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Short Communication

Sample stacking with in-column silylation for less volatile polar compounds using GC-MS

This paper presents sample stacking with in-column silylation (SIS) for quantitative analysis of less volatile polar compounds using gas chromatography-mass spectrometry (GC-MS). This was achieved by the combination of sandwiched in-column silylation and multiple injections (up to 100 times or 100 μL in total). *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was used as a silylating reagent. For the SIS technique, samples were introduced multiple *N* times ($N = 2 \sim 100$) into a capillary column in between BSTFA injections. The quantitative characteristic of SIS technique was studied using bisphenol A (BPA) as a model compound. The sandwiched in-column silylation for the less volatile polar compounds effectively replaced polar hydrogen with trimethylsilyl group to reduce sample adsorption and band broadening. Meanwhile, multiple injections at the SIS technique contributed to increase the sensitivity quantitatively. The capability and limitation of this analytical approach were further investigated with various types of compounds such as hydroxyls, carboxylic acids, and amine.

Keywords: Bisphenol A / In-column silylation / Multiple injections / *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) / Sample stacking
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1 Introduction

Gas chromatography-mass spectrometry (GC-MS) is a powerful analytical technique with high efficiency, sensitivity, and reproducibility for the analysis of volatile compounds [1]. Non-polar volatile compounds are analyzed with excellent chromatographic properties with minimal adsorption and peak tailing by GC [2]. However, polar compounds such as hydroxyl, carboxylic acid, or amine are difficult to analyze by GC because of their adsorption onto column or inlet surfaces [2–4]. This results in poor analytical reproducibility, in part due to carry-over effect [2–4]. This difficulty has been usually overcome by substituting polar functional groups with less polar ones [2–5]. The less polar derivatives of the analytes show enhanced chromatographic sensitivity as well as enhanced reproducibility because of reduction in peak tailing and sample loss. This makes it feasible for a conventional GC-MS to achieve sensitivity at parts-per-billion (ppb) level in the analysis of polar compounds.

Derivatization is usually performed off-line or using on-line condition [6–10]. The latter approach is more efficient in that on-line derivatization is less time consuming, reduces the consumption of both sample and derivatizing reagents, and can be easily automated [9]. On-line derivatization is usually accomplished in a flash-heater (GC injector) or column [7–9]. In the former method, the derivatization occurs at high temperature (ca. 300°C), a condition that can introduce additional by-products during derivatization [8, 9]. In contrast, in-column derivatization applies mild conditions (ca. 50°C) by injecting samples right after derivatizing reagents and thus can reduce undesired by-products [7, 9, 10]. Meanwhile, both methods in a conventional splitless mode usually impose restrictions on injected sample volume (<5 μL) [11].

Silylation is one of the most widely practiced derivatization techniques for hydroxyl, carboxylic acid, amine, thiol, and phosphate functional groups because of high reactivity of silylating reagents and high stability of silylated products [8, 10, 12, 13]. Many silylating reagents with different reactivity such as *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane have been used for on-line or off-line derivatization [9, 12, 13]. Excessive silylating reagents are usually mixed with samples to complete derivatization (> 99%). However, excessive silylating reagents can produce many by-products by reacting with the column surface or unknown contaminants carried over from previous runs [14–16]. These artifacts sometimes make it difficult to properly analyze/quantify samples of interest at trace level. It would be helpful if these artifacts or contaminants could be easily

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Abbreviations: **BA**, Benzyl alcohol; **BPA**, Bisphenol A; **BSTFA**, *N,O*-bis(trimethylsilyl)trifluoroacetamide; **SIS**, sample stacking with in-column silylation; **TMS**, trimethylsilyl.

Color Online: See the article online to view Figs. 1–4 in color.

differentiated from the analyte of interest by selectively concentrating the samples.

This paper reports sample stacking with in-column silylation (SIS) technique to selectively enhance the sensitivity of samples of interest (up to 100 times) for the trace-level analysis of less volatile polar compounds in a GC-MS. The SIS technique was performed by the combination of sandwiched in-column silylation and multiple injections, i.e. the sequential injections of BSTFA, samples ($\times N$), and BSTFA, where N represents number of injections. A mass spectrometer was used to identify and quantify analytes. Bisphenol A (BPA) was chosen as a model compound to evaluate the performance and characteristic of the SIS technique because its analysis requires high sensitivity [17–19]. The capability and limitation of the SIS technique was further investigated with various types of chemicals such as hydroxyls, carboxylic acids, and amines.

2 Materials and methods

2.1 Chemicals and materials

The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA): BPA, BSTFA, benzyl alcohol (BA), 4-*t*-octylphenol, 4-nonyl phenol, 2-naphthol, 4-methoxyphenol, 2,6-di-*t*-butyl-*p*-cresol (DBPC), *p*,α,α-trimethylbenzyl alcohol, naproxen, ibuprofen, 4-*t*-butylbenozic acid, and 2-naphthyl amine. $^{13}C_{12}$- and D_{16}-BPA were from Cambridge Isotope Laboratories (Andover, MA, USA). Ethyl acetate was purchased from Fluka Chemika (Milwaukee, WI, USA).

2.2 Off-line or in-column silylation and GC-MS analysis

Off-line silylation was performed at 60°C for 2 h by mixing 100 μL of BSTFA with 900 μL of samples. In-column silylation with sandwiched configuration was performed by the sequential introduction of BSTFA, samples, and BSTFA into a GC column at 50°C. The sequential multiple injections were performed by sequence programming using Chemstation software (Agilent, Palo Alto, CA, USA). There was no time delay in between each injection.

The analysis was performed using a GC system (6890N, Agilent) equipped with an autosampler (G2614A, Agilent) and coupled to a mass selective detector (5973N, Agilent). A DB-5MS fused-silica capillary column (60 m × 250 μm id with a film thickness of 0.25 μm; Agilent J&W Scientific, Folsom, CA, USA) was used to separate the analytes. Helium was used as a carrier gas at a constant flow rate of 1 mL/min through the column. One microliter of the sample was injected in the splitless mode, if not specified otherwise. A deactivated splitless mode liner (part number: 5062–3587, Agilent) was used to minimize sample breakdown. The purge valve was open at 1 min with flow of 50 mL/min. The GC temperature program for method development was as follows: initial oven temperature at 50°C, hold for 1 min, ramp at 25 to 150°C/min, hold at 150°C for 1 min, ramp at 10°C/min to 270°C, and hold for 8 min. The temperatures of injector, transfer line, and electron impact (EI) ion source were set to 350°C, 280°C, and 230°C, respectively. The electron energy for ionization was set to 70 eV. Mass spectral data were collected in a full scan mode (m/z 100–500) or a selected ion monitoring (SIM) mode, and delayed 7 min after sample injection to exclude chromatographic peaks of solvent, excess BSTFA, and BSTFA by-products, if not specified otherwise.

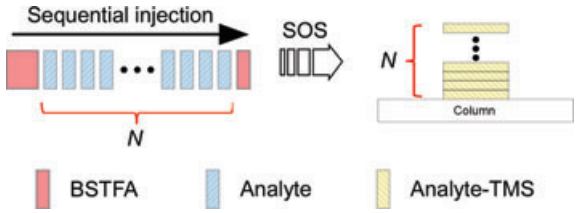

Figure 1. Schematic of SIS technique. The samples are introduced multiple (N) times in between BSTFA injections on a column. TMS derivatives are produced by first and last injection of BSTFA and stacked vertically on the column surface as BSTFA and solvent evaporate.

3 Results and discussion

3.1 Performance of SIS technique

Figure 1 shows the schematic of the SIS technique presented here. The samples are introduced multiple (N) times (N = 2∼100) into a capillary column in between BSTFA injections. The initial BSTFA acts not only as a silylating reagent but also as a blocker to prevent the spread of analytes by converting them to less polar trimethylsilyl (TMS) derivatives. Analytes with higher boiling points than BSTFA are deposited on the rear of BSTFA band because of a temperature gradient at the interface between the injector and the column [20]. The last introduced BSTFA completes silylation reaction. As BSTFA and solvent evaporate, the quantitative accumulation of TMS derivatives is achieved by solvent focusing or thermal focusing [20, 21].

To investigate the analytical performance of SIS technique, we first compared peak profiles of bis(trimethylsilyl)ether derivative of BPA (BPA-TMS) by different silylation methods: (A) off-line silylation, (B) conventional in-column silylation in which BPA is injected right after BSTFA, and (C) SIS technique (Fig. 2). The total injected amount of BPA (or BPA-TMS) was kept at 4 ng to accurately characterize and compare the difference in these three silylation methods. Ethyl acetate was chosen as a common solvent because of good solubility for both BPA and BPA-TMS. Injection of 1 μL BPA-TMS by off-line silylation produced a symmetric peak (data not shown). With increase of injection volume (≥2 μL), however, the peak became asymmetric with a diffuse leading edge (see dotted/red-shaded peak in Fig. 2). Meanwhile, the peak of

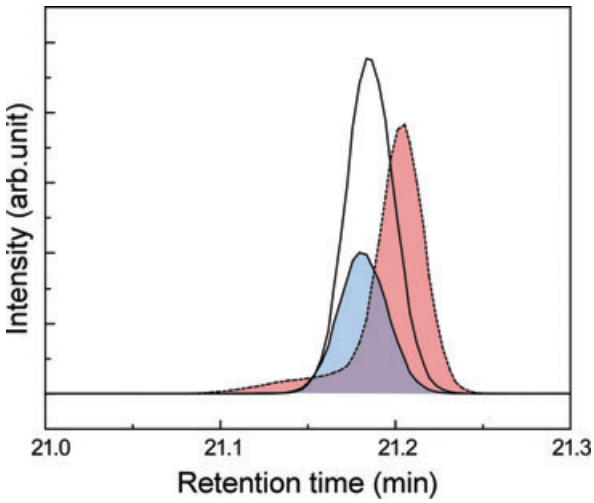

Figure 2. Chromatograms of TMS derivatives of BPA by off-line silylation (dotted, red shaded), conventional in-column silylation (solid, blue shaded), and SIS (solid, unshaded).

BPA-TMS (blue shaded; sold line) by using the conventional in-column silylation [BPA (2 μL)-BSTFA (2 μL)] was much smaller (ca. 50% in height) and eluted slightly earlier than that by off-line silylation. This decrease in the peak height can be explained by the adsorption and the spread of free BPA on the column surface during in-column silylation. However, the peak profile in SIS technique (BSTFA [2 μL]-BPA [1 μL]×2-BSTFA [2 μL]) was symmetric, and its height was highest (not shaded; solid line). This implies that the silylation was complete without any loss of the samples.

3.2 Quantitative characteristic of SIS technique

To further investigate the quantitative characteristic of the SIS technique, we compared the chromatograms of BPA-TMS injected multiple times (2, 4, 6, 8, and 10) at a constant BPA concentration (2 μg/mL). Conventional multiple injections ($N > 2$) of off-line silylated BPA-TMS resulted in significant band broadening and distortion (data not shown). On the contrary, the peaks of BPA-TMS by SIS technique were symmetric and did not show any noticeable band broadening with change in the number of multiple injections (Fig. 3A). Meanwhile, the peak positions were slightly shifted with increase in number of multiple injections, which may be attributed to the sample focusing by repeated solvent evaporation during multiple injections. As shown in Fig. 3B, the peak intensity increased in proportion to the number of multiple injections. The correlation coefficient (R) of the linear regression for the intensity plot as a function of number of injections was very close to 1 (>0.999). In addition, the calibration curve for 10 injections was plotted using broad range of concentrations from 0.01 to 1 μg/mL (data not shown). The calibration plot showed an excellent linearity with a correlation coefficient of 0.999. This was better than our results for a regular injection of off-line silylated BPA-TMS, which usually had a correlation coefficient of 0.99. Furthermore, we compared peak profiles between ten injections of 1×, *i.e.* unchanged sample (1 μg/mL BPA) and 100 injections of tenfold diluted sample (0.1 μg/mL BPA). Figure 3C shows that two peak profiles almost overlapped and demonstrates that the SIS technique worked well up to 100 injections without noticeable distortion or broadening. Thus, SIS technique is a useful in-column concentration technique for quantitative analysis of trace-level BPA.

3.3 Application of SIS technique to other chemicals

The applicability of SIS technique was further tested for polar functional groups, including hydroxyls, amine, and carboxylic acids. Table 1 lists the chemicals studied and their retention times, boiling points at 760 mm Hg, and quality of SIS technique. The chemicals could be categorized into five groups depending on the quality of SIS techniques, which depended on the volatilities of the TMS derivatives. The quality of the SIS technique was rated by using a scale of one (very poor) to five (excellent) on the basis of how well the peak profile of one injection of 1 μg/mL analytes matched that of ten injections of 0.1 μg/mL analytes.

The first group of chemicals with one-point (very poor) quality rarely or only partially reacted with BSTFA under the current experimental conditions. For example, *p*,α,α-

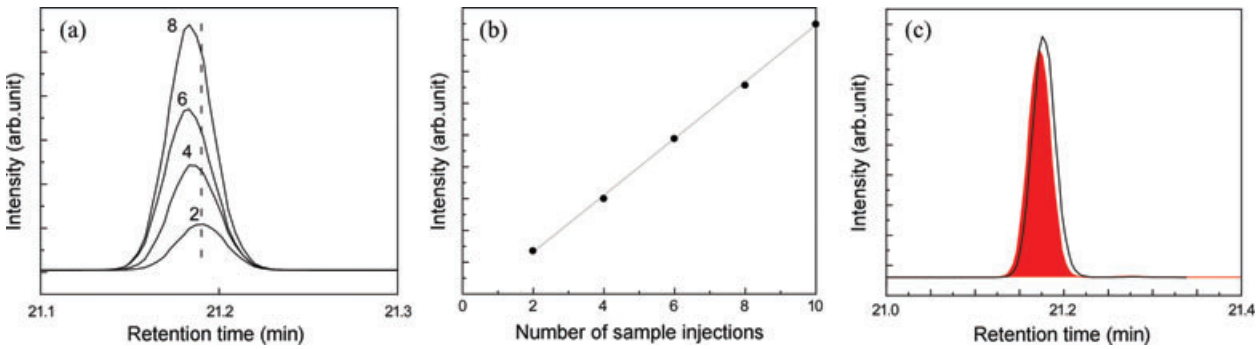

Figure 3. (A) Chromatograms of TMS derivatives of BPA for 2, 4, 6, and 8 injections with SIS technique. (B) Plot of signal intensity as function of number of injections. (C) Chromatograms of TMS derivatives of BPA for ten injections of 1 μg/mL BPA (unshaded) and 100 injections of 0.1 μg/mL BPA (red shaded).

Table 1. Boiling points (bp) at 760 mm Hg and retention times (RT) of studied analytes and their TMS derivatives, and quality of SOS successfulness.

Analyte	bp (°C)	RT of analyte (min)	RT of analyte-TMS (min)	Quality of SOS technique
Benzyl alcohol	205	8.48	9.54	2
p,α,α-Trimethylbenzyl alcohol	205	9.70	N/A	1
4-Methoxyphenol	243	12.09	13.17	3
2,6-di-*t*-Butyl-*p*-cresol	265	13.29	N/A	1
4-*t*-Octylphenol	282	14.48	13.54	4
4-*t*-Butylbenzoic acid	283	13.03	14.20	5
2-Naphthol	285	13.62	14.08	5
4-Nonylphenol	295	17.31	17.72	5
Naphthyl amine	306	14.14	11.33	1
Ibuprofen	320	14.50	13.54	4
Bisphenol A	398	21.18	21.19	5
Naproxen	404	19.53	19.52	5

trimethylbenzyl alcohol and 2,6-di-*t*-butyl-*p*-cresol did not react at all with BSTFA because of steric hindrance (data not shown). 2-Naphthyl amine partially reacted with BSTFA because of strongly basic nature of amine groups. For this group of chemicals, off-line silylation as well as in-column silylation is not recommended using mild condition (50°C for a few minutes).

The second group with two points (poor) did not work well with in-column silylation, but did work well with off-line silylation. Analytes with high volatility, such as benzyl alcohol (BA), are in this group. They were easily spread with solvent along the column during sample injections. As shown in Fig. 4A, the peak of benzyl trimethylsilyl ether (BA-TMS; 0.1 μg/mL; one injection) using off-line silylation had symmetric peak profile (blue shaded), while BA-TMS (1 μg/mL; one injection) by in-column silylation had asymmetric split peaks with broad leading edge (not shaded). In addition, the peak area of in-column silylated BA-TMS is only three times larger than that of off-line silylated BA-TMS, even though the concentration of the former is ten times higher than that of the latter. This means that a lot of free BA was lost before the silylation reaction was complete. The loss of analytes in this group was even worse using conventional in-column silylation than using the SIS technique (data not shown). For this group of chemicals, in-column silylation is not recommended.

In the case of third group with three points (marginal), in-column silylation with one injection worked well, but multiple injections did not work well. Analytes with boiling points less than 280°C belong to this group. Figure 4B shows that chromatograms of TMS derivatives of 4-methoxyphenol for one injection at 1 μg/mL (dotted line; not shaded) and ten injections at 0.1 μg/mL (solid line; red shaded). As the number of injections increased ($N > 3$), the peak distortion became significant.

In case of the fourth group with four points (good), the SIS technique worked with slight band broadening. Ibuprofen and 4-*t*-octylphenol are in this group. The defining characteristic of chemicals in this group is that the TMS derivatives eluted much earlier than their free counterparts while the boiling points of the underivatized chemicals are around 280~320°C (see Table 1). Figure 4C shows the chromatograms of TMS derivatives of 4-*t*-octylphenol for one injection at 1 μg/mL (dotted line; unshaded) and ten injections at 0.1 μg/mL (solid line; red shaded).

The last group with five points (excellent) is very suitable for SIS technique. The analytes in this group have usually boiling point higher than 280°C and elute as early as or earlier than their TMS-derivatives. Figure 4D shows that chromatograms of TMS derivatives of 2-naphthol for one injection at 1 μg/mL (dotted line; not shaded) and ten injections at 0.1 μg/mL (solid line; red shaded). 4-*t*-Butylbenzoic acid, 4-nonlyphenol, and naproxen had similar profiles.

4 Concluding remarks

In summary, this work demonstrates the development of an in-column sample concentration technique by combining in-column silylation with multiple injections in a GC. The successfulness of the SIS techniques depended on the silylation reactivity and volatility of the TMS-derivatives of analytes. In other words, the silylation reaction should be fast enough to be completed within a few minutes under mild

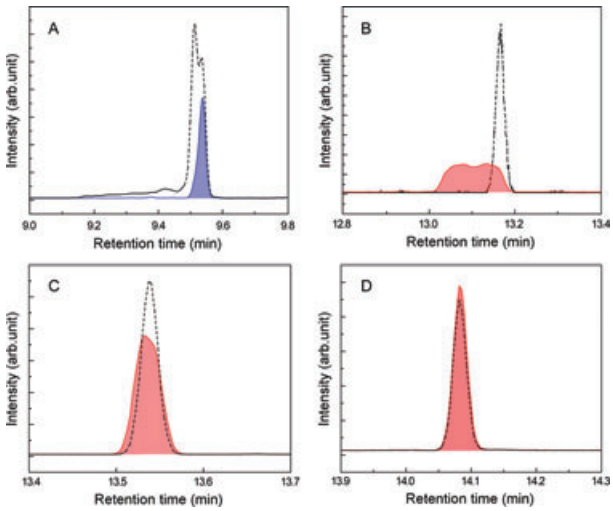

Figure 4. Typical chromatograms for TMS derivatives of (A) benzyl alcohol, (B) 4-methoxyphenol, (C) 4-*t*-octylphenol, and (D) 2-naphthol. In (A), the blue-shaded peak represents off-line silylated BA-TMS (0.1 μg/mL; one injection) while the unshaded peak represents in-column silylated BA-TMS (1 μg/mL; one injection). In (B–D), unshaded peaks represent one injection of 1 μg/mL sample while red-shaded peaks represent ten injections of 0.1 μg/mL sample by SIS technique.

condition, and the analytes should have boiling points higher than 280°C and elute as early as or earlier than their TMS-derivatives. The advantage of SIS technique is that 100-fold in-column concentration of analytes can be achieved without any instrumental modification and readily automated for high-throughput analyses. Additional study using other derivatizing reagents such as *N*-(trimethylsilyl)imidazole and *N*,*O*-bis(trimethylsilyl)acetamide is going on to further extend applicability of this technique.

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