Journal of Chromatography A, 1218 (2011) 1878–1890

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Derivatization and fragmentation pattern analysis of natural and synthetic steroids, as their trimethylsilyl (oxime) ether derivatives by gas chromatography mass spectrometry: Analysis of dissolved steroids in wastewater samples

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ARTICLE INFO

Article history:
Received 4 November 2010
Received in revised form 13 January 2011
Accepted 17 January 2011
Available online 2 February 2011

Keywords:
Steroids
Gas chromatography–mass spectrometry
Oxiamation
Trimethylsilylation
Fragmentation patterns
Aquatic environmental pollutant

ABSTRACT

This paper reports the extension of our multiresidue analysis (MA) procedure with 18 natural and synthetic steroids; permitting the identification and quantification, in total of 81 pollutants from one solution, by a single injection, as their trimethylsilyl (TMS)-oxime ether/ester derivatives, by gas chromatography–mass spectrometry (GC–MS), within 31 min. As a novelty to the field, basic researches, such as fragmentation pattern analysis and derivatization optimization studies were performed for androsterone, transdehydroandrosterone, transandrosterone, mestranol, dihydrotestosterone, ethinylestradiol, testosterone, norethisterone, estriol, 4-androstene-3,17-dione, gestodene, levonorgestrel, etonogestrel, coprostanol, progesterone, cholesterol, medroxy-progesterone-acetate, stigmasterol and β-sitosterol. Results confirmed that (i) the TMS oxime-ether derivatives of the keto steroids provide from 1.40 times (gestodene) up to 4.25 times (norethisterone) higher responses compared to their TMS-ether ones, and (ii) the distribution of syn/anti oximes is characteristic to the ketosteroid species examined. Based on our optimized mass fragmentation, solid phase extraction (SPE) and derivatization studies separations have been performed in the total ion current (TIC) mode, identification and quantification of compounds have been carried out on the basis of their selective fragment ions. Responses, obtained with derivatized standards proved to be linear (hydroxysteroids), or have been calculated from calibration curves (ketosteroids) in the range of 1.88–750 ng/L levels. Limit of quantitation (LOQ) values varied between 1.88 ng/L and 37.5 ng/L concentrations. The most important practical messages of this work are the high androsterone (0.744–4.28 μg/L), transandrosterone (0.138–4.00 μg/L), coprostanol (2.11–302 μg/L), cholesterol (0.308–41 μg/L), stigmasterol (1.21–8.40 μg/L) and β-sitosterol (1.12–11.0 μg/L) contents of influent wastewaters. β-Estradiol (100 ng/L) and estriol (54 ng/L) were found in one influent sample, only. Reproducibilities, characterized with the relative standard deviation percentages (RSD%) of measurements, varied between 1.73 RSD% (β-estradiol) and 5.4 RSD% (stigmasterol), with an average of 4.82 RSD%.

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1. Introduction

Gas chromatography mass spectrometry (GC–MS) of steroids is still a challenge for analytical chemists. Publications selected for the literature overview (except one [1]), appeared in the last decade [2–77].

The relevancy of the topic can be characterized by the fact that steroid profiling proved to be of primary importance in the diagnosis of clinical disorders [4,11,13–22,26,29,50,53,54,64], in the recognition of drug abuses in sports doping control [8,12], in food analysis [2,3,45] and most importantly in the pollutant analysis of environmental water samples [1,5–7,10,23–25,27,28,30,32–44,46–48,52,53,61–63,65–68]: in this last context case studies confirm the unambiguous harm of steroids impairing wildlife [57–60].

As to the review papers [61–64] – comparing the advantages and disadvantages of the relevant GC–MS/(MS) and LC–MS/(MS) steroid analysis protocols – it seems to be clear that GC–MS/(MS) is at least comparable [61–63], however out and away the method of choice [64]. In agreement with the conviction of the present papers' authors [65–68], GC–MS has been characterized very recently as "... a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry..." [64].

The literature overview of steroids' derivatization techniques reveals that in the overwhelming part of proposals the use of various silylating reagents has been preferred, like *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) [1–21], bis-

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doi:10.1016/j.chroma.2011.01.051

(trimethylsilyl)trifluoro-acetamide (BSTFA) [22–45], *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) [46,47], and trimethylsilylimidazole(s) (TMSI) [48–50]. Acylations were performed with pentafluorobenzoyl chloride [51–53] or with heptafluorobutyric anhydride [54,55]. Subsequently to enzymatic oxidation steroids were determined also as hydrazones [56].

In order to define methodological pitfalls selected analytical techniques have been compared, focusing in particular to the optimum silylation conditions of steroids [69–77]. Evaluating the details of these comparisons it turned out that the main uncertainties are associated with the stability of derivatives, depending

(a) on the silylating agents, like MSTFA, BSTFA, and MTBSTFA [69,70,73–76],
(b) on the time and temperature (60 °C, 30 min [69,72,73], 65 °C, 30 min [74], 50 °C, 30 min [71], 85 °C, 100 min [70], microwave: 900 W, 1 min [75], 80 °C, 60 min [76], 60–70 °C, 30 min [77]),
(c) on the optimum solvent of derivatizations, and
(d) on the acquisition protocols applied (GC–MS, GC–MS/(MS) [65]).

Authors of this paper are convinced that

(1) unsatisfactory analytical attention was paid to the distinction, consequently, to the simultaneous identification and quantification of the keto, the keto and hydroxyl and the only hydroxyl group(s) containing steroids, from a single chromatographic run, in shortage of exhaustive mass fragmentation studies,
(2) in several proposals the keto groups' derivatizations are simply neglected [5–7,10,13,16,17],
(3) in others, by means of reductive silylation {MSTFA/NH_4J/dithiothreitol (DTE) ≈ 500–1000/4/2 (v/v/v)}, keto groups were transformed to the corresponding hydroxyl groups containing species: consequently, for sake of distinction, two derivatizations (a reductive and a non reductive one) would be needed [1–4,6,8,11,12,14,15,18–21,60],
(4) the advantage of the analysis of the methyloxime trimethylsilyl derivatives of steroids was, unfortunately, used in few cases, and without basic studies, only [32,48–50,64]. This protocol was applied in the analysis of faecal sterols from catchment waters [32], selected steroids from wastewaters [48], to identify dehydroepiandrosterone and its 7-oxygenated metabolites in human serum [49], to quantify urinary steroids, selectively [50] and to define steroid disorder metabolomes [64].

The goal of this paper was

(1) to give a detailed overview on the fragmentation pattern analysis of 20 selected steroids as their TMS (oxime) ether derivatives applying the optimum two step derivatization protocol (1: oximation; 2: silylation), on basic research level; documenting also the response of the only trimethylsilylated ethers,
(2) to compare derivatization protocols of steroids with the commonly used reagents (MSTFA, BSTFA, MTBSTFA), including the preferred, of our longstanding hexamethyldisilazane + trifluoroacetic acid (HMDS + TFA) one,
(3) to document the reproducibilities of the TMS (oxime) ether derivatives of the selected steroids, along with the corresponding limit of quantitation values from model solutions, and
(4) to confirm the practical utility of the suggested protocol, by an overview of the steroid contents of the influent and effluent wastewater samples obtained from two Hungarian Waste Water Treatment Plants (WWTPs).

2. Experimental

2.1. Instrumentation

The apparatus consisted of a Varian 240 GC–MS/MS system (Varian, Walnut Creek, CA, USA) equipped with a Varian CP-8400 AutoSampler, and with the Septum-equipped Programmable Injector (SPI). The column used was a product of SGE (Victoria, Australia); SGE forte capillary: 30 m × 0.25 mm; df = 0.25 μm. The temperature of the transfer line, ion trap and manifold were, in order of listing 300 °C, 210 °C and 80 °C, respectively.

MS conditions: Electron energy was 70 eV; multiplier offset was 250 eV. The actual parameters of the ITD were defined by the automatic set up mode.
Actual automatic set-up conditions: Mass range: 40–650 amu; the scan rate: 1 scan/second.
Acquisition time: 31 min; solvent delay: 420 s (omitting the acquisition of reagent peaks); peak threshold: 100 count; mass defect: 100 mmu/100 u; background mass: 50 u.

SPE extractions were performed on the Visiprep DL Vacuum manifold for 12 samples (Cat no: 57044) from Supelco (Bellefonte, PA, USA).

Extracts were dried on a Büchi Rotavapor R-200 by means of Büchi Vacuum pump, V-700, both from Büchi (Flawil, Switzerland).

2.2. Materials and reagents

All were of analytical reagent grade. Pyridine, and hydroxylamine·HCl were from Reanal (Budapest, Hungary). Hexane, methanol, ethyl acetate, hexamethyldisilazane (HMDS), bis-(trimethyl-silyl) trifluoroacetamide (BSTFA), *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA), trifluoroacetic acid (TFA) and model compounds such as, androsterone (5α-androstan-3α-ol-17-one), β-estradiol (estra-1,3,5(10)-triene-3,17β-diol), transdehydroandrosterone (androst-5-en-3β-ol-17-one), trans-androsterone (5α-androstan-3β-ol-17-one), mestranol (3-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-17-ol), dihydrotestosterone (5α-androstan-3-one-17β-ol), ethinylestradiol (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol), testosterone (androst-4-en-3-one-17β-ol), norethisterone (19-nor-17α-pregna-4-en-20-yne-3-one-17β-ol), estriol (estra-1,3,5(10)-triene-3,16,17-triol), 4-androstene-3,17-dione (androst-4-en-3,17-dione), gestodene (18a-homo-19-nor-17α-pregna-4,15-dien-20-yne-3-one-17β-ol), levonorgestrel (18a-homo-19-nor-17α-pregna-4-en-20-yne-3-one-17β-ol), etonogestrel (11,18a-dihomo-19-nor-17α-pregna-4,11a-dien-20-yne-3-one-17β-ol), progesterone (pregn-4-en-3,20-dione), coprostanol (5β-cholestan-3β-ol), cholesterol (cholest-5-en-3β-ol), medroxyprogesterone acetate {(6a-homo-pregn-4-en-17α-ol-3-one)-acetate}, stigmasterol (stigmast-5,22-dien-3β-ol) and β-sitosterol (stigmast-5-en-3β-ol) were all from Sigma (St. Louis, MO, USA). Glass microfiber filters (GF/A 125 mm, ∅, Cat no: 1820-125) were from Whatman (Maidstone, UK). Cartridges (Oasis, HLB 6cc), for solid phase extraction (SPE), were from Waters (Milford, MA, USA).

2.3. Sample preparation for pollutants' GC–MS determinations

2.3.1. Solid phase extraction

Cartridges, prior to extractions were treated with 5 mL hexane, 5 mL ethyl acetate, 10 mL methanol and 10 mL distilled water. Before the SPE enrichment, wastewater samples were filtered on glass microfiber paper (Glass microfiber filters (FF/A 125 mm, ∅, Cat no: 1820-125) which was from Whatman (Maidstone, UK). Car-

tridges have been dried in vacuum, and elutions were performed, in order of listing with 5 mL hexane, 5 mL ethyl acetate, and with 10 mL methanol. The unified eluents were reduced in volume, evaporated to dryness by means of a rotary evaporator, at 30–40 °C (further on: extract).

2.3.2. Preparation of the TMS/TBDMS (oxime) derivatives

Model compounds (10 mg/10 mL), weighed with analytical precision, were dissolved in ethanol or in water/ethanol = 1/1 (v/v) solution and further diluted for 10×, 100×, 1000×. Model solutions (10–500 μL) and the extracts were rotary evaporated to dryness at 30–40 °C. The residues were treated with 125 μL pyridine (in case of oximation with 125 μL hydroxylamine·HCl containing pyridine {2.5 g hydroxylamine·HCl/100 mL}) + 225 μL HMDS + 25 μL TFA, or 125 μL pyridine + 250 μL BSTFA, or 125 μL pyridine + 250 μL MSTFA or 125 μL pyridine + 250 μL MTBSTFA in 2–4 mL Reacti vials. Vials were heated in oven, at 50 °C, at 70 °C and at 90 °C for 30 min, 50 min, 90 min and 120 min. Finally as optimum derivatization condition 70 °C and 30 min was selected for oximation and 70 °C and 90 min was selected for trimethylsilylation. 1 μL of the diluted solutions was injected into the GC–MS system.

2.3.3. Separation of the TMS/TBDMS derivatives

Under gradient conditions, the optimized temperature programs, different for both the column and the septum equipped programmable injector (SPI), were as follows:

(a) injections were made at 100 °C, and held at 100 °C for 0.5 min, then heated to 300 °C (200 °C/min), with a 3 min hold at 300 °C,
(b) column temperature starts at 100 °C, held for 1 min, then heated up to 300 °C for 10 °C/min, with a 10 min hold at 300 °C (total elution time 31 min).

3. Results and discussion

3.1. The selection of steroids

The intrinsic properties of 20 steroids (Table 1: including β-estradiol and cholesterol reported also earlier [67], β-estradiol used in this study as a point of reference, as internal standard {IS}), were characterized

(a) with their chemical (Chemical Abstracts Service = CAS number, molecular weight, MW) and
(b) with their chromatographic retention (retention time, t_R) phenomena,
(c) with their characteristic selective fragment ions (SFIs), and
(d) with their response values, expressed as integrator units (Iu)/1 pg of steroids.

The special behavior of the syn/anti oximes is demonstrated in Fig. 1, while the structure of steroids, associated with their elution profile and detailed mass fragmentation behavior, is compiled in Fig. 2a–c. *Note*: it is to be highlighted that experimental data in Table 1, in Fig. 1 and in Fig. 2a–c are based on the evaluation of the TMS (oxime) ether derivatives' responses, obtained under optimum derivatization conditions, following our longstanding working strategy [65–68]; specified derivatization optimization for steroids are detailed in Section 3.2.

The selection of steroids, in order to be target compounds of this derivatization and mass fragmentation study, can be attributed to the facts that

(a) their detailed derivatization/mass fragmentation characteristics, as silyl (oxime) ether derivatives, according to a standard analytical aspect could not be found in the literature [1–64,69–77], as well as
(b) some of them, according to our introductory investigations, could be expected in samples of two Hungarian WWTPs (Section 3.3, Table 5).

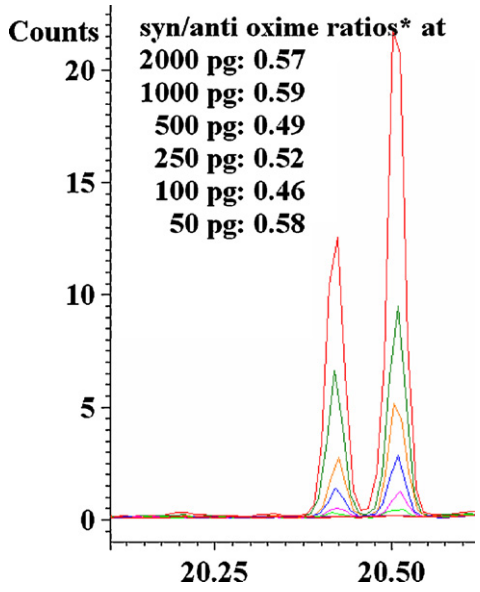

Fig. 1. Norethisterone-oximes-1,2: syn and anti oxime ratios (50–2000 pg) based on the area, obtained from their selective fragment ions; *m/z* values in Table 1 (further details in Section 3.1.1).

3.1.1. Fragmentation pattern analysis and response values of the TMS (oxime) ether derivatives of steroids

On the basis of the joint evaluation of the fragmentation pattern characteristics compiled in Table 1, in Fig. 2a–c, it is clear that

(1) in cases of the keto group(s) containing steroids (Table 1, compounds marked by asterisk), without exception, the two step derivatization protocol (1: oximation, 2: trimethylsilylation) proved to be of primary importance: ketosteroids do form TMS (oxime) ethers (Table 1, data in lines A).
(2) Ketosteroid (oxime) ethers, mostly are eluted in two, as syn and anti oximes, infrequently in unresolved form (androsterones, testosterone, medroxyprogesterone acetate).
(3) The ratios of oximes (Table 1, data in column R^{*oxime}) confirm a wide range of syn/anti ratio values, from 0.26 (progesterone) up to 0.95 (dihydrotestosterone) providing an average reproducibility of 5.7 RSD%.
(4) "...As to the intrinsic properties of the syn/anti ratio values it has been repeatedly proved (in agreement with the phenomenon of the reducing sugar [79,80] and ketoprofen oximes [67]) that these values are characteristic to the oxime species in question and are independent of their amounts analyzed. As expected,

(a) The reproducibility of the completely resolved dihydrostestosterone TMS (oxime) ratios, based on their syn/anti values (calculated from the area of their SFIs, from 5 pg to 2000 pg injected amounts, chromatograms not shown), varied between 0.93 and 0.95, and confirms an excellent average reproducibility of 1.27 RSD%.
(b) The syn/anti ratios of norethisterone-oximes – in spite of the co-elution of norethisterone anti oxime with estriol –, certify an acceptable reproducibility, varying between 0.46 and 0.59, with an average reproducibility of 9.9 RSD% (Fig. 1).

Table 1
Fragmentation patterns of various functional group containing natural and synthetic steroids determined as their trimethylsilyl (oxime) ether derivatives by GC–MS based on their selective fragment ions (SFIs).

Compound	CAS number	MW	Solubility (μg/L)	Derivative	t_R (min)	R*, syn/anti oximes	SFIs (*m/z*)			Response values, lu/pg (RSD%)	Response ratio values	
							$[M]^+$	$[M-15]^+$	Additional ions		R^{*SFI}	R^{**SFI}
1. Androsterone*	53-41-8	290.44	12	A	18.87 –	–	449	434	360; 270; 213	57, 710(5.9)	0.801	2.08
				B	17.88	–	362	347	272, 257	27, 749(1.95)	0.385	
2. β-Estradiol (IS)	50-28-2	272.39	3.60	A/B	19.15	–	416	401	326; 285; 231	72, 059(1.12)	1.00	–
3. Transdehydroandro-sterone*	53-43-0	288.42	64	A	19.41 –	–	447	432	358; 318; 268	21, 254(6.3)	0.295	1.52
				B	18.44	–	360	345	270; 129	13, 983(3.55)	0.190	
4. Transandrosterone*	481-29-8	290.22	20	A	19.50 –	–	449	434	360; 270; 213	49, 787(3.55)	0.691	2.17
				B	18.50	–	362	347	272	15, 419(4.21)	0.214	
5. Mestranol	72-33-3	310.43	3.77*1	A/B	19.62	–	382	367	227; 174	30, 815(5.1)	0.428	–
6. Dihydrotestosterone*	521-18-6	290.44	52,500	A&	19.68 19.86	0.95 (1.27)	449	434	344; 254; 211; 129	32, 266(5.1)	0.448	–
7. Ethinylestradiol	57-63-6	296.40	11	A/B	19.97	–	440	425	285; 231	34, 140(3.47)	0.473	–
8. Testosterone*	58-22-0	288.42	23	A	20.03 –	–	447	432	211; 358; 343	23, 993(3.04)	0.333	1.66
				B	19.37	–	360	345	270; 226	14, 455(3.38)	0.200	
9. Norethisterone*	68-22-4	298.42	7.0	A	20.42 20.52	0.54 (9.9)	457	442	368; 317; 302; 209	28, 021(6.9)	0.389	4.25
				B	19.83	–	370	355	303; 209; 167; 125	6597(1.63)	0.078	
10. Estriol	50-27-1	288.38	441*2	A/B	20.51	–	504	489	414; 386; 324; 311; 296; 270	68, 864(2.29)	0.956	
11. 4-Androstene-3,17-dione*	63-05-8	286.19	58	A	20.64 20.70	0.47 (1.47)	460	445	371; 211	24, 777(8.6)	0.344	3.09
				B	19.16	–	286	271	201; 148; 124	8018(6.5)	0.111	
12. Gestodene*	60282-87-3	310.43	–	A	20.91 21.01	0.45 (5.9)	469	454	440; 380	12, 012(6.8)	0.167	1.40
				B	20.37	–	382	367	353; 338; 325	8580(4.42)	0.119	
13. Levonorgestrel*	797-63-7	312.45	2.05	A	21.13 21.24	0.49 (4.50)	471	456	442; 382; 331	16, 509(8.0)	0.229	2.79
				B	20.62	–	384	369	356; 341; 317	5917(5.2)	0.082	
14. Etonogestrel*	54048-10-1	324.46	7.4	A	21.46 20.57	0.36 (2.70)	483	468	454; 394; 343; 153	12, 828(9.0)	0.178	1.79
				B	20.94	–	396	381	367; 329	7166(4.23)	0.099	
15. Coprostanol	360-68-9	388.67	4×10^{-4}*3	A/B	21.77	–	460	445	370; 257; 215	2229(2.05)	0.031	–
16. Progesterone*	57-83-0	314.47	8.81	A	22.22 22.30	0.26 (14)	488	473	399; 344; 211; 145	20, 855(8.9)	0.289	4.56
				B	20.74**	–	386	371	314; 272; 229**	4573(1.47)**	0.0063	
17. Cholesterol	57-88-5	386.6	95	A/B	22.55	–	458	443	358; 353; 3.29	21, 719(4.46)	0.301	–
18. Medroxyprogesterone acetate**	71-58-9	386.52	2.95	A	23.16 –	–	473	458	371; 280; 225; 209	1259(7.9)	0.017	1.42
				B	21.63**	–	386		283; 301, 244**	850(5.8)**	0.012	
19. Stigmasterol	83-48-7	412.69	1.1×10^{-4}*4	A/B	23.82	–	484	469	394; 379; 255; 129	14, 085(2.48)	0.204	–
20. β-Sitosterol	83-46-5	414.72	0.32	A/B	24.53	–	486	471	396; 381; 255; 129	3697(5.8)	0.049	–

Indications: * = steroids providing oximes; A = TMS (oxime) ethers; B = TMS ethers; MW = average molecular weight of the underivatized compound; – = no data available; $[M]^+$ = molecular ion; ** = measured in their initial form; IS = including in all tests (375 pg/μL); $R^{*oximes}$ = ratios of syn/anti oximes; lu = integrator units; R^{*SFI} = response ratios to β-estradiol; R^{**SFI} = response ratios of the TMS (oxime) ethers to the TMS ethers; & = the TMS ether derivative was not obtained; *1, *2, *3, *4 = calculated/predicted values, taken from Physical Properties database [78].

3.1.2. Fragmentation pattern analysis and response values of the TMS ether derivatives of steroids

As to the response values in general (Table 1, Iu/pg values), they are varying in a wide scale from 72,059 Iu/pg (β-estradiol, data in line A/B) down to 850 Iu/pg (medroxyprogesterone acetate in its initial form, data in line B). Reproducibility values (in parentheses) characterized with the relative standard deviation percentages of analyses varied from 1.12 RSD% (β-estradiol) up to 9.0 RSD% (etonogestrel) with an average of 4.78 RSD%.

(1) Response ratio characteristics indicated by the R^{*SFI} values show the distribution of responses related to the β-estradiol's one. These data, without exception, represent the values of ≤ 1, varying from 0.956 (estriol) down to 0.012 (medroxyprogesterone acetate in its initial form, data in line B), while,
(2) the response values of the TMS (oxime) ether derivatives compared to the TMS ether ones (Table 1, data in the last vertical column, R^{**SFI} values) show considerable advantages, in all cases tested, indicating the values of ≥ 1, varying between 1.40 (gestodene) and 4.56 (progesterone), respectively.

3.1.3. Chromatographic elution, mass spectra and fragmentation phenomena of steroids

Evaluating the fragmentation characteristics and the mass spectra of steroid derivatives (Fig. 2a–c), as general conclusion, it can be stated that steroids being in structural relationship provide unambiguous similarities (*Note*: ring indications (A–D) and C atom numbering are shown at the scheme of androsterone-oxime, only: Fig. 2a).

Fragmentation of the 17-ketosteroids takes place

(a) partly between the C_{12} and C_{13} and between the C_8 and C_{14} bonds resulting in the D ring elimination and the formation of the abundant fragment ions *m/z* 270 (androsterone, transandrosterone) and *m/z* 268 (transdehydroandrosterone),
(b) partly between the C_7 and C_8 and between the C_9 and C_{10} bonds associated with the simultaneous elimination of the C and D rings and with the formation of the fragment ions *m/z* 213 (androsterone, transandrosterone) and *m/z* 211 (dehydroandrosterone), both masses (*m/z* 211, *m/z* 213) reveal of marginal intensities only.

The fragmentation behavior of the 3-ketosteroids (Fig. 2a, compounds: 6, 8, 9, 12, 13, 14) including the 3, 17 diketosteroids (Fig. 2a, compounds 11, 16), in comparison to that of the 17-ketosteroids {(Fig. 2a: compounds (1, 3, 4, 11)}, proved to be considerably different: 3- and 3,17-ketosteroids being in particular stable species.

In all cases of the 3- and 3,17-ketosteroids their abundant masses proved to be their molecular ions ($[M]^{+\cdot}$) and/or their fragment ions, formed by the loss of one methyl group ($M-CH_3]^+$): like in dihydrostestostrone's syn and anti oximes (Fig. 2b, spectra 3A, 3B), in testosterone-oxime (Fig. 2b, spectrum 4B). Similar fragmentation pattern characterizes the spectra in Fig. 2c the syn and anti oximes of gestodene (spectra 1A, 1B), and those of levonorgestrel (spectra 2A, 2B), etonogestrel (spectra 3A, 3B) and progesterone (spectra 4A, 4B).

The TMS ethers of the only hydroxyl group containing steroids, except coprostanol (spectra not shown) certify high stability:

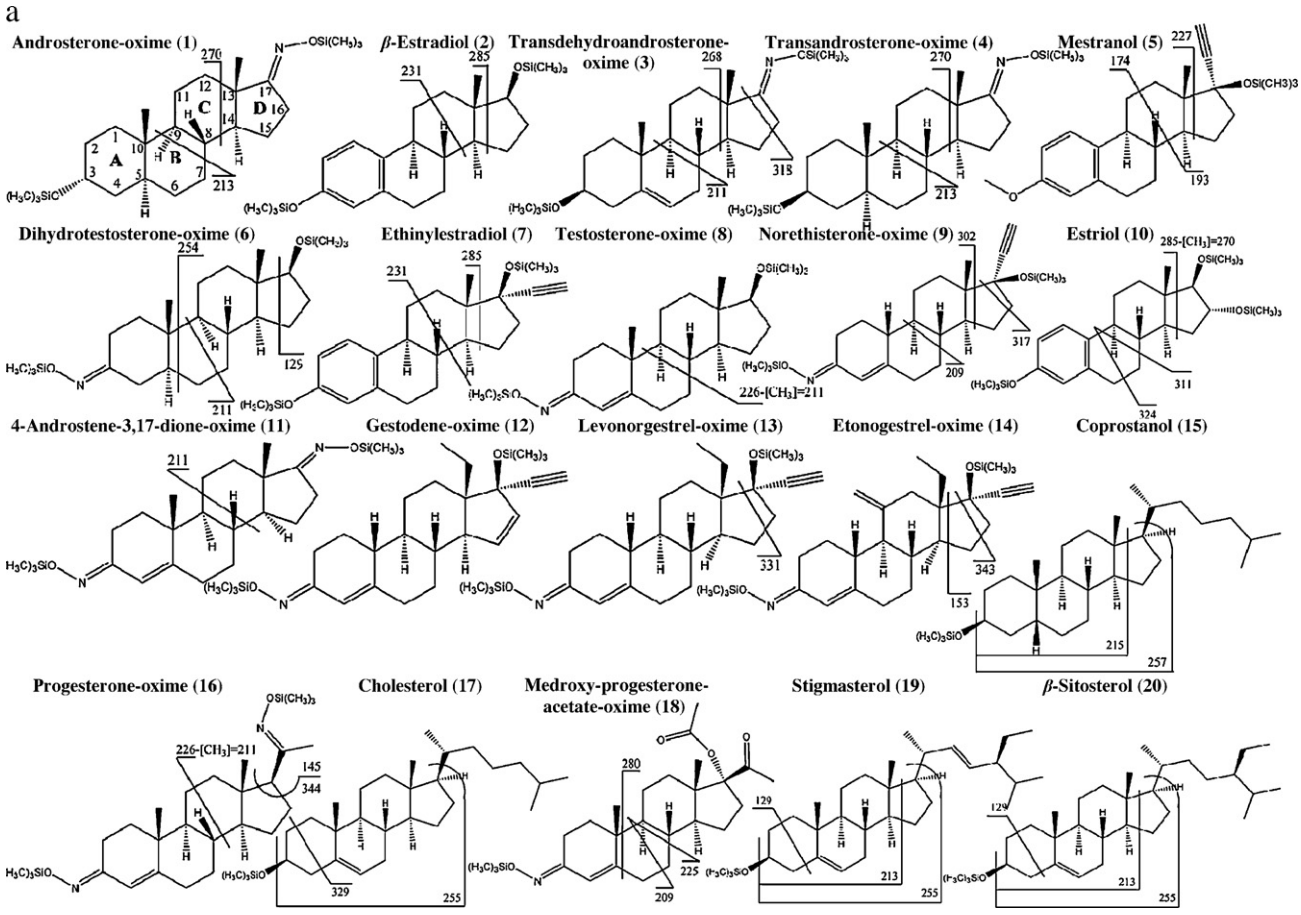

Fig. 2. (a–c) Molecular structure, fragmentation pattern, peak profile and mass spectra of the trimethylsilyl (oxime) ether derivatives of steroids. (a) Molecular structure and fragmentation behavior of steroid derivatives {compounds (1–20)}. (b) Peak profile and mass spectra of selected steroid derivatives {compounds (1–10)}. (c) Peak profile and mass spectra of selected steroid derivatives {compounds (11–16)}.

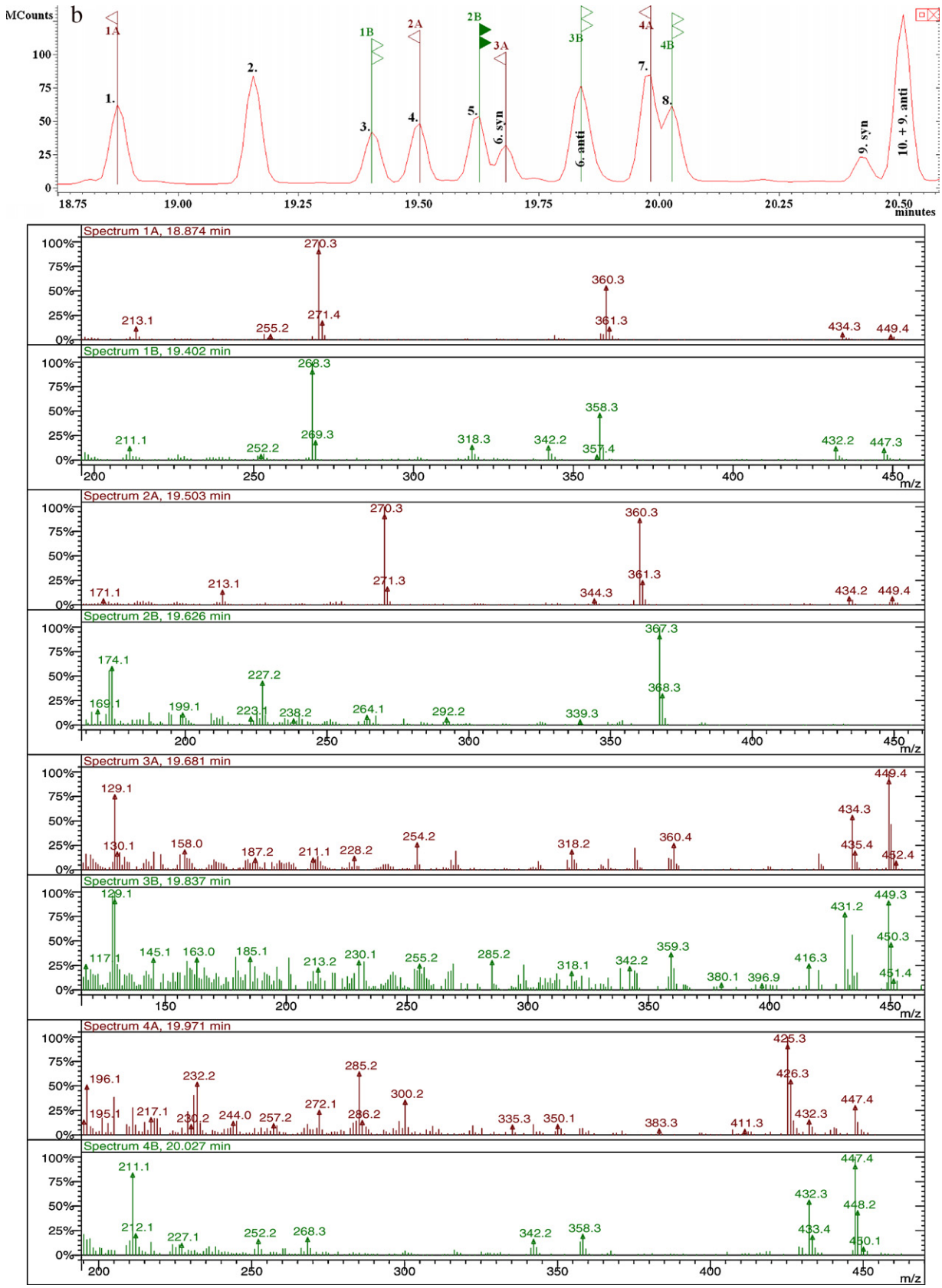

Fig. 2. (*Continued*).

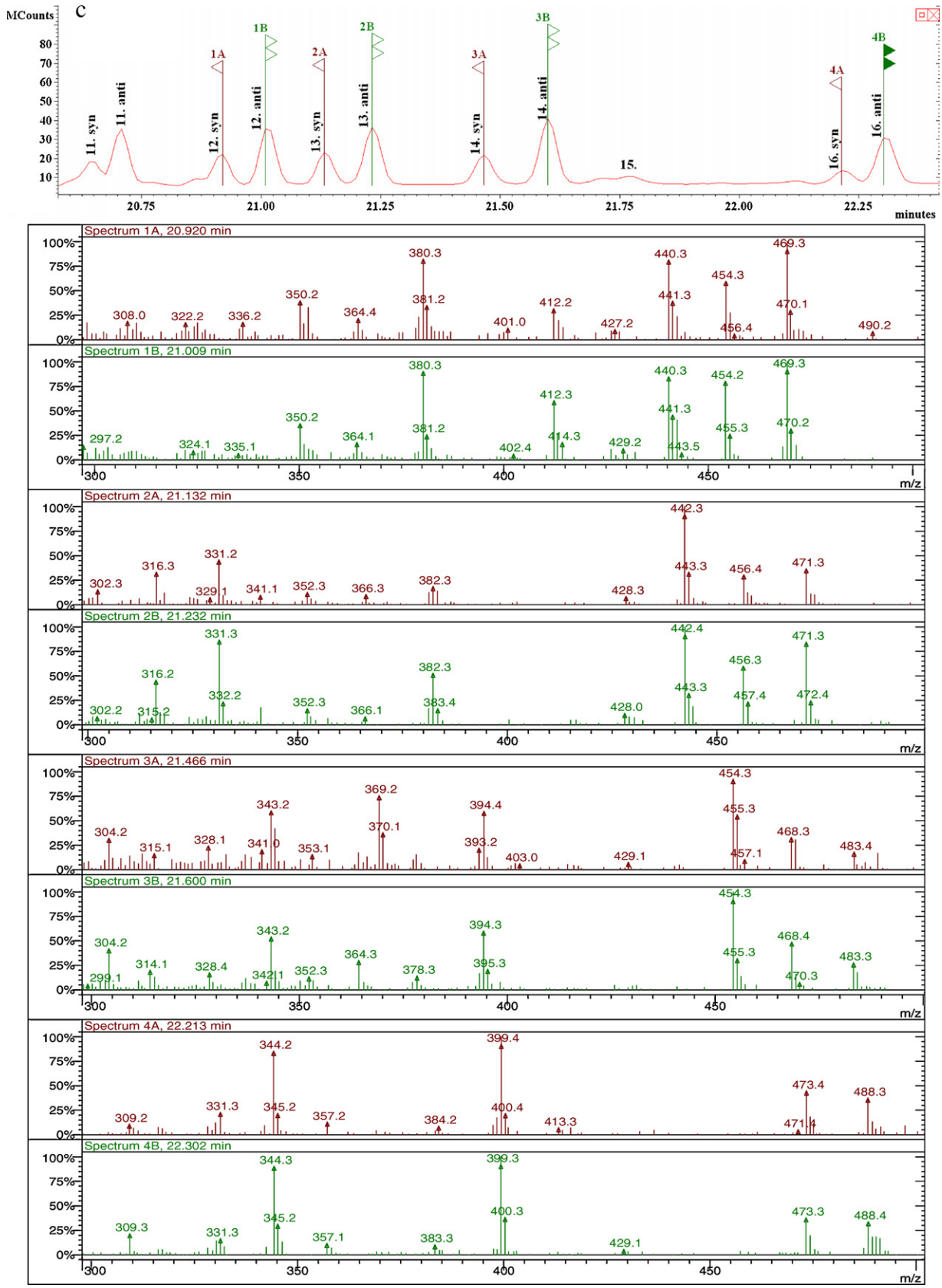

Fig. 2. (*Continued*).

Table 2
Derivatization study of various functional group containing natural and synthetic steroids: response values obtained from model solutions (500 pg of each), depending on the silylating agent, determined on the basis of their selective fragment ions (SFIs) by gas chromatography mass spectrometry (GC–MS), as their trimethylsilyl (oxime) ether derivatives.

Derivatization conditions⇒	Integrator units/injected pg								
Compounds ⇓	HMDS + TFA					MSTFA		BSTFA	
	Av^a	RSD%	Av^b	RSD%	RSD% ($Av^a + Av^b$)	Av^a	RSD%	Av^a	RSD%
Androsterone	37, 603	4.58	35, 034	0.98	4.04	36, 235	8.5	19, 478	3.69
β-Estradiol	45, 204	3.31	46, 239	1.71	2.04	50, 131	3.42	45, 874	2.83
Mestranol	19, 793	3.90	21, 280	0.37	4.94	21, 033	2.00	18, 803	4.17
Ethinylestradiol	22, 865	1.31	21, 437	0.93	1.94	23, 389	0.74	21, 868	1.96
Testosterone	17, 229	1.85	15, 946	1.31	3.89	18, 857	3.35	13, 803	5.4
Estriol	66, 430	3.71	61, 005	0.31	4.40	69, 534	2.78	64, 454	2.65
Norethisterone	11, 989	6.6	11, 590	0.18	2.03	13, 299	4.43	8316	5.5
Gestodene	6769	7.2	6319	1.48	5.1	7121	6.4	4884	4.41
Levonorgestrel	8985	1.35	9314	1.90	1.64	10, 439	5.9	6799	4.25
Etonogestrel	8307	5.2	7957	4.53	5.2	9818	3.45	5768	4.12
Stigmasterol	9280	4.48	8929	0.39	1.50	9053	5.4	11, 265	2.94

Indications: as in Table 1, as well as: Av^a = immediately after dilution; Av^b = 12 h later.

furnishing the corresponding molecular ions ($[M]^{+\cdot}$) and/or their $[M–CH_3]^+$ versions (Table 1 and Fig. 2a). Additionally, common fragmentations are going on between the C_{13} and C_{17} and between the C_{14} and C_{15} bonds leading to the elimination of the D-ring. Depending on the D-rings' specificities the characteristic fragment ions, like *m/z* 285 (Fig. 2a: β-estradiol), *m/z* 227 (Fig. 2b, spectrum 2B: mestranol), *m/z* 285 (Fig. 2b, spectrum 4A: ethinylestradiol), *m/z* 270 (Fig. 2a: estriol), *m/z* 215 (Fig. 2a: coprostanol) and *m/z* 213 (Fig. 2a: stigmasterol and β-sitosterol) are formed, without exception, with high intensities.

3.2. Derivatization and reproducibility studies of steroids

As indicated in Sections 3.1–3.3 mass fragmentation treaties have been performed under optimum derivatization conditions performing the two step of our longstanding derivatization protocol. However, to prove its general utility even in the case of steroids, in the light of considerably different literature data [69–77] derivatization optimization studies had to be re-examined, in detail.

At first, under strictly the same experimental conditions, which means applying the same reagent excess, temperature and reaction time, the silylating reagents had to be varied (Section 3.2.1), thereafter, with the selected reagent, reaction time and temperature were optimized (Section 3.2.2).

In the knowledge of optimum derivatization protocol, which proved to be the procedure of our longstanding one, reproducibility studies as a function of the amounts of the derivatized steroids, associated with LOQ values were documented (Section 3.2.3).

3.2.1. Derivatization and stability studies of selected steroids depending on the silylating reagents

In this treatise, subsequently to the oximation step, HMDS + TFA, MSTFA, BSTFA and MTBSTFA have been compared by the derivatization of selected representatives of steroids.

On the basis of these experiences (Table 2) we could confirm that

(1) the responses of the TMS (oxime) derivatives of steroids (with exceptions of the BSTFA derivatized, considerably lower responses providing species: Table 2, last two vertical columns) proved to be more or less comparable.

The stability of the HMDS + TFA derivatized steroids (similarly to all TMS-derivatives, data not shown) has been tested as a function of time (Table 2, response values like $Av^a + Av^b$ and their RSD percentages, data in the first five vertical columns). The stability behavior of these species has been characterized with their RSD percentages which varied between 0.18 RSD% and 7.2 RSD%. The proof of the convincing stability feature of the derivatized, diluted species was inevitably necessary in order for their, at least one night long storage in the autosampler vials, prepared for injections (the overall stability of undiluted species was compiled in Table 4).

(2) MTBSTFA reacts with the hydroxyl-steroids only: providing unsatisfactory derivatization with low response values. In the case of β-estradiol, the total of responses of the monosubstituted and disubstituted TBDMS-derivatives proved to be less than the half of the TMS-ones, while ethinylestradiol furnishes a single TBDMS derivative, however, with a half response of the corresponding TMS-species.

As to the selection of the silylating agents, out of the four reagents tested, for our further studies trimethylsilylation with HMDS + TFA was preferred. Since,

(a) this reagent ensures the same efficiency as MSTFA and BSTFA (Table 2),
(b) MTBSTFA, in accordance also with our experiences does not react with the sterically hindered groups of steroids [6,69,73], and in addition
(c) HMDS + TFA combination is the most cost-effective, and of many sided proved of our longstanding silylating reagent.

3.2.2. Reaction time and temperature dependence of the derivatization of selected steroids: optimization of the oximation and the silylation steps, vice versa

Reaction time and temperature versions with selected representatives of steroids applying the preferred derivatization protocol (step 1: oximation with $NH_2OH·HCl$ in pyridine; step 2: silylation with HMDS + TFA) are compiled in Table 3.

(1) Temperature and reaction time variations in order to optimize the oximation step have been performed under the same trimethylsilylation conditions (Table 3, data in the first five vertical columns), while.
(2) Optimum temperature and reaction time selection were carried out under the same oximation conditions (Table 3, data in the 6–9 vertical columns).

On the basis of these vice versa varied approaches it has been confirmed, that except for the use of 50 °C (italic printed data

Table 3
Optimization of the two steps derivatization of natural and synthetic steroids: response values obtained from model solutions, depending on the reaction time and temperature, both of the oximation ($NH_2OH{\cdot}HCl$) and that of the trimethylsilylation steps (HMDS+TFA), determined on the basis of their selective fragment ions (SFIs) by gas chromatography mass spectrometry (GC–MS), as their TMS (oxime) ether derivatives.

Derivatization conditions ⇒	Integrator units/injected pg										
Compounds ⇓	Oximation*					Trimethylsilylation**					
	°C			time, min		°C			time, min		Av***
	50	70	90	60	90	50	90		60	120	
Androsterone	57,925 (5.7)	58,953 (10)	58,235 (7.2)	58,965 (6.0)	59,477 (5.1)	*52,479 (7.5)*	57,306 (11)		56,595 (5.7)	55,594 (0.76)	57,881 (1.79)
β-Estradiol	74,401 (4.27)	71,707 (6.2)	73,341 (3.85)	73,252 (1.90)	72,380 (1.77)	72,901 (6.1)	69,000 (8.9)		72,197 (4.2)	69,890 (2.83)	72,119 (1.77)
Mestranol	30,858 (2.19)	31,752 (8.2)	30,774 (0.13)	32,637 (1.16)	31,649 (2.40)	30,858 (4.33)	30,128 (8.5)		30,638 (0.22)	29,894 (0.23)	31,021 (2.13)
Ethinylestradiol	32,536 (4.13)	34,892 (6.8)	34,694 (7.8)	36,123 (1.16)	35,311 (5.4)	34,226 (8.2)	32,053 (9.2)		34,646 (6.1)	32,338 (3.01)	34,091 (3.48)
Testosterone	23,486 (11)	23,650 (6.8)	24,038 (1.82)	23,586 (4.54)	22,192 (1.48)	24,299 (7.4)	24,921 (10)		23,082 (1.16)	14,639 (3.15)	15,071 (2.48)
Estriol	64,602 (7.6)	66,030 (6.0)	67,578 (6.5)	65,531 (1.67)	64,876 (4.15)	67,373 (5.1)	65,275 (6.5)		65,490 (1.23)	64,902 (3.05)	64,740 (1.27)
Norethisterone	*24,979 (6.1)*	27,930 (6.7)	25,761 (4.76)	29,396 (2.99)	28,589 (3.85)	26,486 (1.17)	25,422 (16)		25,391 (10)	26,175 (4.47)	26,892 (4.87)
Gestodene	11,890 (6.1)	11,790 (10)	10,927 (6.4)	12,450 (3.00)	12,097 (6.9)	11,896 (2.05)	10,705 (8.0)		11,796 (6.0)	10,432 (1.05)	11,554 (5.0)
Levonorgestrel	16,159 (4.52)	16,310 (8.0)	15,956 (0.83)	16,318 (1.54)	15,521 (1.90)	15,570 (1.84)	14,989 (11)		15,374 (7.8)	15,076 (0.32)	15,696 (2.77)
Etonogestrel	10,649 (5.3)	10,729 (7.2)	10,007 (5.3)	11,087 (3.85)	10,143 (4.62)	9837(8.4)	10,003 (10)		9735 (6.1)	10,027 (2.54)	10,246 (3.74)
Stigmasterol	12,601 (3.58)	11,953 (4.62)	14,049 (3.50)	12,480 (7.0)	12,367 (12)	14,268 (2.19)	12,369 (10)		12,593 (8.0)	12,987 (2.28)	12,974 (5.0)

Indications: as in Tables 1 and 2, as well as: * = trimethylsilylations were performed uniformly, at 70 °C for 90 min; ** = oximations were performed uniformly, at 70 °C for 30 min; *italic printed data* = omitted from the average; Av*** = obtained from two separate tests and two injections of each; () = in parentheses relatives standard deviation percentages.

for norethisterone oximation and for androsterone trimethylsilylation), for both derivatization steps, all other reaction conditions provided satisfactory responses: averages of data obtained from all conditions (Table 3, last vertical column) varied between 1.27 RSD% and 6.9 RSD%, respectively.

In conclusion, remaining on the safe side, and taking also into consideration our previous experiences [65–68], as optimum reaction temperature for both steps, the 70 °C, as optimum reaction times the 30 min for oximation, and the 90 min for trimethylsilylation have been defined.

3.2.3. Reproducibility, calibration and stability studies as a function of the amounts of the derivatized steroids from model solutions: limit of quantitation values (LOQ) and recovery data

In the frame of these investigations response values of various amounts of 20 steroid derivatives, in the range of 1.88–750 ng/L levels, have been evaluated from model solutions, in two separate tests and from three injections of each (Table 4).

Response values revealed, that

(1) Calibration properties of derivatives proved to be associated with their initial molecular structure; it means,
   - hydroxysteroids (Table 4, compounds 2, 5, 7, 10, 15, 17, 19, 20) provided, without exception, linear responses, with excellent reproducibilities, varying between 1.73 RSD% (β-estradiol) and 5.4 RSD% (stigmasterol), respectively.
   - ketosteroids have been evaluated partly from linear responses (Table 4, compounds 1, 3, 4, 8, 9, 12), partly from calibration curves (11, 13, 14, 16, 18). In both cases reproducibilities characterized with their relative standard deviation percentages were acceptable. Average reproducibility values of linear responses furnishing ketosteroids ranged from 2.31 RSD% (gestodene) up to 5.8 RSD% (dihydrotestosterone). Ketosteroids' reproducibility evaluated on the basis of calibration curves depends on the absolute response of the ketosteroid derivative and on its actual amount to be determined. The worst reproducibility was obtained from medroxyprogesterone-acetatate oxime (maximum response: 1508 integrator units/pg; RSD%: between 2.7 and 23 RSD%), while the best characteristics from progesterone oxime (maximum response: 30,041 integrator units/pg; RSD%: between 2.1 and 8.5 RSD%).

(2) Stability of derivatives saved in the refrigerator were followed within a period of 75 days (Table 4, data in the fifth vertical column (93.8 ng/L concentrations of compounds). Injections were made from the same stock solutions in consecutive three cases (July 08, July 28 and September 16, all in 2010); these responses, even calculated from calibration curves, provide a standard deviation between 0.13% and 5.9%, with an average of 2.78 RSD%.

(3) LOQ values vary between 1.88 ng/L and 37.6 ng/L concentrations ($s/n \geq 10$), without diluting the 375 μL stock solution of the TMS (oxime) ether derivatives of steroids.

(4) In the case of wastewater samples (Table 5) to precede the fast contamination of the injector system the injection of the diluted stock solutions (from 2-fold up to 10-fold) is preferred. Consequently, in these cases the LOQ values are changed proportionally.

(5) Recoveries, characterized with the relative standard deviation percentages (RSD%), obtained from fortified effluent wastewater samples (added amounts of steroids ranged in the 1–2 μg/L concentrations), varied between 79% (mestranol) and 106% (etonogestrel), with an average recovery of 95%. The low solubilities of coprostanol, cholesterol, stigmasterol and β-sitosterol resulted in their low average recovery (34%), calculated from their one by one values (coprostanol,

Table 4
Reproducibility in the quantitation of various amounts of natural and synthetic steroids from model solutions, determined as their trimethylsilyl (oxime) ether derivatives by gas chromatography mass spectrometry, based on their selective fragment ions (SFIs).

Compounds	Derivatized (ng/L)											LOQ (ng/L)	Injected pg**	Recovery, %
	1.88	3.76	18.8	37.6	93.8			187.5	375	750	Av*			
					July 08	July 28	Sept 16							
	Integrator units/pg (%, RSD)													
1. Androsterone	<LOQ	*10,875 (3.71)*	*34,062 (1.77)*	*44,235 (2.29)*	*49,550*	*49,158 (1.66)*	*50,742*	57,710 (4.30)	61,074 (2.50)	61,658 (2.96)	60,147 (2.70)	3.76	10	101 (2.86)
2. β-Estradiol (IS)	72,326 (0.83)	71,555 (1.55)	71,561 (1.84)	75,445 (5.46)	71,517	69,237 (1.85)	71,498	74,627 (4.18)	72,035 (30)	70,789 (21)	72,059 (1.73)	1.88	5	95 (1.85)
3. Transdehydr-oandrosterone	<LOQ	<LOQ	*9008 (5.78)*	*13 139 (15)*	*15,971*	*16,174 (2.22)*	*15,487*	19,231 (4.26)	21,292 (3.50)	23,239 (4.26)	21,254 (6.3)	18.8	50	107 (1.63)
4. Transandrosterone	<LOQ	<LOQ	*21,177 (2.82)*	*34,092 (5.7)*	*38,820*	*40,630 (3.67)*	*37,798*	44,366 (4.71)	50,977 (2.88)	54,017 (2.23)	49,787 (3.55)	18.8	50	96 (1.66)
5. Mestranol	32,443 (7.3)	34,708 (5.1)	30,714 (5.4)	34,711 (4.43)	28,726	29,232 (1.30)	28,499	29,553 (3.30)	29,582 (0.11)	29,982 (2.71)	30,815 (5.1)	1.88	5	79 (2.09)
6. Dihydrotestosterone	<LOQ	<LOQ	*22,413 (3.51)*	*25,272 (8.9)*	29,279	30,439 (1.96)	28,994	31,798 (2.52)	33,260 (1.12)	34,545 (3.13)	31,386 (5.8)	18.8	50	86 (3.31)
7. Ethinylestradiol	29,550 (3.40)	33,992 (1.58)	35,473 (6.9)	34,205 (0.67)	34,484	35,666 (1.86)	35,573	34,745 (2.55)	34,974 (3.37)	35,698 (2.14)	34,140 (3.47)	1.88	5	90 (4.61)
8. Testosterone	<LOQ	<LOQ	*36,705 (2.33)*	*33,793 (8.5)*	25,470	23,941 (4.0)	26,998	23,993 (8.1)	24,178 (1.03)	24,699 (1.62)	24,838 (3.21)	18.8	50	89 (2.39)
9. Norethisterone	<LOQ	*11,301 (4.30)*	*21,235 (3.16)*	*24,369 (1.04)*	26,000	25,284 (1.84)	26,720	28,021 (4.07)	28,707 (4.48)	30,792 (0.98)	27,587 (5.7)	3.76	10	100 (1.50)
11. 4-Androstene-3,17-dione	<LOQ	<LOQ	*9585 (3.68)*	*9860 (2.80)*	*15,609*	*16,243 (5.9)*	*14,439*	*21,573 (2.10)*	*24,967 (5.2)*	*27,792 (7.8)*	Calibr. curve	18.8	50	92 (5.8)
12. Gestodene	<LOQ	<LOQ	*8045 (8.0)*	*8879 (4.37)*	*10,115*	*10,013 (1.22)*	*10,259*	12,012 (1.05)	12,032 (2.40)	12,680 (2.35)	12,241 (2.31)	18.8	50	91 (4.21)
13. Levonorgestrel	<LOQ	<LOQ	*12,423 (3.07)*	*13,142 (9.6)*	*14,853*	*14,315 (3.51)*	*15,357*	*16,509 (6.0)*	*17,433 (2.38)*	*19,403 (1.34)*	Calibr. curve	18.8	50	101 (3.61)
14. Etonogestrel	<LOQ	<LOQ	*9625 (2.05)*	*9953 (6.0)*	*12,100*	*11,447 (4.89)*	*12,624*	*12,828 (1.66)*	*14,247 (1.40)*	*15,608 (4.06)*	Calibr. curve	18.8	50	106 (4.1)
15. Coprostanol	<LOQ	<LOQ	2374 (15)	2245 (14)	2162	2155 (0.17)	2160	*2659 (6.55)*	2304 (4.35)	2204 (1.93)	2229 (2.05)	18.8	50	31 (1.79)
16. Progesterone	*10,899 (8.5)*	*11,381 (2.69)*	*9525 (5.7)*	*12,407 (2.97)*	*15,617*	*15,874 (3.67)*	*14,790*	*20,855 (3.15)*	*25,041 (6.5)*	*30,041 (2.10)*	Calibr. curve	1.88	5	95 (3.20)

Table 4 (*Continued*)

Compounds	Derivatized (ng/L)											LOQ (ng/L)	Injected pg**	Recovery, %
	1.88	3.76	18.8	37.6	93.8			187.5	375	750	Av*			
					July 08	July 28	Sept 16							
17. Cholesterol	<LOQ	<LOQ	22,513 (4.41)	23,381 (13)	20,109	20,058 (0.13)	20,099	22,158 (3.70)	20,892 (2.68)	21,249 (6.8)	21,719 (4.46)	18.8	50	27 (10)
18. Medroxyprogesterone-acetate**	<LOQ	<LOQ	<LOQ	*639 (23)*	*961 (5.6)*		–	*1259 (9.6)*	*1520 (6.6)*	*1508 (2.70)*	Calibr. curve	37.6	100	102 (10)
19. Stigmasterol	<LOQ	<LOQ	14,702 (5.8)	15,854 (6.0)	13,162	13,611 (2.62)	12,613	15,237 (4.16)	13,623 (1.21)	13,398 (4.26)	14,025 (5.4)	18.8	50	40 (5.3)
20. β-Sitosterol	<LOQ	<LOQ	3634 (5.3)	3910 (5.7)	3758	3932 (5.2)	3538	3730 (4.26)	3605 (3.77)	3544 (0.87)	3639 (2.77)	18.8	50	36 (1.42)

Indication: as in Tables 1–3, as well as: Av* = from two separate derivatizations and 3 injections of each; italic printed data were omitted from the mean; ** = taking into account that the 1 μL sample was injected from 375 μL stock solution (ng/L: injected pg × 375); LOQ = limit of quantitation = s/n ≥ 10; calibr. curve = calibration curve. *Note*: wastewater samples' stock solutions (375 μL) were 10-fold diluted, to avoid fast contamination and choking of the insert: in these cases LOQ values are 10-fold higher.

Table 5
Dissolved natural steroid contents of influent (infl) and effluent (effl) wastewater samples (0.5 L), determined as their trimethylsilyl (oxime) ether derivatives by GC–MS, based on their selective fragment ions (SFIs).

Steroids	Steroids obtained from Hungarian waste waters treatment plants (WWTPs) (μg/L)																
	Dél-Pest		Telki		Dél-Pest			Telki		Dél Pest		Telki			Telki	
	December 2009		January 2010		February 2010		April 2010	May 2010		June 2010		July 2010			September 2010	
	infl	effl	infl	effl	infl	effl	infl	infl	effl	infl	effl	$infl^{0.5}$	$infl^{1.0}$	effl	infl	effl
Androsterone	4.09 (5.1)	<LOQ	0.74 (0.05)	<LOQ	3.96 (10.0)	<LOQ	3.25 (1.48)	1.08 (6.2)	<LOQ	<LOQ	<LOQ	2.17 (2,48)	2.21 (0.75)	<LOQ	4.28 (1.68)	<LOQ
Transandrosterone	1.70 (4.23)	<LOQ	<LOQ	<LOQ	0.78 (1.25)	<LOQ	0.138 (0.601)	1.87 (1.07)	<LOQ	<LOQ	<LOQ	4.00 (5.5)	3.53 (6.2)	<LOQ	2.91 (1.11)	<LOQ
Androsterone-3,11-ol-17-one*	1.04 (10)	<LOQ	0.058 (7.2)	<LOQ	1.04 (1.05)	<LOQ	0.63 (0.056)	1.09 (1.00)	<LOQ	0.57 (2.44)	<LOQ	4.37 (4.13)	4.50 (3.96)	<LOQ	<LOQ	<LOQ
β-Estradiol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.100 (3.65)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Estriol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.054 (15)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Coprostanol	180 (3.26)	16 (8.3)	188 (3.24)	2.11 (4.75)	302 (1.37)	15 (1.93)	100 (0.737)	144 (3.51)	20.0 (5.4)	45.0 (6.2)	6.40 (4.34)	44.0 (1.96)	31 (0.21)	<LOQ	20 (2.72)	4.16 (5.5)
Removed**	164 {91}		186 {99}		287 {95}		–	124 {86}		39 {87}			{100}		16 {80}	
Cholesterol	21 (7.4)	0.308 (3.19)	10.0 (0.69)	0.437 (5.7)	37 (7.3)	1.39 (4.8)	13 (3.77)	8.50 (3.52)	0.96 (4.13)	6.70 (4.80)	0.369 (2.14)	41.0 (5.6)	25.0 (1.18)	0.79 (13)	15 (5.65)	2.88 (7.0)
Removed**	20.7 {99}		9.6 {96}		35.6 {96}		–	7.5 {89}		6.3 {94}		40.2{98} or 24.2 {97}			12 {80}	
Stigmasterol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	8.40 (4.94)	1.21 (5.6)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Removed**	–	–	–	–	–	–	–	–	–	7.19 {86}		–	–	–	–	–
β-Sitosterol	<LOQ	<LOQ	10.0 (0.85)	1.12 (2.81)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	7.00 (5.6)	<LOQ	<LOQ	<LOQ	<LOQ	11 (7.6)	4.38 (12)
Removed**	–	–	8.9 {89}		–	–	–	–	–	7.0 {100}		–	–	–	6.22 {60}	

Indications: as in Tables 1–4, as well as, * = identified according to their SFIs (details in the text); () = in parentheses relative standard deviation percentages; $infl^{0.5}$ and $infl^{1.0}$ = performed from 0.5 L and from 1.0 L wastewater samples, in two separate parallels. *Note*: identifications were performed from 2-fold up to 10-fold diluted, 375 μL stock solutions; ** = removed under the wastewater treatment process; {} = expressed in the percentages of the corresponding pollutants found in the influent samples.

31%; cholesterol, 27%; stigmasterol, 40% and β-sitosterol, 36%, respectively).

3.3. The steroid content of two Hungarian wastewater treatment plants' influent and effluent samples

Under a 10 month period (from December 2009 to September 2010) influent and effluent wastewater samples from two WWTPs (Dél-Pest, Telki) have been analyzed (Table 5).

Results revealed that

(a) androsterone, and androsterone-3,11-ol-17-one[#], out of eight cases in seven, while transandrosterone, out of eight cases in six, were present in influent samples: their concentrations varied between 0.74 and 4.28 μg/L (androsterone), 0.138 and 4.00 (transandrosterone) and 0.058 and 4.50 μg/L (androsterone-3,11-ol-17-one), respectively. Effluent samples do not contain androsterones. [#](Note: androsterone-3,11-ol-17-one {MW = 306}, as its double TMS-ether (oxime) derivative was unambiguously identified on the basis of its molecular ion $\{[M]^{\ddagger} = m/z\,537\}$ and on its selective fragment ion $\{[M–CH_3]^+ = m/z\,522\}$.

(b) β-Estradiol (0.100 μg/L) and estriol (0.054 μg/L) were found in a single sample, only (Table 5: WWTP Dél-Pest, April 2010).

(c) The high coprostanol (20–302 μg/L) and cholesterol (6.7–47.3 μg/L) contents of influent wastewater samples were considerably decreased under the wastewater treatment process: removal efficiencies varied between 80% and 100% respectively with an average of 90%. (*Note*: The measured coprostanol and cholesterol concentrations taking into consideration their 27–31% recoveries, all of their values are to be multiplied by ≥3.)

(d) Stigmasterol and β-sitosterol, because of their low water solubility and moderate response characteristics were found in the overwhelming part of samples below their LOQ values.

4. Conclusion

(1) Detailed literature overview was presented to clear up the reality, the theoretical and practical importance of the proposed protocols for the simultaneous identification and quantification of the only hydroxy, the only keto and both the hydroxy and keto groups simultaneously containing steroids.

(2) On the basis of an exhaustive mass fragmentation and derivatization study it was shown that in order of convincing distinction and reliable identification and quantification of the various hydroxy- and ketosteroids, the two step derivatization protocol is obligatory: consisting as the first step the oximation of the keto group(s), followed as the second step the trimethylsilylation of the hydroxyl group(s).

(3) Fragmentation pattern characteristics and the mass spectra of steroid derivatives confirmed unambiguous structural relationship in terms of similarities and differences:

The TMS ether derivatives of hydroxy steroids and the TMS (oxime) ether species of 3-ketosteroids proved to be in particular stable providing molecular ions and molecular ions formed by the loss of one methyl group as abundant fragment ions, while the 17 ketosteroids' main characteristic fragment ions are originated from the D ring elimination of the steroid skeleton.

(4) Based on these experiences the practical utility of the proposal was shown by the identification and quantification of the androsterone, transandrosterone, androsterone-3,11-ol-17-one, β-estradiol, estriol, coprostanol, cholesterol stigmasterol, and β-sitosterol contents of two Hungarian WWTPs, applying our optimized protocol, evaluating these pollutants on the basis of their selective fragment ions (eight times under a 10 months period of time).

(5) As to the efficiency of the two Hungarian WWTPs, it is worthy of mention that comparing the steroid contents of the influent and effluent samples, the removal of androsterones, β-estradiol, estriol and any other steroids is quantitative, while the removal of cholesterol, coprostanol and phytosterols varied between 60% and 100%.

Acknowledgements

This work was supported by the National Office for Research and Technology (CD FILTER, Project No. OM-00371/2008) and by the National Committee for Technical Development (SHENZHEN,Project No. OMFB-01676/2009.)

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