Because the sterol composition in human waste differs from those of other animals, the sterol composition provides the potential for discriminating between sources.

For the determination of fecal sterols with gas chromatographic (GC) analysis [3,5], a prior derivatization step is necessary for improving their chromatographic performance. Acetic anhydride has been used to derivatize fecal sterols, in which acetylated sterols were produced [6]. Compared to acetylation, silylation is far more common and versatile method employed to derivatize fecal sterols [6]. *N*-*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) or *N*-methyl-*N*-trimethylsiyltrifluoroacetamide (MSTFA) was used as silylation reagent, which lead to the formation of trimethylsilyl (TMS) derivatives. Catalysts such as trimethyl-chlorosilane (TMCS), together with trimethylsilyl-imidazole (TMSI) are usually added to enhance derivatization process performance. In these silylation procedures, reactions usually took place at 60 **°C** or 70 **°C** for 60 minutes, making it a tedious procedure. In addition, moisture content can affect the accuracy and reproducibility of analysis. To address the above problems, injection-port derivatization [7-8] was selected to simplify the derivatziation procedure and enhance the efficiency of organic compounds analysis.

Furthermore, as it shortens derivatization time, degradation of analytes due to exposure to moisture is much reduced, if not eliminated. Injection-port derivatization has been widely applied in the analysis of organic compounds [7-8].

N-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA), another silylation reagent, has been widely used as derivatiaztion reagent for the analysis of organic compounds containing hydroxyl or/and carboxyl functional groups. In general, compared to other silylated derivatives, TBS derivatives formed by MTBSTFA have superior properties such as more specific mass fragmentation, higher m/z values in EI mass spectra, greater hydrolytic stability [9].

Normally, trimethylsilyldiazomethane (TMSD) was employed as methylated reagent for the analysis of organic acids at room temperature [10].

There is no report about using MTBSTFA or TMSD as derivatization reagent for the analysis of fecal sterols so far. Injection-port derivatization procedure has not been employed for the derivatization of fecal sterols. In this work, three derivatization reagents: BSTFA, MTBSTFA and TMSD were employed to conduct the derivatization reaction for analysis of four fecal sterols, with their analytical sensitivities on GC-MS to be investigated and compared. Two derivatization procedures (including water bath and injection-port derivatization procedure) were investigated when BSTFA and MTBSTFA were applied, respectively.

II. Experimental Procedures

A. Standards and Reagents

5β-Cholestan-3β-ol (coprostanol, ≥98%), 5α-cholestan-3α-ol (epicholestanol), cholesterol (≥99%) were bought from Aldrich. 5α-Cholestan-3β-ol (cholestanol) was supplied by Supelco (Bellefonte, PA, USA). Methylene chloride was bought from J.T. Baker (Philipsburg, NJ, USA). The chemical structures of all the studied analytes are illustrated in Fig. 1. Stock solutions (1 mg/mL of each analyte) were prepared in methylene chloride separately. A mixture of working standards containing each compound at 10 μg/mL was prepared by diluting the stock solution in methylene chloride.

Derivatization reagent *N,O*-bis(trimethylsilyl)trifluoro-acetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), *N-(tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) and trimethylsilydiazomethane (TMSD) were purchased from Fluka (Buchs, Switzerland).

B. Derivatization

TMS derivatives of fecal sterols were prepared by the addition of 1 mL 1ppm fecal sterols mixtures (dissolved in methylene

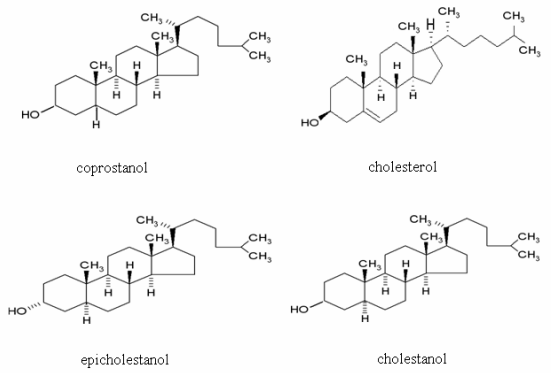

Fig. 1 Chemical structures of fecal sterols

chloride) and 100 μL BSTFA (1% TMCS) to a 2 mL amber GC autosampler vial. To apply the injection port derivatization process, the vial was capped, vortexed and injected into GC injection-point for derivatization. To apply the water bath derivatization process, the vial was heated for 1 h at 60 **°C** after vortexing, followed with drying under N_2 flow. Then the fecal sterols derivatives were reconstituted into 1 mL in methylene chloride and introduced into GC for analysis.

TBS derivatives of fecal sterols were prepared in the similar way via two derivatization procedures. The derivatization reagent of MTBSTFA was employed instead of BSTFA (1% TMCS).

Methylated derivatives of fecal sterols were carried out by the addition of 1 mL 1ppm fecal sterols mixtures (dissolved in methanol) and 100 μL TMCS to a 2 mL amber glass sample vial. Derivatization procedures were performed for 2h at 25 **°C**. Then the reaction mixtures were introduced into GC-MS system for analysis.

C. GC-MS Analysis

Analysis of the fecal sterol derivatives were carried out on an Agilent Series 6890 GC coupled to a 5973 MS detector. The GC was fitted with a HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25-μm). Helium was used as the carrier gas at 1.0 ml/min. The GC oven temperature was as follows: 70°C for 2 min; then 20 °C/min to 270°C, held for 1 min; finally 5°C/min to 300°C, held for 6 min. 150 and 230°C was set as the GC-MS quadropole and the MS source temperature, respectively. Electron impact (EI) mass spectra were obtained at acceleration energy of 70 eV. A 2.0 μL aliquot of extract was injected in the splitless mode. Fragment ions were analyzed over 35-550 m/z mass range in the full scan. The filament delay time was set as 15 min.

III. Results and Discussion

TMS Derivatives with BSTFA

Silyl derivatives are produced by the displacement reaction of active protons as a nucleophilic attack of a more electronegative heteroatom upon the silicon atom of the silylating reagent [11].

Fig. 2A displays the EI mass spectrum for the TMS derivatives of coprostanol and the proposed fragmentation pattern. The major fragmentation peaks of trimethylsilyl derivatives of fecal sterols included $[M]^+$ (m/z 460) and $[M\text{-}CH_3]^+$ (m/z 445) due to the loss of the methyl group from the derivative [12-13]. In addition, the base ion occurring at the m/z 370 for coprostanol trimethylsilyl derivatives was formed from the loss of $[(CH_3)_3\text{-Si-O}]$ [12-13]. Furthermore, as shown in Fig. 2A, the characteristic ion of m/z 355 was observed in the mass spectrum of trimethylsilylated coprostanol, possibly resulting from the further loss of methyl group from derivatives.

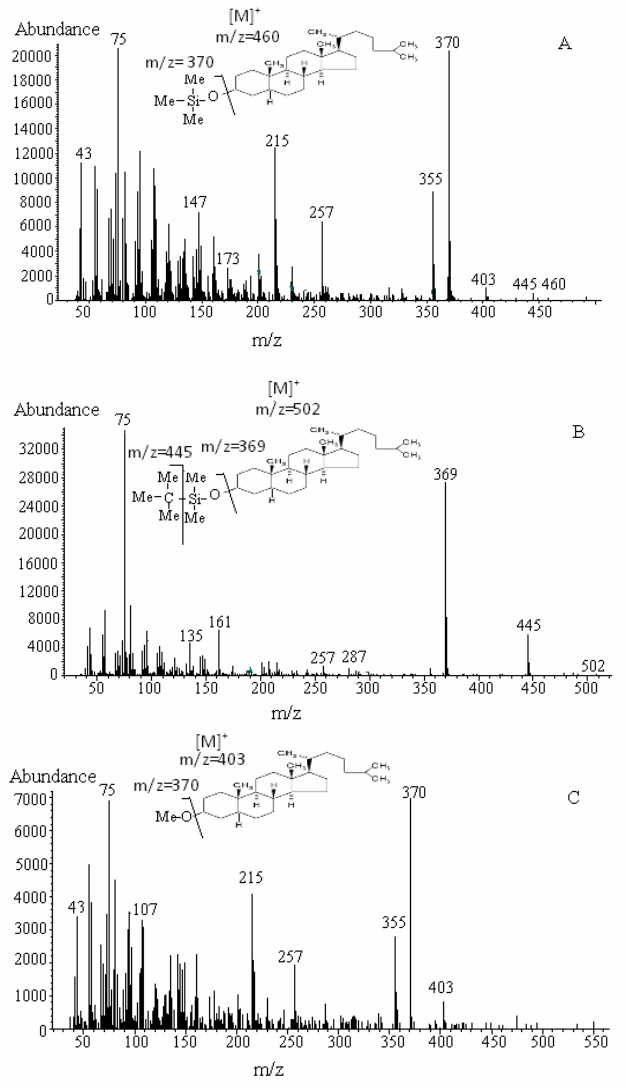

Fig. 2 Mass spectra for coprostanol derivatives. (A) Mass spectrum for trimethylsilyl coprostanol derived from BSTFA. (B) Mass spectrum for tert-butyldimethylsilyl coprostanol derived from MTBSTFA. (C) Mass spectrum for methylated coprostanol derived from with TMSD

TBS Derivatives with MTBSTFA

Fig. 2B shows the EI mass spectrum for the TBS derivatives of coprostanol and the proposed fragmentation pattern. The diagnostic ions for TBS derivative of coprostanol included the molecular ion with m/z 502 $[M]^+$, and m/z 445 $[M\text{-}57]^+$ due to the loss of the *t*-butyl group form the derivatives (as seen from Fig. 2B) [12-13]. In addition, it is found that the m/z 369, as a base ion, possibly resulted from the loss of the fragment $[(CH_3)_3\text{-C-Si-}(CH_3)_2\text{-O}]$ from the TBS derivatives [12-13].

Methylated Derivatives with TMSD

The mass fragmentation of methylated derivatives of coprostanol and the proposed fragmentation pattern is shown in Fig. 2C. The major peak, m/z 370 $[M\text{-}33]^+$, was observed, due to the loss of $[CH_3\text{-O}]$ from the methylated derivative. Other diagnostic ions for methylated derivative of coprostanol included m/z 355, possibly due to the further loss of methyl group from derivatives. It is also observed that derivative contained the molecular ion with m/z 403 $[M]^+$ (as illustrated in Fig. 2C) [14].

Optimization of Derivatization Conditions

The derivatization conditions were preliminarily optimized by using different derivatization reagents (BSTFA, MTBSTFA, TMSD) under different derivatization processes. And the results were demonstrated in Fig. 3. For TMS derivatives of fecal sterols (derived from BSTFA), there was no difference in derivatization efficiencies, when derivatizations were performed at 60°C for 1h, or GC high-temperature injection-port, respectively. The same phenomenon was observed when MTBSTFA was employed as derivatization reagent. It is easily understood since the high reaction temperature can help to overcome the energy barrier of the silylation and steric hindrance, thus leading to reaction time much shortened in GC-injection-port derivatization process.

Among three derivatization reagents, BSTFA provided the highest sensitivity for the analysis of epicholestanol, cholesterol and cholestanol with GC-MS, followed by TMSD (performed at 25°C, 1h). MTBSTFA demonstrated the poorest sensitivity. However, for coprostanol, different trends were found. MTBSTFA provided the highest sensitivity, followed by BSTFA. TMSD showed the lowest sensitivity for coprostanol.

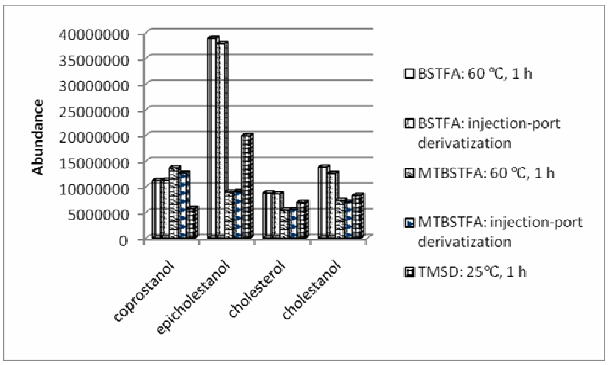

Fig. 3 GC-MS intensities of fecal sterols derivatives derived from BSTFA, MTBSTFA and TMSD under different derivatization processes. Fecal sterols derivatives concentration: 1 μg/ml

VI. Conclusion

Three chemical reagents of BSTFA, MTBSTFA, and TMSD have been successfully applied for the derivatization and determination of fecal sterols by GC-MS, with the characteristic fragments of their derivatives being identified in their mass spectrum, respectively. Among the three reagents: BSTFA, MTBSTFA, and TMSD, most of TMS derivatives from BSTFA demonstrated the highest sensitivities, followed by methylated derivatives from TMSD. TBS derivatives from MTBSTFA showed the poorest sensitivities. In addition, the study on two derivatization processes with BSTFA or MTBSTFA as probes indicated that the simple injection-port derivatization process could achieve the same efficiency as that in the tedious water bath derivatization procedure.

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