

# Stability of 7-ketocholesterol, 19-hydroxycholesterol, and cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol during storage: Derivatization issues

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## Abstract

Cholesterol oxide products (COPs) may be present as contaminants in standards or evolve during storage of standard solutions or during analysis. If evolved during storage or analysis, they are considered artifacts. It is paramount that standard oxides, often needed for routine analyses, remain stable under varying conditions of storage and analysis. An investigation was undertaken to determine if solutions of 7-ketocholesterol (7-keto), 19-hydroxycholesterol (19-OH) and cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (triol) remained stable over 0, 1, 3, 5 and 7 d when stored at 23  $\pm$  2°C, 4°C, and -20°C. Chromatographic results, expressed as LS means, allowed for adjustment of the impact of injection number, a confounding variable. LS means for tms ethers of 7-keto and 19-OH significantly increased by day. Temperatures for storage times of 7-keto were significantly different with LS means increasing as temperature increased. For the triol tms ether, LS means significantly increased at 23  $\pm$  2°C and 4°C when compared to -20°C; but no discernible trend was observed. Overall, as injection number increased, more derivatized compounds and less underivatized compounds were detected. Injection number was significant for 7-keto tms, underivatized 7-keto, combined 7-keto, 19-OH tms, the related 19-OH peak, and the triol tms ether. For triol, significance for the interaction of day and liner, but not liner, was indicated. Injection number, liner, or day when the liner was changed did not significantly affect the triol related peak.

**Keywords:** 7-ketocholesterol (7-keto); 19-hydroxycholesterol (19-OH); Cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (triol); Injection number; Liner, Stability

## Introduction

If cholesterol oxide products (COPs) are initially present with standards, they are considered contaminants, but if quantities increase when stored in solution or during analysis, they are considered artifacts [1,2]. The presence of artifacts complicates results of analysis because it is difficult to accurately quantify, identify, and distinguish naturally occurring COPs that are in samples due to metabolism, possible disease states, or food processing conditions from the same COPs that might be generated during storage and analysis. Ultimately, underestimation of the parent compound or overestimation of the artifact, presumed to be present in the sample initially as a COP, may occur.

Inter-laboratory comparisons for quantifications of COPs in various matrices are often difficult to interpret due to differences in methodology. Although some methods have been compared, most do not include artifact monitoring [3-10]. The most ambitious comparisons have been those of the Lausanne Workshop consisting of mostly EU scientists [11,12]. While artifacts were considered in some methodologies, artifact monitoring was not a central part of the harmonization study. Thus, more research should be conducted to reveal how artifacts, present in samples or produced during analysis, contribute to results. It is paramount that standard oxides, often needed for routine analyses, remain stable under varying conditions of storage and analysis. Therefore, in the present study, an investigation was undertaken to determine if solutions of standard cholesterol oxides - 7-ketocholesterol (7-keto); 19-hydroxycholesterol (19-OH), and cholestane-3 $\beta$ -5 $\alpha$ ,6 $\beta$ -triol (triol) - were stable for 7 days at 23  $\pm$  2°C, 4°C, and -20°C. Due to changing and unforeseen experimental conditions, issues of injections number and changing of injection liners were encountered. The authors determined that these issues, rarely published, were important to consider in chromatography separation.

Thus, a discussion on how injection conditions (injection number and changing of injection liner) may affect results is included.

## Experimental Procedures

Cholestane-3 $\beta$ -5 $\alpha$ ,6 $\beta$ -triol (triol) and  $\beta$ -sitosterol ( $\beta$ S) were obtained from Sigma (St. Louis, MO, USA). 7-Keto; 19-OH; were from Steraloids (Newport, RI, USA). Toluene (Omni-Solv) was obtained from EMD Chemicals (Gibbstown, NJ, USA). Anhydrous pyridine was from Aldrich (Milwaukee, WI, USA). Sylon BTZ was from Supelco (Bellafonte, PA, USA). Silanized inserts and amber vials were purchased from National Scientific (Duluth, GA, USA).

The experimental design was a 3 x 5 x 3 factorial with 3 standards of 7-ketocholesterol (7-keto), 19-hydroxycholesterol (19-OH), and cholestane-3 $\beta$ -5 $\alpha$ ,6 $\beta$ -triol (triol); 5 sampling times during the days of storage (0, 1, 3, 5, and 7) and 3 storage temperatures at 23°C  $\pm$  2°C, 4°C and -20°C. Storage and temperature conditions were typical of those used during preparation of standards and samples for analyses. The experiment was performed on triplicate samples in low light and duplicated. In silanized amber vials, 5 mg/mL of each stock solution was prepared by addition of toluene and flushing with nitrogen, followed by serial dilution with the same solvent. Aliquots (500  $\mu$ L) of 0.12 mg/mL dilutions were placed in silanized amber vials, flushed with nitrogen, capped, stored in the dark and subjected to varying time/

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temperature conditions.

At each sampling time, 500  $\mu$ L aliquots of 0.05 mg/mL  $\beta$ S (surrogate for recovery) in toluene prepared daily were added to each sample, then, dried completely under nitrogen. Dried samples, redissolved in 50  $\mu$ L of anhydrous pyridine and 50  $\mu$ L of Sylon BTZ [(3:2:3 (N,O Bis (trimethylsilyl) acetamide: trimethylchlorosilane: trimethylsilylimidazole)], were derivatized for 30 minutes at 23°C  $\pm$  2°C. Following derivatization, 50  $\mu$ L of 0.5 mg/mL 5-ene-7-one in toluene (prepared daily as an instrument internal standard), was added to each sample. Samples were transferred to silanized inserts in autosampler vials and 1  $\mu$ L aliquots were analyzed by a Varian 3400 GC-flame ionization detection (FID) (Varian, Walnut Creek, CA, USA) with a 30 m, 0.25 mm x 0.25  $\mu$  DB-5ms capillary column containing a fused 10 m guard column (Agilent, Palo Alto, CA, USA) and a Varian 8200 CX autosampler (Varian, Walnut Creek, CA, USA). Helium, 99.999% pure, was used as a carrier gas. Injector temperature was 290°C; detector temperature was 330°C. The split ratio was 30:1. The temperature program was 100°C for 1 min, 100-260°C with a ramp of 20°C/min, 260-273°C with a ramp of 5°C/min, 273°C held for 12 min, 273-290°C with a ramp of 5°C/min, and 290°C held for 5 min. Typical standard curves ( $R^2 > 0.95$  or greater, Figure 1) for each compound were used for quantification.

Peak identification of compounds was confirmed by GC-MS using a Thermofinnigan GCQ Plus ion trap (Thermo Electron Corp, Waltham, MA, USA) with an electron energy of 70 eV. Ion source temperature was 200°C; transfer line temperature was 275°C. A 17 min hold at 273°C with a 30 m, 0.25 mm x 0.25  $\mu$  DB-5 ms column (Agilent, Palo Alto, CA, USA) was used with the otherwise similar temperature profile delineated above. Helium, 99.999% pure, was the carrier gas.

7-Keto and 19-OH were stored during the same week. For these compounds, silanized GC vial inserts were inadvertently left out of the GC vials on days 0 and 1. Therefore, day 1 samples were re-run using inserts and inserts were used on subsequent days. On day 7, samples were re-run after a new injector liner was installed. For triol, silanized GC vial inserts were used throughout the experiment. The injector liner was routinely replaced after analysis on day 3 and prior to analysis on day 5.

For the statistical analysis of 7-keto and 19-OH, general linear models [13] were utilized with factors of day, temperature, injection number, and the interaction of day and temperature. Injection number was included as a covariate to account for changing liner conditions. Analyses of variance (ANOVAs) were performed. Wilk-Shapiro statistics were calculated and used to assess normality of the residual errors. When normality was violated due to the presence of outliers, Winsorizing was used. When normality was violated due to skewness, Box-Cox transformations were used. Levene ANOVAs were used to assess the validity of the constant residual variance (homoscedasticity) assumption. If the Levene test was significant ( $p < 0.05$ ), then a weighted ANOVA was performed. For triol, general linear models were utilized with factors of temperature, injection number, liner, interaction between liner and temperature, and interaction between day of the analysis and changing of the liner. Statistical significance throughout was at least at 95% confidence level,  $p < 0.05$ .

Because temperature treatments were not employed on day 0 samples, means for six peak area ratios (FID area of the compound divided by the FID peak of the internal standard) for day 0 were obtained, and along with injection number, was applied to each temperature condition. Least squares means (LS means) were calculated in the general linear models and were presented as results rather than

arithmetic means of peak area ratios. LS means allow for adjustment of the impact of possible confounding variables such as injection number. *Post hoc* comparisons among LS means were based on a Tukey Kramer adjustment. Relevant F statistics and probabilities are included in the text and are not provided in tabular form.

## Results and Discussion

In our study, COPs were present both as genuine COP standards and as initial contaminants. Table 1 lists standards, identified underivatized compounds, and unidentified related compounds. As noted above, GC-MS was used to identify compounds with percent match at  $>90\%$ .

### Identification of peaks

In our study, it was discovered that incomplete derivatization occurred even though suitable solvents were employed (Table 1). Shan et al. [14] suggested liner condition as a possible cause of incomplete derivatization [14]. Park and Addis noticed discrepancies between triol bis- and tris-tms ether spectra [15]. With the derivatization conditions employed in their previous work, Park and Addis determined that triol was derivatized to a bis-tms ether [15,16]. The derivatized triol tms ether peak observed in our study was a bis-tms ether also. However, the triol related peak remained an unknown. It did not appear to be a mono-tms ether or underivatized triol. The triol related peak eluted after triol bistms ether, so seemed to be more polar and less derivatized. When triol was derivatized with pyridine and Sylon BTZ at 70°C [16], the bis-tms ether peak decreased as the tris-tms ether peak increased.

For 19-OH, there were two peaks. The 19-OH tms ether peak was a bis-tms ether. The 19-OH related peak was not identified as underivatized 19-OH. It is possible that the 19-OH related peak was a mono-tms ether of 19-OH; however, this possibility requires confirmation.

### Storage and temperature effects

LS means for 7-keto compounds are shown in Figure 1a. The tms ether of 7-keto significantly increased ( $F = 118.67, p < 0.0001$ ) by day. Overall, underivatized 7-keto, significantly decreased ( $F = 144.54, p < 0.0001$  over time. When LS means for derivatized and underivatized 7-keto were analyzed together, there was a significant increase ( $F = 19.24, p = 0.0001$ ) over time. 7-Keto cholesta-3,5-dien-7-one (7-keto 3,5-7-one) was initially present as a contaminant of 7-keto on day 0 with no correlation with storage time ( $F = 0.44, p = 0.5144$ ). Because 7-keto 3,5-7-one did not increase over time and derivatized 7-keto was not lost over time, the former was not generated as an artifact of the latter. Temperature was significant ( $F = 10.45, p = 0.0006$ ) for 7-keto tms ether with LS means that increased as temperature increased (Table 2, Figure 2a). LS means also significantly increased with the interaction of days in storage and temperature ( $F = 4.39, p = 0.0028$ ). Unlike derivatized 7-keto, there was no significant temperature effect for underivatized 7-keto ( $F = 0.41, p = 0.6724$ ), for the combined compounds ( $F = 0.71,$

Stored Standard	Standard Peaks		Contaminant Peaks	
	Derivatized	Underivatized	Underivatized	Unknown
7-keto <sup>a</sup>	7-keto tms ether	7-keto	7-keto 3,5-7-one <sup>b</sup>	
19-OH <sup>c</sup>	19-OH tms ether			19-OH Related
Triol <sup>d</sup>	Triol tms ether			Triol Related

<sup>a</sup>7-ketocholesterol, <sup>b</sup>7-keto cholesta-3,5-dien-7-one, <sup>c</sup>19-hydroxycholesterol and <sup>d</sup>Cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol

**Table 1:** Peaks observed from the original standards after storage and derivatization.

$p = 0.4975$ ), or for 7-keto 3,5-7-one ( $F = 0.77$ ,  $p = 0.4741$ ) (Table 2).

As shown in Figure 2b, 19-OH tms ether significantly ( $F = 78.78$ ,  $p < 0.0001$ ) increased while the 19-OH related peak significantly ( $F = 43.34$ ,  $p < 0.0001$ ) decreased by day. 19-OH tms ether and its related compound were not significantly affected by storage temperature ( $F = 1.05$ ,  $p = 0.3681$  and  $F = 0.20$ ,  $p = 0.8176$ , respectively, Table 2).

The triol tms ether peak did not increase over time, nor did the triol related peak decrease over time (Figure 2c). For the triol tms ether, temperature was significant ( $F = 12.03$ ,  $p = 0.0005$ ), on day 3. This observation plus the unusually high standard deviations for days 0, 3 and 5 may be associated with day within liner effects as discussed below. No consistent trend for all days was observed as seen for the 7-keto tms ether (Table 2).

The lack of significance among LS means for temperature across underivatized 7-keto and the related compounds of 19-OH and the triol suggested that storage temperature had no effect on the stability

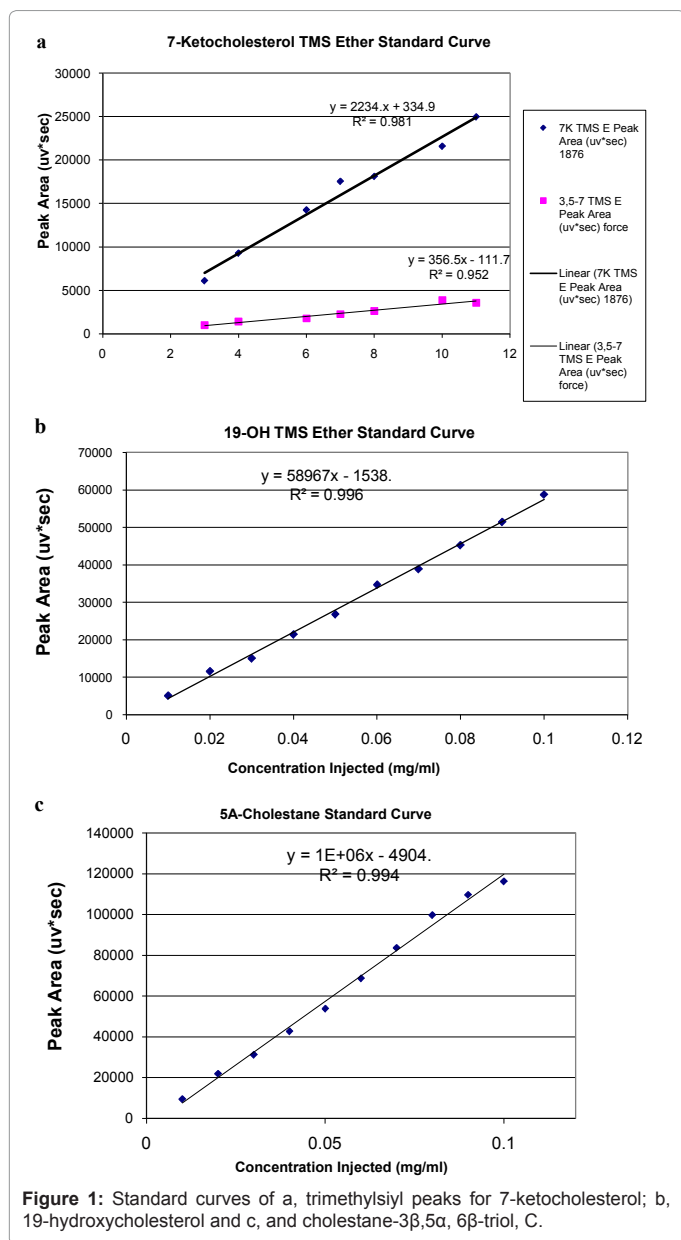


Figure 1: Standard curves of a, trimethylsilyl peaks for 7-ketocholesterol; b, 19-hydroxycholesterol and c, and cholestane-3 $\beta$ ,5 $\alpha$ , 6 $\beta$ -triol, C.

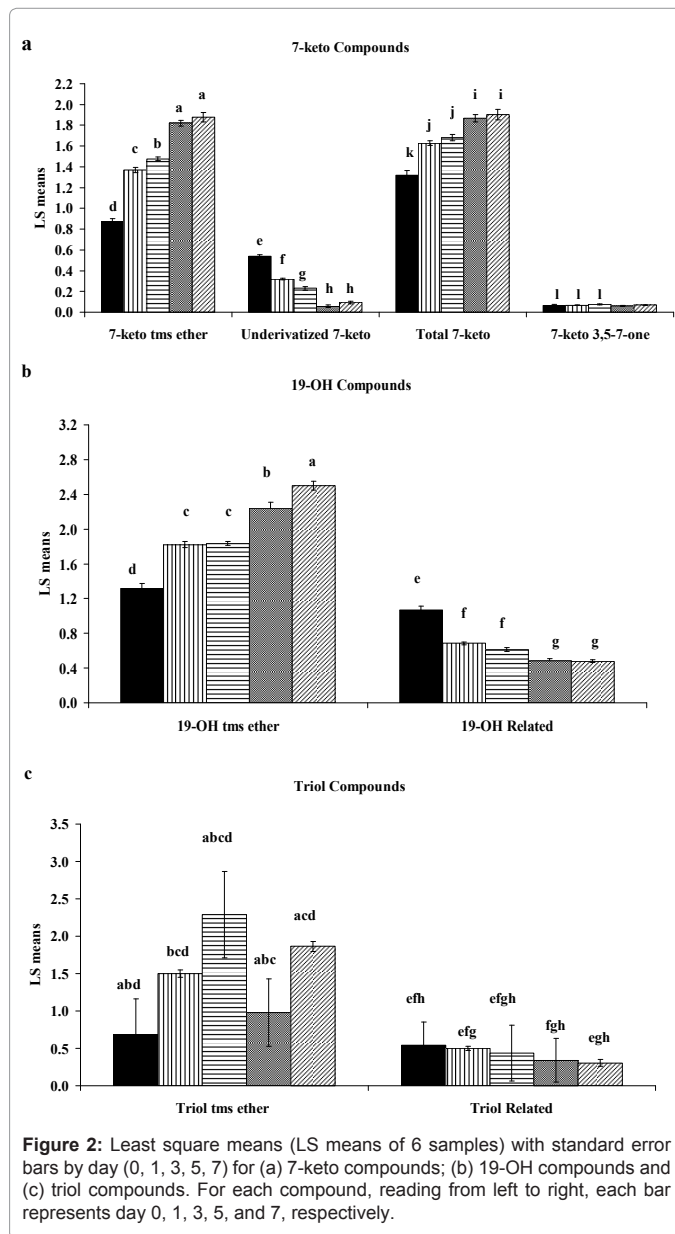


Figure 2: Least square means (LS means of 6 samples) with standard error bars by day (0, 1, 3, 5, 7) for (a) 7-keto compounds; (b) 19-OH compounds and (c) triol compounds. For each compound, reading from left to right, each bar represents day 0, 1, 3, 5, and 7, respectively.

of these compounds. No increase of original contaminants indicated stability of all initial parent compounds.

### Injection effects (liner and number of injections)

Because all standards were stored underivatized, similar temperature effects were expected for the tms ethers, underivatized compounds or related compounds. Samples were analyzed in the same order each day; so, time from derivatization to injection was similar. To further investigate the apparent changes due to temperature, data for changes in the liner over time and injection number were included in the general linear model and totals from derivatized and underivatized conditions were compared to LS means of each separate condition. Results suggested that the significance of LS means for derivatized compounds (tms ethers) may not reflect only storage temperature effects, but also interfering effects of injection number which was significant for 7-keto tms, underivatized 7-keto and the combined 7-keto compounds at  $F = 10.92$ ,  $p = 0.0032$ ;  $F = 10.42$ ,  $p = 0.0049$ ; and  $F = 6.07$ ,  $p = 0.0195$ , respectively. Injection number was not significant

COP Standard	Compound	Temperature	LS Mean <sup>a</sup>			
			Day 1	Day 3	Day 5	Day 7
7-keto <sup>a</sup>	7-keto tms ether	22+2° C	166.5	178.8	218.3	228.7
		4° C	157.6	169.8	206.6	213.0
	7-keto	-20° C	148.5	161.1	203.4	206.4
		-	59.3	42.7	10.4	17.6
	Total 7-keto	-	123.4	127.5	141.6	144.3
	7-keto 3,5-7-one <sup>b</sup>	-	99.4	112.2	90.6	101.2
19-OH <sup>c</sup>	19-OH tms ether	-	138.1	138.9	169.9	189.6
		-	63.8	57.3	45.1	44.7
Triol <sup>d</sup>	Triol tms ether	22+2° C	221.7	330.8	144.4	263.8
		4° C	217.1	334.6	143.6	277.2
		-20° C	218.8	337.1	141.1	275.6
	Triol related	-	92.0	80.0	62.7	56.3

<sup>a</sup>7-ketocholesterol, <sup>b</sup>7-keto cholesta-3,5-dien-7-one, <sup>c</sup>19-hydroxycholesterol (19-OH), and <sup>d</sup>Cholestane-3 $\beta$ -5 $\alpha$ ,6 $\beta$ -triol

**Table 2:** Stability of COPs over time and temperature: Percent least squares means (LS means of six samples) relative to Day 0 (with value of 100.0). For the for 7-keto tms ether only, temperature was significant ( $F = 10.45$ ,  $p = 0.0006$ ) with LS means that decreased as temperature decreased; a significant interaction ( $F = 4.39$ ,  $p = 0.0028$ ) between day and temperature was noted. For the triol tms ether, temperature ( $F = 12.03$ ,  $P = 0.0005$ ) was significant on day 3.

( $F = 3.27$ ,  $p = 0.0811$ ) for 3,5-7-one present in 7-keto samples. There was a significant injection number effect for 19-OH tms ( $F = 19.41$ ,  $p = 0.0002$ ) and its related peak ( $F = 4.44$ ,  $p = 0.0468$ ). Results for triol tms ether were significant for injection number ( $F = 8.12$ ,  $p = 0.0107$ ) and day the liner was changed ( $F = 19.29$ ,  $p < 0.0001$ ) but insignificant ( $F = 1.25$ ,  $p = 0.2776$ ) for liner. The triol related peak was not significantly affected by injection number ( $F = 3.51$ ,  $p = 0.0774$ ), liner ( $F = 0.70$ ,  $p = 0.4136$ ), or day the liner was changed ( $F = 0.47$ ,  $p = 0.6331$ ).

### Derivatization and injection liner conditions

Overall, tms ethers for triol, 7-keto and 19-OH experienced similar derivatization trends. As derivatized compounds increased over time, the corresponding underivatized species or unknown decreased over time. For 7-keto and 19-OH, (a) LS means for day 0 were significantly lower for derivatized compounds and significantly higher for underivatized compounds compared to all other days (Figure 1a, b and c) and injection number was significant for all derivatized, underivatized and related compounds except for the triol related peak. Generally, these findings indicated an association between confounding effects of liner changes/increased injection number and increased derivatized compound or decreased underivatized compound. Over time as injection number increased, more derivatized compound and less underivatized compound was detected in the present study. Previously in our work, derivatization under similar conditions resulted in single products (calculated from standard curves and identified by GC-MS), so it was unusual to see the prevalence of multiple derivatization products [17].

Incomplete derivatization may have several causes. Derivatization of COPs is commonly performed to increase volatility and thermal stability for improved GC analysis. For COPs, the most common derivatization reaction is silanization of hydroxyl groups to tms ethers. Keto groups are not silanized, although enolizations have been demonstrated depending on the presence and location of double bonds to keto groups. Also, epoxide groups are not silanized.

Variance in consistency of Syton BTZ between batches can result in incomplete silanization of triol and 25-hydroxycholesterol at room temperature for 30 minutes [15,17]. In our study, analyte and recovery internal standard concentrations were chosen in order to detect possible minute artifact quantities; this may have resulted in a sub-optimal ratio of silylating reagent to hydroxyl groups, resulting in incomplete derivatization [14]. Multiple GC peaks from derivatized

COPs have been reported, previously [14,15,17], but not from the compounds used in this study. One possibility to mitigate our observation of multiple peaks was to use strong derivatizing agents for more complete derivatization; however, they may decompose some labile COPs, although not generally including 7-keto [14].

As consistent trends of increased derivatized and decreased underivatized compounds over time were demonstrated, observed results cannot be attributed solely to the causes of incomplete derivatization delineated above. Possibly, changes in ratios of derivatized and underivatized compounds may have been caused by unidentified continual changes in the conditions of the injector liner. Other possible factors affecting our samples include injector bias or discrimination of polar underivatized compounds during sample transfer onto the column and further derivatization in the hot injector.

### Conclusion

Simultaneous lack of new peak generation or increase of an original contaminant indicated stability of all initial parent compounds. Caution should be taken when storing standards and biological samples because, although not often reported, they may be affected by storage time and temperature. Due to the extensive clean-up necessary when analyzing COPs in a small number of biological samples, investigators should be aware of possible incomplete derivatizations presumably caused by injection and liner interferences.

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