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Sensitive Determination of Natural and Synthetic Steroidal Hormones of a Free and Conjugated Form in Surface Water by Gas Chromatography-Mass Spectrometry

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A method based on the TMS derivatives and acidic hydrolysis was developed for the simultaneous determination of free and conjugated steroidal hormones in surface water. A silylation of five natural and two synthetic steroidal hormones was achieved with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide/NH_4I (1000:3) under catalysis of dithioerythritol for 60 min at 80 ºC. TMS derivatives of the steroid hormones containing multifunctional groups offer a single derivative product under this condition. The accuracy of the analytes was in the range of 87 to 110% at a concentration of 20 and 50 ng/L with relative standard deviations of less than 10%. The method detection limit was in the range of 0.01 to 0.02 ng/L for surface water. Natural steroidal hormones were detected in a concentration range of 0 to 1.03 ng/L in free form and 0 to 14.6 ng/L in conjugated form, respectively. We found that most of the natural hormonal steroids exist in conjugate forms (43 to 100%) in river water.

Key Words : GC-MS, Acidic hydrolysis, Natural and synthetic steroidal hormones, Water

Introduction

Natural steroidal hormones, such as estrone (E1), 17β-estradiol (E2), estriol (E3), progesterone (P) and testosterone (T), as well as synthetic steroidal hormones, such as 17α-estradiol (α-E2) and 17α-ethinyl estradiol (EE2) have attracted a great deal of scientific and public attention in recent years because of their prevalence in surface waters and their endocrine-disrupting effects.[1-12] These compounds can mimic the action of physiological estrogens at estrogen receptors to cause reproductive impairment in animals and humans.[1-6] In many regions, the aquatic environment is contaminated by xenoestrogens from domestic and industrial waste discharge.[7-12] Concentrations of steroidal compounds are usually detected at low levels of ng/L. Even at low concentration levels, several steroids have extremely high physiological activity and can still have dramatic effects on the hormonal system of organisms in an aquatic environment.[13]

Many analytical procedures have been proposed for the determination of low ng/L levels for natural or synthetic steroidal compounds in water. Most of these procedures are based on the determination of 17β-estradiol, estrone, estriol, androgens or progesterone in environmental water by enzyme immunoassay,[14-20] high-performance liquid chromatography (HPLC)[21,22] and high-performance liquid chromatography-mass spectrometry (LC-MS).[23-26]

Many researchers have attempted trimethylsilylation in order to improve the gas chromatography-mass spectrometry (GC-MS) behavior of these analytes.[27-34] Silylation is the most common and versatile method used to derivatize organic compounds containing the hydroxyl group; it enhances GC-MS properties with the reduced polarity, enhanced volatility and increased thermal stability necessary for optimal sensitivity and the resolution of various compounds. For compounds such as E1, P and T, which contain two functional groups, a single derivative product is preferred for the establishment of the best analytical method. Zuo[34] reported on the generation of a single product of a di-TMS derivative of EE2 by using in pyridine, but did not apply the method to a real environmental sample.

The purpose of the present experiment is to develop a sensitive and simultaneous determination method of free and conjugated forms of the natural steroidal hormones (E1, E2, E3, P and T) and synthetic steroidal estrogen (α-E2 and EE2) in environmental water. The derivatization is performed by the reaction of steroidal hormones with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA)/NH_4I (1000:3) under the catalysis of dithioerythritol.

Experimental

Chemicals and Reagents. The following chemicals were purchased from Sigma (St. Louis, MO, USA) : estrone, 17α-estradiol, 17β-estradiol, estriol, 17α-ethinyl estradiol, progesterone, testosterone, testosterone-d_3, dithioerythritol, NH_4I, MSTFA. For the reagents, we used analytical grade of potassium carbonate, potassium hydroxide, potassium bis-hydrogen phosphate, sodium sulfate, hydrochloric acid and sodium chloride (Sigma, St. Louis, MO, USA). For the solvents, we used methanol and methylene chloride (E. Merck, Darmstadt, Germany).

Water Sampling. Surface water samples were collected from 5 basins of Kum river in Korea and without headspace in 1 L brown glass bottle containing 3 drop of 2 M HCl. Sewage samples were collected from influent water to sewage treatment facilities (Hongseung, Chungnam) and without headspace in 1 L brown glass bottle containing 3 drop of 2 M HCl.

Hydrolysis. A 500 mL sample of surface water was placed in a 1000-mL round flask. We added 50 mL of conc HCl to the solution, and boiled the sample for 2 h in Soxlet. The solution was neutralized with 50 mL of 12 N NaOH.

Extraction Procedure. A 500 mL sample of surface water was placed in a 1000-mL separating funnel. We added 25 μL of a testosterone-d_3 internal standard solution (500 μg/L in acetonitrile) to the solution and extracted the sample two times with 20 mL of methylene chloride by subjecting the mixture to mechanical shaking for 10 min. The total organic phase was evaporated in a vacuum rotary evaporator and eventually dried finally in nitrogen stream to dryness.

Derivatization. A dry residue is dissolved with 70 mL of silylating reagents (MSTFA + 0.3% NH_4-I + 0.8% dithioerythritol (*v*/*w*/*w*)), and the tubes are heated at 80 °C. At appropriate times, a 2 mL sample of the solution is injected in the GC system.

Gas Chromatography-Mass Spectrometry. All the mass spectra were obtained with an Agilent 6890/5975 instrument. The ion source was operated in an electron ionization mode (EI; 70 eV, 230 °C). Full-scan mass spectra (*m*/*z* 40-800) were recorded for analyte identification. Separation was achieved with an HP fused-silica capillary column with cross-linked 5% phenyl methylsilicone (DB 5); the column has a length of approximately 30 m, an inner diameter of 0.25 mm and a film thickness of 0.25 mm. Samples were injected in a splitless mode. The flow rate of the helium was 1.0 mL/min. The operating parameters were as follows: injector temperature, 310 °C; transfer line temperature, 300 °C; oven temperature, programmed from 150 °C at 12 °C·min^{-1} to 310 °C (hold for 4 min). The ions that selected for monitoring by SIM were *m*/*z* 414, 399 and 309 for E1, *m*/*z* 416, 285 and 410 for α-E2 and E2, *m*/*z* 504, 345 and 311 for E3, *m*/*z* 440, 425 and 300 for EE2, *m*/*z* 458, 443 and 459 for P, *m*/*z* 432, 433and 417 for T, and *m*/*z* 435, 436 and 420 for Tes-d_3 (internal standard).

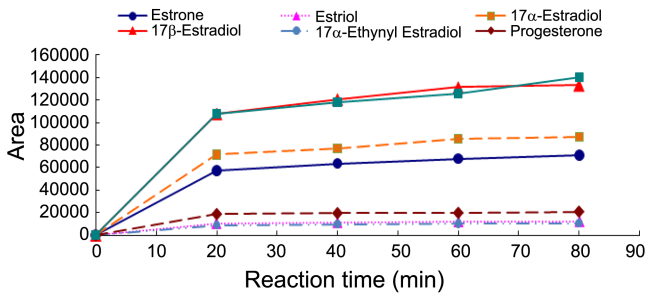

Figure 1. Time course of the reaction of E1, α-E2, E2, E3, EE2, P and T with MSTFA/NH_4-I (1000:3) containing 0.1 mg of dithioerythritol.

Calibration and Quantification. Calibration curves for E1, α-E2, E2, E3, EE2, P and T were established by extraction and derivatization after the addition of 0.05, 0.1, 1.0, 10, 25 and 50 ng of standards and 12.5 ng of the internal standard in 500 mL of milli-Q water. The concentration range of the calibration curves is from 0.1 to 100 ng/L. The ratio of the peak area of the standard to that of the internal standard is used in the quantification of the compound.

Results and Discussion

Derivatization. E1, α-E2, E2, E3, EE2, P and T contain more than one derivatizable functional group. E1 has an

Table 1. Mass characteristic ions of steroid derivatives

Derivative	M^+	M^+-CH_3	M^+-OTMS+H	M^+-HOTMS	M^+-OTMS-CH_4	M^+-TMSO-CH_2-CH_3	D-ring cleavage	$[TMS]^+$	Others
di-TMS-E1	414(87.2)	399(55.1)	–	–	309(16.6)	–	285(4.8)	73(100)	155(8.0) 231(4.3)
di-TMS-α-E2	416(94.1)	401(7.1)	–	326(10.6)	309(3.5)	298(9.4)	285(72.4)	73(100)	129(32.4) 232(24.1)
di-TMS-β-E2	416(100)	401(9.3)	–	326(11.0)	309(4.0)	298(9.3)	285(87.3)	73(74.1)	129(33.9) 232(26.0)
tri-TMS-E3	504(76.9)	489(9.3)	–	414(7.7)	399(5.0)	386(26.4)	285(23.7)	73(100)	311(36.8)
di-TMS-EE2	440(26.7)	425(100)	–	350(4.6)	335(3.6)	322(5.1)	285(45.6)	73(96.4)	345(39.6) 231(29.2) 300(26.7)
di-TMS-P	458(63.5)	443(46.5)	370(7.1)	–	353(4.1)	–	–	73(100)	157(32.9)
di-TMS-T	432(100)	417(13.1)	–	342(1.3)	327(2.0)	–	–	73(96.7)	247(9.4) 129(9.8) 208(11.1)
di-TMS-T-d_3	435(100)	420(16.9)	–	345(1.5)	330(2.4)	–	–	73(99.4)	131(15.7) 209(17.5)

( ) = relative abundance

acidic hydroxyl group of the aromatic ring and a carbonyl group, which stands in equilibrium with the enolic hydroxyl group. E2 and E3 contain an acidic hydroxyl group of the aromatic ring and one or two less acidic hydroxyl group of the aliphatic ring, respectively. EE2 contains an acidic hydroxyl group of the aromatic ring and a less acidic, more sterically hindered hydroxyl group of the aliphatic ring. P contains two enolic carbonyl groups. T contains an enolic carbonyl group and a less acidic hydroxyl group of the aliphatic ring. The reactivity of the steroids was tested with various reactive functional groups and a silylating reagent. The silylating reagent, MSTFA, which includes 0.3% NH_4-I/dithioerythritol as a catalyzing agent was tested in terms of reactivity and repeatability of the derivatives. The derivatives were analyzed at reaction times of 20, 40, 60 and 90 min.

The reaction rate of steroids with MSTFA/NH_4-I (1000:3, *v*/*w*) containing 0.1 mg of dithioerythritol was determined by detecting the products (Figure 1). The steroids showed a relatively rapid and complete reaction with MSTFA/NH_4-I (1000:3) containing 0.1 mg of dithioerythritol. A ketone group of E1 and T, and two ketone groups of P were conv-

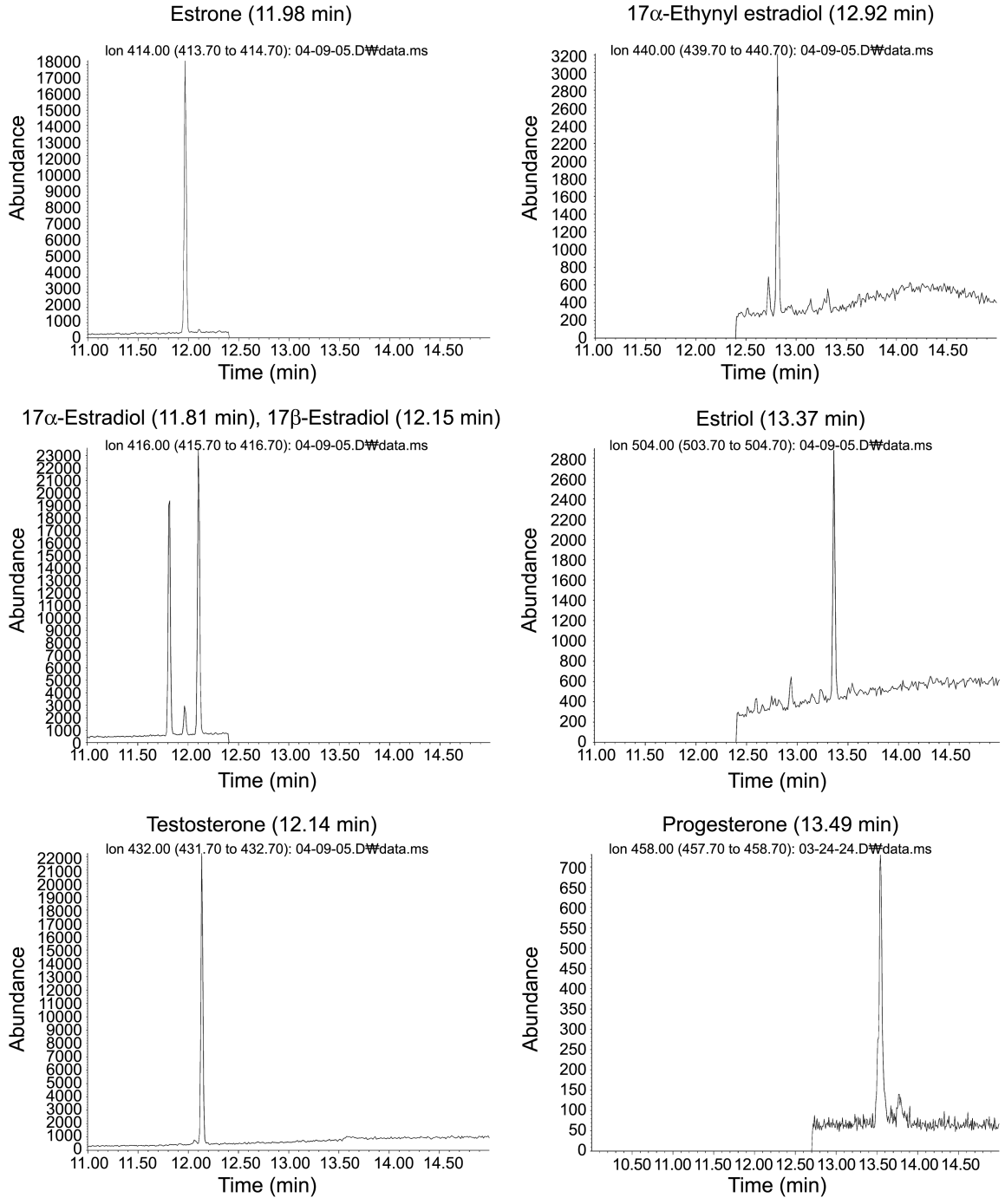

Figure 2. GC-MS chromatogram of the derivatives after the extraction of steroidal hormones in water spiked in concentration of 0.5 ng/L.

Table 2. Calibration curves of the target compounds

Compound	Retention time	Linear equation	Correlation coefficient
di-TMS-E1	11.98	y = 0.057x − 0.033	0.997
tri-TMS-E3	13.37	y = 0.002x + 0.003	0.979
di-TMS-17α-E2	11.81	y = 0.071x − 0.012	0.997
di-TMS-E2	12.15	y = 0.080x − 0.032	0.997
di-TMS-EE2	12.92	y = 0.009x − 0.002	0.997
TMS-P	13.49	y = 0.010x + 0.041	0.994
di-TMS-T	12.14	y = 0.059x + 0.116	0.996

erted to TMS-enol in 60 min at 80 °C. The repeatability of the derivatives measured by RSD was 1.9 to 5.6%.

Mass Spectrometry. The mass fragmentation of the di-TMS-E1, di-TMS-α-E2, di-TMS-E2, tri-TMS-E3, di-TMS-EE2, di-TMS-P, di-TMS-T and di-TMS-T-d_3 under electron ionization at 70 eV is summarized in Table 1. The molecular ion and the fragment ions formed by the loss of a methyl group, HO-TMS, O-TMS + CH_4 and TMS-O-CH_2-CH_3 from the molecular ion were characteristic. The fragment ion was formed by D-ring cleavage in the molecular ion.

Chromatography. Figure 2 shows a chromatogram of the di-TMS-E1, di-TMS-α-E2, di-TMS-E2, tri-TMS-E3, di-TMS-EE2, di-TMS-P, di-TMS-T and di-TMS-T-d_3. The use of nonpolar stationary phase was found to be efficient for the GC separation of the derivatives. The peaks are symmetrical and no tailing can be seen. The derivatives also fail to show any adsorption effects in the GC system. Table 2 shows the retention times of di-TMS-17α-E2, di-TMS-E1, di-TMS-E2, di-TMS-T-d_3, di-TMS-T, di-TMS-EE2, tri-TMS-E3, di-TMS-P. There are no extraneous peaks observed in a chromatogram of a sample at the retention times of the analytes.

Hydrolysis. Hydrolysis of steroid hormones has generally been performed by enzymatic method, but it is hard to apply this method in case of large volume sample. Therefore, acidic hydrolysis method was selected for the hydrolysis of

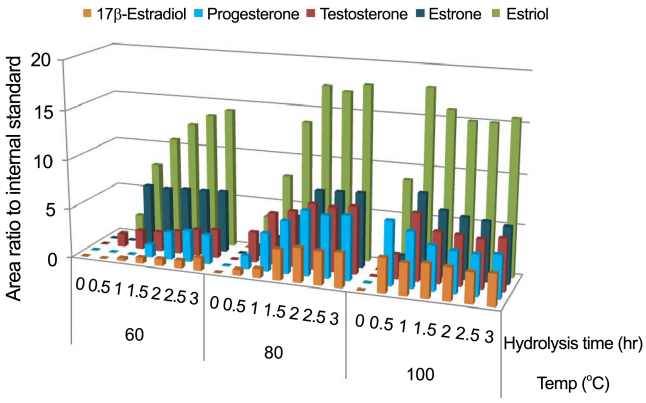

Figure 3. Efficacy of acidic hydrolysis of natural conjugated steroids in relation to various hydrolysis time and temperature conditions.

conjugated steroids in large volume of river water. Influent to sewage treatment facilities, which contains a considerable amount in mixture state of conjugated natural steroids, was used to obtain optimal acidic hydrolysis conditions. To identify the experimental conditions for obtaining acidic hydrolysis of conjugated steroids, we evaluated the best reaction conditions by reacting the samples at 60, 80 and 100 °C for periods ranging from 0, 30, 60, 90, 120, 150 and 180 min. The minimum amount of HCl needed to complete hydrolysis was evaluated in three acid morality (0.5, 1.0 and 2.0 M). As a result, an optimal hydrolysis condition of conjugated steroids was attained when the analytes were reacted for 2 h at 80 °C (Figure 3). At the time, the optimal morality of the HCl solution was 1.0 M. The hydrolysis in 0.5 M HCl solution was not complete even in 180 min, otherwise that in 2.0 M HCl solution was not reproducible.

Extraction and recovery. In spite of improvements in the conditions of other alternative extraction techniques, liquid-liquid extraction (LLE) is still an efficient technique for routine analysis of steroids in water. Several water samples at a concentration of 2.0 μg/L were prepared, and the

Table 3. Intra laboratory precision and accuracy results of the steroid analysis

Compound	Spiked conc. (ng/L)	Measured conc. (ng/L)					Mean ± SD (RSD%)
E1	20	23.2	22.3	21.6	21.2	22.4	22.1 ± 0.8 (3.5)
	50	55.3	52.1	54.6	48.1	46.9	51.4 ± 3.8 (7.3)
E3	20	20.1	16.9	19.4	21.2	19.7	19.4 ± 1.6 (8.1)
	50	44.5	40.5	45.8	45.7	41.4	43.6 ± 2.5 (5.7)
α-E2	20	24.2	23.4	19.6	19.4	20.7	21.5 ± 2.2 (10.2)
	50	56.4	52.3	55.4	57.0	45.3	53.3 ± 4.8 (9.0)
E2	20	21.8	21.5	19.9	19.6	21.1	20.8 ± 1.0 (4.6)
	50	55.6	53.4	58.3	50.2	45.9	52.7 ± 4.8 (9.1)
EE2	20	21.8	22.1	21.8	22.1	23.1	22.2 ± 0.6 (2.5)
	50	55.5	55.8	56.6	46.3	49.0	52.6 ± 4.7 (8.9)
P	20	20.6	22.1	21.6	22.4	22.6	21.9 ± 0.8 (3.6)
	50	51.8	50.7	50.7	48.6	49.7	50.3 ± 1.2 (2.4)
T	20	22.4	22.6	22.5	21.7	23.2	22.5 ± 0.5 (2.4)
	50	54.9	54.5	55.9	55.1	56.3	55.3 ± 0.7 (1.3)

Table 4. Comparison of analytical methods for determining steroidal compounds in water

Ref	Matrix (Sample Vol)	Preparation method	Instrument	Method detection limit (ng/L)						
				E1	α-E2	E2	E3	EE2	P	T
[18]	River water	SPE	ELISA	1.25	–	–	–	–	–	–
[19]	River water	–	ELISA	–	–	2.3	4.3	–	–	–
[20]	River water	DLLME	LC	200	–	100	–	–	–	–
[22]	River water	MIP	LC-ESMS	–	–	1.8	–	–	–	–
[23]	River water	SPE	LC-MS/MS	–	–	–	–	–	0.16	–
[25]	River water	SPE	LC-MS/MS	0.4	–	0.5	2.0	1.0	–	–
[26]	River water	Acylation and Silylation, SBSE	GC-MS	–	–	0.5	–	–	–	–
[28]	River water	SPE, Silylation	GC-MS	1.7	–	3.4	–	0.8	–	–
[30]	River water	SPE, Silylation	GC-MS/MS	0.3	–	0.6	1.0	1.0	–	–
[32]	River water	SPE, Silylation	GC-MS	–	–	5	10	–	–	–
This study	River water	LLE, Silylation	GC-MS	0.01	0.01	0.01	0.02	0.02	0.02	0.01

DLLME: dispersive liquid-liquid microextraction. CEI: chemiluminescence enzyme immunoassay. MIP: molecularly imprinted polymers. FEI: fluorometric enzyme immunoassay. SBSE: stir bar sorptive extraction

Table 5. Analytical results of natural and synthetic steroidal hormones in river water (n = 5)

Compound	Analytical results (ng/L)				
	Free form		Conjugated form		
	Detected conc range	Mean ± SD	Detected conc range	Mean ± SD	Conjugated form (%)
E1	0-1.03	0.61 ± 0.50	0-14.55	0.76 ± 5.67	56
E3	ND	–	0-1.45	0.35 ± 0.56	100
α-E2	ND	–	ND	–	–
E2	0-0.34	0.28 ± 0.14	0-0.35	0.21 ± 0.12	43
EE2	ND		ND	–	–
P	0-0.44	0.14 ± 0.18	0-3.25	0.13 ± 1.28	48
T	0-0.61	0.26 ± 0.23	0.03-0.61	0.27 ± 0.22	51

relative recovery was calculated in terms of the percentage of derivatives recovered. The recovery values of E1, α-E2, E2, E3, EE2, P and T are in the range of 102 and 103%.

Validation of the assay. A linear relation with an average correlation coefficient of 0.97 was found in our examination of a typical standard curve. We used a least squares fit to compute a regression line of the peak area ratios of di-TMS-E1, di-TMS-17α-E2, di-TMS-E2, tri-TMS-E3, di-TMS-EE2, di-TMS-P and di-TMS-T to di-TMS-TES-d_3 on concentration. Table 2 shows the lines of the best fit and the correlation coefficients for the steroids.

Table 3 confirms that the reproducibility of the assay is very good. For five independent determinations at 20 and 50 ng/L, the coefficient of variation is less than 10%.

The method detection limit is defined as 3.14 times the standard deviation of replicate determinations (n = 7) of samples spiked at a concentration of 0.01 ng/L in river water, in which analytes were not detected. Our results from this method were 0.01 to 0.02 ng/L for a 0.5 L sample of water. The high sensitivity of the derivative by EI-MS (SIM) permits the determination of di-TMS-E1, tri-TMS-E3, di-TMS-17α-E2, di-TMS-E2, di-TMS-EE2, di-TMS-P and di-TMS-T at concentrations well below those reported previously. Table 4 compares various analytical methods for determining steroids in water.

Real sample analysis. We applied a method of analyzing target compounds in five surface water samples. The five water samples were analyzed directly without hydrolysis and with acidic hydrolysis. E1, E2, E3, EE2, P and T were detected in a concentration range of 0 to 1.03 ng/L in free form and 0 to 14.6 ng/L in conjugated form as shown in Table 5, respectively. We found that most of the natural hormonal steroids exist in conjugate forms (43 to 100%) in river water. Until now, the concentration of steroids in natural water was reported only as free form and was considered a risk assessment factor. At this point, we suggest that the conjugated forms of the natural steroids must be considered in terms of their endocrine-disrupting effects.

Conclusion

A sensitive and simultaneous method has been developed for the analysis of natural and synthetic steroids in surface water. The silylation derivatives of steroids containing multi-functional groups with MSTFA/NH_4I (1000:3) containing dithioerythritol have good chromatographic properties and offer a single derivative product. The extraction of these compounds from surface water with methylene chloride

yields a high rate of recovery with a small degree of variation. The quantification of steroids is excellent. The linear calibration curves cover a range of 0.1 to 100 ng/L, and the method detection limits are 0.01 to 0.02 ng/L for a 0.5 L sample of water. In the present study, the suggested method enables the successful determination of trace amounts of natural and synthetic steroidal compounds and can be used for routine analysis of drinking water.

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