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Short communication

Enhancement of chemical derivatization of steroids by gas chromatography/mass spectrometry (GC/MS)

John A. Bowden[a], Dominic M. Colosi[a], Diana C. Mora-Montero[a], Timothy J. Garrett[b], Richard A. Yost[a,*]

[a] Department of Chemistry, University of Florida, PO Box 117200, Gainesville, FL 32611, United States
[b] Biomedical Mass Spectrometry Laboratory, Department of Medicine, University of Florida, PO Box 100322, Gainesville, FL 32610, United States

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ABSTRACT

Steroid derivatization was investigated by varying the experimental parameters (reagent, reaction time, and reaction temperature) to determine the optimal conditions for individual steroids, and for larger subsets. Three methods of derivatization enhancement were also investigated: the use of sonication, the use of a microwave heating, and the addition of solvents to the reaction mixture. On a comprehensive level, derivatization using *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was most efficient, while the application of solvent addition and microwave heating, in several cases, provided a clear enhancement. In addition, generalized rules for steroid derivatization are described.

1. Introduction

Gas chromatography/mass spectrometry (GC/MS) is well-suited for the identification of a large number of potential steroids and metabolites due to its high chromatographic resolution capacity and reproducible ionization efficiency [1–3]; however, its success is often dictated primarily by the efficiency of the derivatization procedures employed prior to injection of the sample. Derivatization alters functional groups in an effort to make the compound more amenable to standard GC/MS analysis, by increasing the volatility and/or thermal stability of a compound. Although derivatization can be time-consuming, it permits for the profiling of additional compounds by allowing both polar and non-polar steroids to be successfully separated. Although carbonyl groups typically present no analytical challenge to GC/MS analysis, hydroxyl groups require modification [4]. Since steroids contain various combinations of carbonyl and hydroxyl groups, the comprehensive analysis of varying steroids depends on the selective derivatization of hydroxyl groups. The application of selective derivatization allows for the minimization of artifacts and undesirable derivatization products [5].

The most commonly used derivatization methodology for steroids is silylation, where active hydrogens on hydroxyl groups are replaced with trimethylsilyl (TMS) groups [6]. The important conditions to be optimized for the silylation of steroids are the reaction time and temperature [7–9], in order to provide conditions that do not promote undesired derivatization [5], but are still capable of driving the reaction to the desired completion (typically 60–70 °C for 30–90 min) [7,9–15]. In addition, there have been several advances towards improving the derivatization process, including solvent enhancement [4,16–19], and alternative heating methods, such as microwave-accelerated derivatization (MAD) [20–24]. MAD generates derivatization conditions comparable to traditional methods in shorter time periods. The use of sonication-assisted derivatization (SAD) has also been discussed [3] as a method to improve derivatization efficiency; however, there are no studies that explicitly investigate its potential. It is clear that for any effective derivatization, especially for comprehensive analyses, enhancing techniques should be considered and optimized.

Current research in steroid analysis by GC/MS focuses on multi-analyte detection, targeting either small groups of related steroids or steroids within specific classes [7,8,16,20,21,25–30]. However, the lack of established protocols or standard methods for reliable steroid derivatization is often a deterrent to steroid analysis by GC/MS [31]. To our knowledge, only a few research studies have investigated the optimization of the derivatization conditions [7–9,16,20,27]; however, these examinations were restricted to investigating only a small set of steroids rather than investigating on a comprehensive level.

* Corresponding author. Tel.: +1 352 392 0557; fax: +1 352 392 4651.
E-mail address: ryost@chem.ufl.edu (R.A. Yost).

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In this study, derivatization of a large suite of diverse steroids was systematically optimized using GC/MS by performing a detailed investigation of three silylating reagents over a series of reaction times and temperatures. The role of derivatization enhancers was also examined, specifically the enhancing effects offered by the use of solvents and non-traditional heating methods, such as MAD and SAD. The procedures examined not only describe the ideal derivatization strategies for investigating steroids comprehensively, but also give insight into the ideal reaction conditions required for steroids on an individual level. Several guidelines for efficient steroid derivatization are also discussed.

2. Experimental

2.1. Chemicals, reagents, and solutions

The natural and synthetic steroids testosterone (T), 17β-estradiol (E_2), estrone (E_1), androstenedione (AE), ethinylestradiol (EE_2), 17-methyltestosterone (17-MT), progesterone (P), pregnenolone (PREG), cholesterol (CHOL), corticosterone (CORT), and dihydrotestosterone (DHT), and the non-steroidal synthetic estrogen, diethylstilbestrol (DES) were acquired from Sigma (St. Louis, MO). The surrogate used for the evaluation of the derivatization reactions was dichlorodiphenyldichloroethylene-p,p (DDE, EPA Research Triangle Park, NC). All chemicals were of a purity grade higher than 98%, except for DHT (97.5%) and 17-MT (97%). Stock solutions of each steroid and the steroid mixture were made to 100 μg mL^{-1} in analytical grade methanol (Fisher Scientific, Fair Lawn, NJ) and stored at −20 °C.

The reagents used were derivatization grade *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (BSTFA/TMCS, Supelco, Bellefonte, PA), *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) (Pierce, Rockford, IL), and *N*,*O*-bis-(trimethylsilyl)-acetamide (BSA) (Pierce). The solvents used in the reactions were anhydrous dimethylformamide (DMF, Sigma), anhydrous acetonitrile (ACN, Sigma), and extra-dry pyridine (PYR, Acros Organics, Morris Plains, NJ).

2.2. GC/MS analysis

The GC/MS analysis was performed using a Trace GC 2000 gas chromatograph/quadrupole ion trap mass spectrometer with an AS3000 autosampler (Thermo Finnigan, San Jose, CA). The column used was a Zebron ZB-5 30 m × 0.25 mm capillary column with a film thickness of 0.25 μm (Phenomenex, Torrance, CA). The ion source and transfer line temperatures were set to 200 and 300 °C, respectively. The injector was set to splitless injection at a temperature of 280 °C with a split flow of 50 mL min^{-1}. The temperature program was set to begin at 120 °C for 2 min, elevated at 15 °C min^{-1} to 250 °C, and finally increased by 5 °C min^{-1} to 300 °C (maintained for 5 min). The carrier gas used was ultra-high-purity helium (99.999%) at a flow rate of 1 mL min^{-1}. The data acquisition software used was Xcalibur 1.4. Electron ionization was used at 70 eV and the mass spectrometer was set to full scan, *m*/*z* 50–600.

2.3. Derivatization experiments

2.3.1. Derivatization overview

Each sample contained the 12 selected steroids and the surrogate and was evaporated to dryness using ultra-high-purity nitrogen to represent sample conditions that normally exist following solid-phase extraction. The residue was reconstituted with a volume of 200 μL of derivatizing reagent (with surrogate to a final concentration of 5 μg mL^{-1}) and vortexed. The block and water bath reactions took place in a Thermolyne type 16500 Dri-Bath and a Fisher Scientific Isotemp Immersion Circulator (Model 70, Pittsburgh, PA), respectively.

The characteristic ions selected for peak area identification, shown in Table 1, consisted of the base peak and the molecular ion for each steroid and the surrogate. The relative response factor (RRF) for each of the products formed was calculated by dividing the peak area of the steroid product by that of the peak area of the surrogate (derivatization inactive).

2.3.2. Derivatization: time and temperature optimization

Each derivatization parameter was examined by running triplicate samples (200 μL each) and pooling them post-heating into a single vial (600 μL total). The pooled sample was then analyzed in triplicate using GC/MS. Samples were pooled to evaluate the enhancement effects on the reactions (an average of three separate reactions) while limiting the number of samples injected. Optimization of the derivatization procedure for the steroid standard mix consisted of examining reaction times and temperatures using three silyl reagents: BSTFA/TMCS, MSTFA, and BSA. The derivatization times and temperatures investigated were 15, 30, 45, and 60 min, and 40, 55, 70, and 90 °C. The optimal time and temperature combination for each reagent was then used for the subsequent enhancement comparisons.

2.3.3. Derivatization enhancing experiments

Solvent enhancement was examined by adding solvent and derivatizing reagent (1:1) to a total volume of 200 μL for derivatization.

MAD was evaluated by comparing the RRF values obtained to those obtained using traditional thermal (block) heating. The MAD was performed using a 1000-W half-time convection/microwave

Table 1
Targeted derivatives and their characteristic ions and retention times.

Steroid	Abbreviation	Functional groups	Molecular weight	Target derivative	Characteristic ions	Retention time (min)
Surrogate	DDE	None	318	Underivatized	246, *318*	10.93
Diethylstilbestrol	DES	2C–OH	268	Di-TMS	*412*, 217	12.12
Dihydrotestosterone	DHT	C=O, C–OH	290	Mono-TMS	*362*, 347	14.36
Estrone	E_1	C=O, C–OH	270	Mono-TMS	*342*, 257	14.49
Androstenedione	AE	2C=O	286	Underivatized	148, *286*	14.68
17β-Estradiol	E_2	2C–OH	272	Di-TMS	*416*, 285	14.90
Testosterone	T	C=O, C–OH	288	Mono-TMS	*360*, 226	15.02
17-Methyltestosterone	17-MT	C=O, C–OH	302	Underivatized	229, *302*	15.06
Pregnenolone	PREG	C=O, C–OH	316	Mono-TMS	298, *388*	15.63
Ethinylestradiol	EE_2	2C–OH	296	Di-TMS	425, *440*	16.02
Progesterone	P	2C=O	314	Underivatized	124, *314*	16.42
Cholesterol	CHOL	C–OH	386	Mono-TMS	*458*, 368	19.46
Corticosterone	CORT	2C=O, 2C–OH	346	Di-TMS	*490*, 475	21.57

Italics indicate molecular ion (*m*/*z*), while the other ion listed is the base peak. The surrogate chosen was DDE due to its derivatization inactivity.

Table 2
RRF values obtained using various time, temperature, and derivatizing reagent combinations.

	MSTFA			BSTFA + 1% TMCS					BSA
	55 °C			55 °C		70 °C			90 °C
	15 min	30 min	45 min	15 min	30 min	15 min	30 min	60 min	30 min
Underivatized-AE	0.12 ± 0.09	0.22 ± 0.01	0.16 ± 0.02	0.26 ± 0.04	0.26 ± 0.05	0.25 ± 0.06	0.24 ± 0.04	0.16 ± 0.02	0.05 ± 0.01
Underivatized-17-MT	0.08 ± 0.02	0.16 ± 0.02	0.21 ± 0.02	0.27 ± 0.02	0.29 ± 0.08	0.26 ± 0.05	0.27 ± 0.04	0.14 ± 0.02	0.05 ± 0.00
Underivatized-PROG	0.13 ± 0.02	0.18 ± 0.03	0.15 ± 0.03	0.25 ± 0.04	0.25 ± 0.06	0.24 ± 0.07	0.24 ± 0.05	0.12 ± 0.02	0.03 ± 0.00
Mono-TMS-CHOL	0.50 ± 0.03	0.49 ± 0.07	0.50 ± 0.11	0.53 ± 0.16	0.53 ± 0.15	0.57 ± 0.26	0.48 ± 0.12	0.52 ± 0.04	0.27 ± 0.01
Mono-TMS-DHT	0.15 ± 0.02	0.16 ± 0.02	0.13 ± 0.02	0.13 ± 0.03	0.13 ± 0.03	0.13 ± 0.03	0.13 ± 0.04	0.13 ± 0.03	0.06 ± 0.01
Mono-TMS-E_1	2.24 ± 0.11	2.59 ± 0.18	2.20 ± 0.38	2.60 ± 0.52	2.40 ± 0.61	2.85 ± 0.98	2.88 ± 0.76	2.68 ± 0.04	1.30 ± 0.14
Mono-TMS-PREG	0.43 ± 0.04	0.49 ± 0.02	0.44 ± 0.08	0.53 ± 0.12	0.56 ± 0.20	0.57 ± 0.23	0.53 ± 0.13	0.55 ± 0.10	0.18 ± 0.01
Mono-TMS-T	0.40 ± 0.05	0.46 ± 0.04	0.44 ± 0.08	0.34 ± 0.03	0.32 ± 0.10	0.31 ± 0.10	0.31 ± 0.07	0.18 ± 0.02	0.14 ± 0.00
Di-TMS-CORT	ND	ND	0.06 ± 0.01	0.06 ± 0.03	0.03 ± 0.01	0.03 ± 0.02	0.04 ± 0.02	0.09 ± 0.03	0.05 ± 0.00
Di-TMS-DES	2.04 ± 0.30	2.39 ± 0.14	2.43 ± 0.30	1.89 ± 0.12	1.78 ± 0.51	1.68 ± 0.56	1.91 ± 0.35	2.73 ± 0.57	3.08 ± 0.64
Di-TMS-E_2	2.18 ± 0.36	2.13 ± 0.11	1.88 ± 0.24	1.65 ± 0.45	1.57 ± 0.61	1.57 ± 0.66	1.84 ± 0.49	1.56 ± 0.35	1.22 ± 0.10
Di-TMS-EE_2	0.67 ± 0.10	0.68 ± 0.04	0.69 ± 0.14	0.07 ± 0.02	0.06 ± 0.01	0.08 ± 0.04	0.12 ± 0.03	0.09 ± 0.03	0.20 ± 0.06

ND, not detected. The derivatization of di-TMS-DES resulted in two chromatographic peaks; the resulting areas for both peaks were summed. The standard deviation of the mean is also shown; values of 0.00 indicate a standard deviation less than 0.005.

oven (Apollo Worldwide, Palm Beach, FL). Initial experiments (data not shown) investigated several microwave powers (500–1000 W) and irradiation times (0.5–2 min). In terms of derivatization effectiveness on a comprehensive level, the ideal conditions were as follows (for each reagent): 900 W for 0.5 and 1 min (BSTFA/TMCS and MSTFA) and 900 and 1000 W for 1 min (BSA). Thus, these were the microwave settings investigated in this study.

SAD was examined by comparing the best overall method for each reagent using the block heater to the RRFs obtained using a heated sonicated water bath. The derivatization reactions in the water bath were run both with and without sonication to isolate and evaluate the potential improvements achieved using sonication. The only deviation was that the water bath temperature for the BSA reaction was at 80 °C instead of 90 °C, due to the temperature constraints of the water bath heater.

3. Results and discussion

3.1. Derivatization optimization

3.1.1. Importance of derivatization time and temperature

The steroid mixture was selected to evaluate the effects of the possible combinations of functional groups (carbonyl and hydroxyl) at various locations on the steroid structure. Even though the steroids analyzed were all present at the same concentration, they exhibited a wide range of RRFs. Due to keto–enol tautomerism, derivatization efficiency, and the tendency of carbonyl groups to derivatize under harsh conditions, a balance in reaction time and temperature was required. The selection of target derivatives was based on the optimal product (underivatized or derivatized) for each steroid that would be the best compromise for allowing comprehensive profiling for the wide polarity range of steroids examined. Two steroids, PROG and AE, contain only carbonyl groups and did not require derivatization, while the predominant product observed for 17-MT was also underivatized due to the steric hindrance of the hydroxyl group at the 17-position. The remaining hydroxylated steroids were sufficiently derivatized (except di-TMS-CORT). The derivatization of di-TMS-EE_2 was difficult to predict, most likely due to the triple bond near the hydroxyl group. Shareef et al. concluded that this may be due to a breakdown of the EE_2-derivative [16]; however, this may also be due to more favorable conditions for the production of mono-TMS-EE_2. Di-TMS-DES had two GC peaks (two isomers); the two peak areas were summed prior to RRF determination.

In general, derivatization conditions beyond 60 min and above 70 °C (90 °C for BSA) did not provide any added benefit in the RRFs obtained. In addition, temperatures of 40 °C or below were not adequate for efficient derivatization at the reaction times tested in this study. The optimal conditions (reagent, reaction time, and temperature) for obtaining the greatest RRF value for each individual steroid are displayed in Table 2.

Steroid derivatization using BSTFA/TMCS was most successful at producing the highest RRF values in the range of 55–70 °C for 15–30 min. As shown in Table 2, 7 of the 12 steroids analyzed displayed the highest RRF using BSTFA as the reagent (including the three underivatized steroids, PROG, AE, and 17-MT). This is a key result in the effort to reduce sample complexity by avoiding the derivatization of compounds which are amenable to GC/MS. However, most reactions using BSTFA/TMCS exhibited the highest level of irreproducibility. It should be noted that conditions beyond 30 min using BSTFA/TMCS for the three underivatized compounds (PROG, AE, and 17-MT) exhibited a reduced RRF value, likely due to the formation of artifacts (data not shown). The optimal conditions using BSTFA/TMCS for profiling the multi-class mixture was determined to be 70 °C for 30 min.

For the derivatization of the steroid mixture using MSTFA, temperatures higher than 55 °C and reaction times longer than 30 min exhibited no increase in RRF values. As shown in Table 2, 4 of the 12 targeted derivatives had the highest RRF values using the MSTFA reagent: di-TMS-EE_2, mono-TMS-E_2, mono-TMS-T, and mono-TMS-DHT. However, the RRF values for the underivatized steroids PROG, AE, and 17-MT, were typically lower than the levels achieved using BSTFA/TMCS. It should be noted that although derivatization with BSTFA/TMCS provided greater RRF values, derivatization with MSTFA often yielded comparable RRF values with a dramatic increase in reproducibility. The best overall reaction condition for using MSTFA reagent was 55 °C for 30 min.

The RRF values obtained with the BSA reagent were in almost all cases lower compared to those obtained with the other two silyl reagents, particularly for the underivatized steroids. As shown in Table 2, RRF values generated using BSA were lower (some by a factor of 10) than the RRF values achieved using the other reagents. Di-TMS-DES, the most easily derivatized species of the study, yielded a 2× higher RRF with BSA. The best reaction condition for the multi-class steroid analysis using BSA was 90 °C for 30 min.

3.1.2. Derivatization reagent

For the derivatization of steroids with two or more hydroxylated groups, MSTFA is the best reagent choice. BSTFA is the more appropriate reagent choice for the derivatization of mono-hydroxylated compounds or with samples containing steroids with only carbonyl

Table 3
The RRF changes due to various derivatization enhancements using solvent addition (1:1, solvent to reagent).

	BSTFA + 1% TMCS			MSTFA			BSA		
	DMF	Pyridine	Acetonitrile	DMF	Pyridine	Acetonitrile	DMF	Pyridine	Acetonitrile
Underivatized-AE	0.8 ± 0.2	1.3 ± 0.2	1.6 ± 0.2	0.7 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	1.5 ± 0.0	1.4 ± 0.1	1.3 ± 0.0
Underivatized-17-MT	0.3 ± 0.0	0.5 ± 0.0	1.0 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	1.7 ± 0.1	1.3 ± 0.3	1.3 ± 0.1
Underivatized-PROG	1.1 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	0.7 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	1.6 ± 0.0	1.5 ± 0.1	1.2 ± 0.1
Mono-TMS-CHOL	1.5 ± 0.2	1.4 ± 0.0	1.3 ± 0.1	1.1 ± 0.0	0.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	1.1 ± 0.0	0.9 ± 0.0
Mono-TMS-DHT	1.8 ± 0.1	2.0 ± 0.0	2.3 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	1.6 ± 0.2	1.9 ± 0.1	1.0 ± 0.1
Mono-TMS-E_1	0.9 ± 0.1	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.3 ± 0.0	1.0 ± 0.1
Mono-TMS-PREG	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.1	1.0 ± 0.0
Mono-TMS-T	1.7 ± 0.1	1.8 ± 0.0	1.9 ± 0.1	0.8 ± 0.0	0.7 ± 0.0	1.0 ± 0.0	1.3 ± 0.1	1.4 ± 0.2	1.1 ± 0.1
Di-TMS-DES	1.3 ± 0.0	1.0 ± 0.0	1.2 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1
Di-TMS-E_2	1.6 ± 0.0	1.5 ± 0.2	1.5 ± 0.2	0.9 ± 0.0	0.9 ± 0.1	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
Di-TMS-EE_2	12.0 ± 1.4	9.1 ± 1.6	2.8 ± 1.5	1.3 ± 0.2	1.1 ± 0.1	1.2 ± 0.2	0.9 ± 0.1	0.8 ± 0.0	0.7 ± 0.0

Values are averages of three injections, normalized to the block heating method without enhancement. Values greater than 1 indicate an increase in RRF over the block method (without enhancement); values less than 1 indicate a decrease in RRF. The averages are shown ± the standard deviation of the mean (also normalized). Error of 0.0 indicates an error below the significant figures shown. Di-TMS-CORT was not effectively derivatized with any of the enhancing experiments and was not included in the tables. The derivatization of di-TMS-DES resulted in two chromatographic peaks; the resulting areas for both peaks were summed.

functional groups. Both MSTFA and BSTFA were comparable in their ability to derivatize the multi-class steroid mixture; however, the optimal reaction condition for MSTFA (at 55 °C for 30 min) offers the best compromise, due to its ability to derivatize a wide variety of steroids with acceptable reproducibility.

3.2. Derivatization enhancers

3.2.1. Use of solvents

The RRF values for the steroid products obtained by adding a solvent (1:1 to each silyl reagent) during the derivatization reaction were evaluated against the RRF values generated for derivatization reactions without solvent. Table 3 presents the change in RRF for enhanced derivatization compared to those obtained with the tradition block derivatization method.

BSTFA/TMCS, with the solvents DMF, ACN, and PYR, generally showed an increase in RRF values over those generated without the aid of solvents (Table 3). The underivatized steroids, AE and PROG, had higher RRF values with the addition of solvent (except for AE with DMF). However, underivatized 17-MT exhibited low RRF values (using DMF and PYR), implying that its derivatization was promoted. ACN was the most effective solvent in preventing the derivatization of carbonyl steroids, and thus had the largest RRF values for these compounds. The RRF values for di-TMS-EE_2 exhibited dramatic increases with the addition of DMF and PYR. It has been suggested that those solvents prevent the breakdown of di-TMS-EE_2 [9,16,32].

MSTFA reactions employing solvents (Table 3) resulted in essentially the opposite effect in RRF value change when compared to BSTFA/TMCS. A comparison of the RRF values showed no benefit when compared to the conventional solvent-less block heating method. No solvent enhancement was observed with MSTFA. Solvent addition during reactions using MSTFA actually lowers the RRF values in comparison to the solvent-less method.

The RRF values obtained using BSA with solvents generally exhibited either an increase or no change (Table 3). Pyridine provided slightly better RRFs for the derivatization of mono-TMS steroids, while DMF was the best at protecting the underivatized steroids.

Overall, the addition of solvent during the derivatization process was shown to be beneficial in creating a more effective reaction for both BSA and BSTFA/TMCS. For BSTFA/TMCS, the use of PYR, ACN, and to a lesser extent DMF, generated higher RRF values indicating an improved derivatization reaction. In addition to increased RRF values, solvent usage during the derivatization reaction reduced the amount of reagent needed, thus reducing the cost per sample.

3.2.2. Microwave-accelerated derivatization (MAD)

The RRF values for all three reagents generated in the microwave were compared to the RRF values obtained using the traditional block heater in Table 4. The microwave experiment was designed solely to analyze the change in RRF values and not to compare RRF values at similar reaction temperatures (between microwave and block heater).

Table 4
Comparison of the RRF changes due to various derivitization enhancements with microwave heating.

	BSTFA + % TMCS		MSTFA		BSA	
	Microwave 30 s	Microwave 1 min	Microwave 30 s	Microwave 1 min	Microwave 1 min	Microwave 1 min[a]
Underivatized-AE	1.6 ± 0.0	2.3 ± 0.0	1.1 ± 0.0	1.5 ± 0.1	1.5 ± 0.3	1.6 ± 0.2
Underivatized-17-MT	1.6 ± 0.0	2.3 ± 0.1	1.1 ± 0.0	1.8 ± 0.6	1.5 ± 0.1	1.6 ± 0.1
Underivatized-PROG	1.4 ± 0.1	2.2 ± 0.0	1.0 ± 0.1	1.5 ± 0.0	1.5 ± 0.2	1.5 ± 0.2
Mono-TMS-CHOL	0.7 ± 0.0	1.8 ± 0.2	1.2 ± 0.1	1.4 ± 0.1	1.1 ± 0.0	1.1 ± 0.0
Mono-TMS-DHT	1.0 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.5 ± 0.1	1.6 ± 0.2
Mono-TMS-E_1	0.8 ± 0.0	1.4 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.2 ± 0.1	1.3 ± 0.1
Mono-TMS-PREG	0.8 ± 0.0	1.6 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.0	1.2 ± 0.0
Mono-TMS-T	4.5 ± 0.0	2.8 ± 0.3	1.0 ± 0.0	1.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Di-TMS-DES	1.0 ± 0.2	0.7 ± 0.0	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.0
Di-TMS-E_2	0.7 ± 0.1	1.4 ± 0.1	0.7 ± 0.0	1.1 ± 0.1	1.2 ± 0.0	1.3 ± 0.1
Di-TMS-EE_2	0.7 ± 0.1	2.0 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	1.6 ± 0.1	1.4 ± 0.0

Values are averages of three injections, normalized to the block heating method without enhancement.

[a] Microwave at 1000 W, rather than 900 W, to compensate for the lower reactivity of BSA. Values greater than 1 indicate an increase in RRF over the block method (without enhancement); values less than 1 indicate a decrease in RRF. The averages are shown ± the standard deviation of the mean (also normalized). Error of 0.0 indicates an error below the significant figures shown. Di-TMS-CORT was not effectively derivatized with any of the enhancing experiments and was not included in the tables. The derivatization of di-TMS-DES resulted in two chromatographic peaks; the resulting areas for both peaks were summed.

Table 5
Comparison of the RRF changes due to the application of sonication and water bath heating.

	BSTFA + 1%TMCS		MSTFA		BSA	
	Water bath	Sonication	Water bath	Sonication	Water bath	Sonication
Underivatized-AE	1.1 ± 0.1	1.0 ± 0.0	1.2 ± 0.1	1.0 ± 0.0	1.2 ± 0.0	0.9 ± 0.1
Underivatized-17-MT	1.1 ± 0.1	0.9 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	1.3 ± 0.0	1.0 ± 0.1
Underivatized-PROG	1.2 ± 0.0	1.1 ± 0.0	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.0	1.2 ± 0.1
Mono-TMS-CHOL	0.9 ± 0.1	0.9 ± 0.1	1.5 ± 0.1	1.4 ± 0.0	1.3 ± 0.1	1.2 ± 0.1
Mono-TMS-DHT	1.0 ± 0.0	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	1.3 ± 0.2	0.2 ± 0.0
Mono-TMS-E_1	0.9 ± 0.1	0.9 ± 0.0	1.6 ± 0.0	1.5 ± 0.1	1.1 ± 0.0	1.0 ± 0.0
Mono-TMS-PREG	1.0 ± 0.0	1.0 ± 0.1	1.3 ± 0.1	1.2 ± 0.0	1.1 ± 0.0	1.2 ± 0.1
Mono-TMS-T	1.2 ± 0.1	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.1	1.2 ± 0.0	1.1 ± 0.1
Di-TMS-DES	0.9 ± 0.0	0.9 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	0.9 ± 0.0
Di-TMS-E_2	1.0 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
Di-TMS-EE_2	1.1 ± 0.0	1.3 ± 0.0	1.7 ± 0.0	1.6 ± 0.1	1.1 ± 0.3	0.7 ± 0.1

Values are averages of three injections, normalized to the block heating method without enhancement. Values greater than 1 indicate an increase in RRF over the block method (without enhancement); values less than 1 indicate a decrease in RRF. The averages are shown ± the standard deviation of the mean (also normalized). Error of 0.0 indicates an error below the significant figures shown. Di-TMS-CORT was not effectively derivatized with any of the enhancing experiments and was not included in the tables. The derivatization of di-TMS-DES resulted in two chromatographic peaks; the resulting areas for both peaks were summed.

MAD with BSTFA/TMCS, MSTFA, and BSA at 900 W for at least 1 min provided higher RRF values than those generated using the traditional block derivatization for all 11 steroids, except for di-TMS-EE_2 with BSA. However, the MAD reaction at 900 W for 30 s was not as effective as the 1 min microwave reaction for both MSTFA and BSTFA/TMCS (except for mono-TMS-T with BSTFA/TMCS). The underivatized steroids had higher RRF values with use of microwave heating, highlighting the unique potential of microwave heating to provide strong enough heating conditions for the effective derivatization of steroids, yet still providing an environment which does not force the derivatization of carbonyl groups. The application of lower wattages was tried with the domestic microwave oven; however, the RRF values were well below the RRF values obtained using the thermal heating block (even when using long irradiation times, data not shown).

Silyl reagents, combined with microwave heating, can be a successful technique for the rapid derivatization of a variety of different steroids. Furthermore, as shown here, the application of MAD often provided higher RRF values when compared to traditional heating methods with a drastic reduction in derivatization time (1 min compared to 30 min).

3.2.3. Sonication-assisted derivatization (SAD)

The concept of SAD is based on the promoted agitation of compounds in a heated solution, thus potentially increasing the frequency of interaction between the steroids and the derivatization reagent. Sonication enhancement was examined by comparing the RRF values generated in a sonicated water bath to those obtained using (1) the same time and temperature on a block heater and (2) the water bath without sonication.

The RRF values employing SAD for all three silylating reagents exhibited an increase when compared to those generated using a traditional block heater (Tables 3–5). However, SAD yielded very similar RRF values to those obtained for derivatization in a water bath without sonication, indicating little or no benefit of sonication for the derivatization of steroids. The data do, however, highlight the potential of water bath heating for more efficient heating medium.

3.3. Derivatization guidelines

Most publications on steroid research employing GC/MS omit any discussion pertaining to the determination of the derivatization reaction settings that are employed and often the reaction conditions are not included in enough detail to elucidate whether optimal conditions were employed for all reactants. The objective of this study was to construct general guidelines for the diagnostic prediction of future steroid derivatizations (with or without enhancement). Several generalizations outlined in this study are presented:

- *Comprehensive derivatization*: Although reactions employing BSTFA/TMCS often result in the highest RRF, reactions employing MSTFA generally produced comparable RRF values with better reproducibility.
- *Carbonyl protection*: The best reagent for selectively avoiding the derivatization of carbonyl functional groups was BSTFA/TMCS. The application of enhancers, specifically microwave heating and solvent addition, also helped avoid carbonyl derivatization.
- *Reaction time and temperature*: For both BSTFA/TMCS and MSTFA, derivatization conditions beyond 60 min and above 70 °C did not provide any added advantage in the RRF values obtained. In addition, temperatures of 40 °C or below were not adequate for efficient derivatization for any of the reagents analyzed at the reaction times tested in this study. For comprehensive derivatization, the ideal conditions determined for each reagent were 70, 55, and 90 °C (for 30 min) using BSTFA/TMCS, MSTFA, and BSA, respectively.
- *Solvent addition*: In general, reactions employing solvents (1:1) exhibited an overall improvement in RRF values when using BSTFA/TMCS and BSA (with a few minor exceptions). One side effect of solvent enhancement is its ability to force a derivatization forward; thus, careful consideration needs to be applied in regards to which steroid derivatization product is being monitored. Solvent addition (DMF, PYR, ACN) is detrimental to MSTFA reactions.
- *Microwave heating*: Reactions performed using a microwave were generally enhanced for all the reagents examined in this study. Microwave reactions were best using 900 W for 1 min, when compared to traditional thermal heating methods. Careful consideration needs to be placed to ensure the sample does not evaporate under intense heating in the microwave.

4. Conclusion

This investigation was focused on the optimization and enhancement of methodologies for the derivatization of a wide polarity range of steroids in a single chromatographic analysis. The application of solvent and the use of microwave heating were found, in most instances, to be more efficient than the optimized traditional heating methods. The use of solvent was found to be most effective with BSTFA/TMCS, resulting in a general increase in RRF values with a reduced amount of reagent needed per sample. Microwave-accelerated derivatization at 900 W for 1 min provided

an increase in RRF values for all the steroids examined in the study and reduced the derivatization time (1–30 min). Sonication-assisted derivatization did not enhance the derivatization of the steroids, but did highlight the potential of water bath heating for derivatization. The results have defined more accurately the optimal derivatization conditions needed for derivatization on three levels: (1) for each individual steroid, (2) for groups of related steroids, and (3) for the comprehensive profiling of a suite of unrelated steroids. Future investigations will include a rigorous examination of the combinatorial effects of the derivatization enhancing techniques and its application to biological samples.

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