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Development of Noninvasive Method for the Automated Analysis of Nine Steroid Hormones in Human Saliva by Online Coupling of In-Tube Solid-Phase Microextraction with Liquid Chromatography–Tandem Mass Spectrometry

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Abstract: Accurate measurement of steroid hormones is crucial to elucidate new mechanisms of action and diagnose steroid metabolism-related diseases. This study presents a simple, sensitive, and automated analytical method for nine representative steroid hormones. The method involves on-line coupling of in-tube solid-phase microextraction (IT-SPME) with liquid chromatography–tandem mass spectrometry (LC–MS/MS). The steroid hormones were extracted and enriched on a Supel-Q PLOT capillary column using IT-SPME. Subsequently, they were separated and detected within 6 min using a Discovery HS F5-3 column and positive ion mode multiple reaction monitoring system via LC–MS/MS. Calibration curves of these compounds using each stable isotope-labeled internal standard (IS) showed linearity with correlation coefficients greater than 0.9990 in the range of 0.01–40 ng/mL, with limits of detection (S/N = 3) of 0.7–21 pg/mL. Moreover, intra- and inter-day variations were lower than 8.1 and 15% (n = 6), respectively. The recoveries of these compounds from saliva samples were in the range of 82–114%. The developed IT-SPME/LC–MS/MS method of steroid hormones is a highly sensitive, specific, and non-invasive analytical method that allows extraction and enrichment with no organic solvents, and enables direct automated online analysis by simply ultrafiltrating a small sample of saliva.

Keywords: steroid hormones; saliva; noninvasive sampling; in-tube solid-phase microextraction; liquid chromatography–tandem mass spectrometry; online automated analysis; sample preparation

Citation: Hitomi, T.; Kataoka, H. Development of Noninvasive Method for the Automated Analysis of Nine Steroid Hormones in Human Saliva by Online Coupling of In-Tube Solid-Phase Microextraction with Liquid Chromatography–Tandem Mass Spectrometry. *Analytica* **2024**, *5*, 233–249. https://doi.org/10.3390/analytica5020015

Academic Editor: Marcello Locatelli

Received: 16 April 2024
Revised: 28 April 2024
Accepted: 4 May 2024
Published: 9 May 2024

1. Introduction

Steroid hormones, which play a central role in many biological functions throughout life [1–5], have been reported to be associated with a variety of diseases, including stress [6], autism spectrum disorders [7], Alzheimer's disease [8] and cognitive dysfunction [9] in old age, Parkinson's disease [10], diabetes [11], cardiovascular disease [12], cancer [13–15], primary aldosteronism [16], hypogonadism [17,18], ovarian disorders [19–21], Cushing syndrome [22–24], congenital adrenal hyperplasia [25], and hypoadrenal and hyperadrenal insufficiencies [26]. Therefore, quantitation of steroid hormones is crucial for determining physiological concentration ranges that allow differentiation between normal fluctuations and pathological levels. However, concentrations of steroid hormones may fluctuate widely due to biological factors such as physical activity, sex, age, stress, and diet [2,27–29]. Moreover, certain steroids, like adrenal and estrogenic hormones, are influenced by circadian and menstrual rhythms. Hence, these factors must be considered when collecting specimens [2,27,30–32]. Steroid hormones are measured in various biological substances, including blood, urine, saliva, hair, cerebrospinal fluid, semen, breast milk, and amniotic fluid [1–4,6,30]. It is usual to measure real-time levels in blood and saliva, daily to hourly average levels in urine, and long-term levels in hair [33]. Blood

is the most commonly used clinically. However, its collection is invasive, and the majority of steroid hormones present are bound to proteins such as albumin and globulin, with only trace amounts in bioactive free form [18,30]. Saliva is a simple noninvasive specimen for hormone analysis. It can be easily and repeatedly collected [19,34] with most steroid hormones present in their free form [30]. Although not all steroid hormones have been studied, a strong correlation between blood- and saliva-obtained steroids is evident [4,8,9,27,35–39], and saliva is an excellent alternative for measuring endogenous steroids instead of serum or plasma [27,30,34,40,41]. However, in most saliva analyses reported so far, measurement was limited to specific hormones including progesterone, testosterone, and cortisol [11,18–25,35–40]. Simultaneous quantification of multiple steroid hormones is an important means of comprehensive profiling of in vivo steroids [1–4,33]. However, hormone concentration in saliva is very low, at 10% or less of that in blood [39,42], making the development of specific, accurate, and sensitive methods an important issue.

The main methods of measuring steroid hormones are immunoassay (IA) and mass spectrometry (MS) [2–5,23,31]. IA is routinely used in clinical testing as a cost-effective, simple technique for measuring a single analyte. However, IA cannot simultaneously analyze multiple analytes, lacks sensitivity and specificity due to cross-reactivity with structurally similar compounds, and is not easily reproducible [2,5,19,43,44]. In contrast, MS-based methods provide structural information on the analytes, resulting in higher specificity, and enable simultaneous quantification of a large number of analytes in conjunction with chromatography [2,5,18,19,31,33,44]. Currently, GC tandem MS (GC–MS/MS) and liquid chromatography–tandem MS (LC–MS/MS) are highly reliable steroid-measurement methods that can replace IA due to their excellent robustness, sensitivity, and selectivity [2,18,31]. Although GC–MS/MS is highly sensitive and accurate, it requires laborious derivatization to convert it to volatile compounds, rendering it expensive when measuring a large number of analytes. LC–MS/MS is a highly useful tool in routine clinical diagnostics because it can directly detect steroids and their metabolites with high sensitivity and accuracy in small samples in little time [2,31,45]. However, there are several limitations to the use of LC–MS/MS methods. For example, to measure steroid hormones present in trace amounts in complex biological matrices, sample pretreatment to remove coexisting substances such as proteins that clog the injector and column frit is essential [1,2]. Also, the concentration range of different steroid hormones in the matrix is wide, and ionization efficiency differs by compound, making simultaneous detection difficult with widely varying sensitivity [1,2]. Furthermore, in the exhaustive analysis of multiple compounds, the greater the number of compounds, the smaller the scanning speed becomes, resulting in reduced sensitivity and other problems [4]. Therefore, various studies have been conducted to overcome these problems. The main extraction methods are liquid–liquid extraction (LLE), supported liquid extraction (SLE) and solid-phase extraction (SPE) [2,44–46], but LLE requires large amounts of organic solvents and has disposal issues [44]. Recently, in addition to SPE, miniaturized sample preparation methods such as dispersion–liquid–liquid microextraction (DLLME) [47–49], micro-SPE [44], and solid-phase microextraction (SPME) [50–52] have been reported. Conversely, the electrospray ionization (ESI) method of LC–MS/MS is strongly dependent on the structure of each steroid and mobile phase composition, which affects the sensitivity and specificity of the ions, and may be affected by matrix interference [2]. However, it has been reported that ion suppression and enhancement effects can be reduced by reducing the flow rate and increasing ionization efficiency [33,53].

Many methods to determine steroid hormones in saliva via LC–MS/MS have been reported [18,22–25,27,30,39–42,54,55], but all require tedious sample pretreatment. Consequently, we introduced an in-tube SPME (IT-SPME) method allowing simple sample extraction and enrichment. The IT-SPME method is easily set up by incorporating open-tube fused silica capillaries with coated inner surfaces as an extraction device into an LC autosampler. It requires almost no organic solvent and enables automated analysis based on an on-line column-switching technique [56]. We have developed IT-SPME/LC–MS/MS methods for various biological components, including hormones and metabolites

in saliva [51,57–63]. Comprehensive profiling of steroid hormones is important to facilitate understanding of new mechanisms of action of steroid hormones and for accurate diagnosis of steroid-related diseases [30,44,55,64]. Therefore, in this study, we optimized IT-SPME conditions for nine representative steroid hormones and developed a novel highly sensitive analytical method coupled with stable isotope dilution LC–MS/MS to establish a non-invasive and simple method for the analysis of trace amounts of salivary steroids.

2. Materials and Methods

2.1. Reagents and Standard Solutions

The standard and internal standard (IS) used in this study are shown in Figure 1. Estrone (E1), 17β-estradiol (E2), and estriol (E3) were from Sigma-Aldrich Japan (Tokyo, Japan); pregnenolone (Preg), progesterone (Prog), cortisol (CRT), and testosterone (TES) were from Nacalai Tesque (Kyoto, Japan); dehydroepiandrosterone (DHEA) was from Tokyo Kasei Kogyo (Tokyo, Japan); and aldosterone (Ald) was from Toronto Research Chemicals Inc. (TRC, North York, ON, Canada). Their stable isotope-labeled compounds including E1-d_4 (isotopic purity 98.4%, TRC), E2-d_4 (isotopic purity 97.6%, TRC), E3-d_3 (isotopic purity 98.1%, TRC), Preg-d_4 (isotopic purity 97.5%, TRC), Prog-d_9 (isotopic purity 96.6%, TRC), Ald-d_4 (isotopic purity 95%, TRC), CRT-d_4 (isotopic purity 98.5%, TRC), TES-d_3 (isotopic purity >98%, Sigma-Aldrich), and DHEA-d_2 (isotopic purity 97%, CDN Isotope Inc., Quebec, Canada) were used as IS. The standard and IS compounds were dissolved in LC–MS grade methanol to make a 0.1 mg/mL solution, then tightly capped and stored at 4 °C. Three groups of stored standard solutions of these steroid hormones were prepared according to detection sensitivity. Group 1 (Prog, TES), Group 2 (E2, Ald, CRT, DHEA), and Group 3 (E1, E3, Preg) were methanol solutions containing 0.5 μg/mL, 2.5 μg/mL, and 10 μg/mL of each compound, respectively. Working standard solutions containing the nine steroid hormones were prepared from the stock solutions via 100-fold pre-dilution with LC–MS grade distilled water prior to use to obtain 5 ng/mL, 25 ng/mL, and 100 ng/mL for groups 1, 2, and 3, respectively. In addition, stable isotope-labeled compounds of the nine steroid hormones were similarly diluted to obtain nine IS mixed solutions. All were tightly capped and stored at 4 °C. LC–MS grade acetonitrile and distilled water used as mobile phases were from Kanto Chemical (Tokyo, Japan); all other chemicals were of analytical reagent grade.

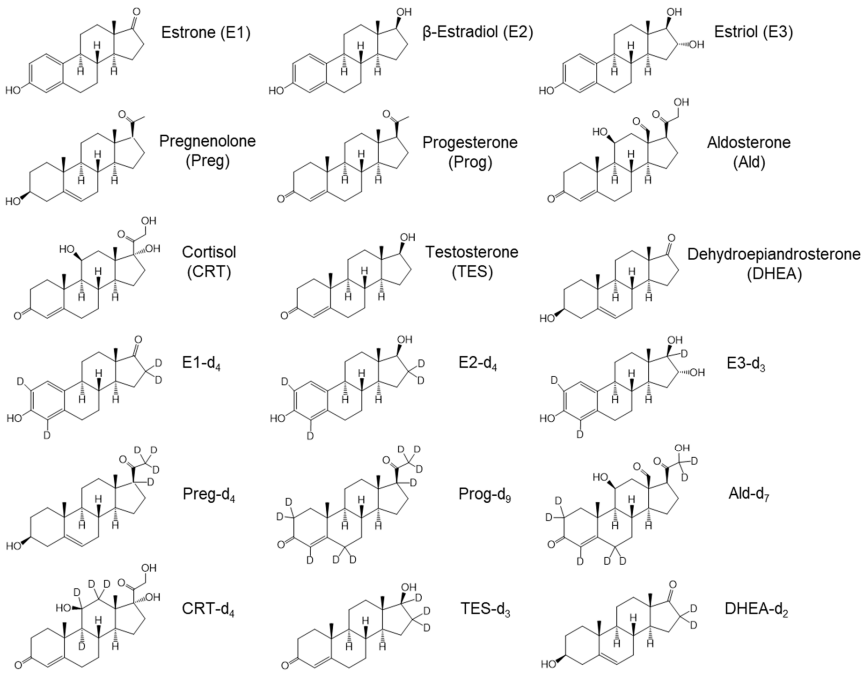

Figure 1. Structures of steroid hormones and their stable isotope-labeled internal standards.

2.2. LC–MS/MS Analysis

LC–MS/MS analysis was performed using Model 1100 series LC system (Agilent Technologies, Boeblingen, Germany) and API 4000 triple quadruple mass spectrometer (Applied Biosystems, Foster City, CA, USA). LC separation was performed on a Discovery HS F5-3 column (100 mm × 2.1 mm, particle size of 3 µm; Supelco, Bellefonte, PA, USA). The column was maintained at 40 °C with an isocratic mobile phase of 0.2% formic acid/acetonitrile (45/55, *v*/*v*) and flow rate of 0.2 mL/min. ESI–MS/MS conditions included the following: turbo ion spray voltage and temperature of 5500 V and 600 °C, respectively; ion source gas GS1 and GS2 flows of 50 and 80 L/min, respectively; a curtain gas (CUR) flow of 30 L/min; collision gas (CAD) flow of 4 L/min and dwell time of 55.5 msec. Other parameters including multiple reaction monitoring (MRM) transitions in positive ion mode are shown in Table 1. Quantification was performed via MRM of the protonated precursor molecular ions $[M+H]^+$ and the related product ions for each compound. Quadrupoles Q1 and Q3 were set at unit resolution (Table 1). LC–MS/MS data analysis was performed using Analyst Software 1.6.2 (Applied Biosystems).

Table 1. MRM transitions and setting parameters for steroid hormones and their internal standards.

Compound	Mass Transition (*m*/*z*)	DP [1] (V)	EP [2] (V)	CE [3] (V)	CXP [4] (V)
E1	271.2 → 253.5	70	10	15	10
E2	255.3 → 159.4	70	10	25	10
E3	271.2 → 253.5	70	10	15	10
Preg	317.5 → 159.5	70	5	30	15
Prog	315.5 → 97.2	75	5	30	10
Ald	361.4 → 315.5	80	5	25	10
CRT	363.0 → 120.9	70	10	30	10
TES	289.0 → 109.0	70	10	35	10
DHEA	289.4 → 271.4	40	10	13	10
E1-d_4	275.3 → 257.5	70	10	15	10
E2-d_4	259.4 → 161.4	70	10	25	10
E3-d_3	274.3 → 256.4	70	10	15	10
Preg-d_4	321.2 → 159.6	70	5	30	10
Prog-d_9	324.5 → 100.3	75	5	30	10
Ald-d_4	368.4 → 322.4	80	5	30	10
CRT-d_4	367.4 → 121.4	70	10	30	10
TES-d_3	292.0 → 109.4	70	10	35	10
DHEA-d_2	291.4 → 273.5	20	10	30	15

[1] Declustering potential (V); [2] entrance potential (V); [3] collision energy (V); [4] collision cell exit potential (V).

2.3. In-Tube SPME

IT-SPME using a capillary tube as extraction device was performed as described [51,59]. The schematic diagram of the IT-SPME system is shown in Figure 2. The GC capillary (60 cm × 0.32 mm i.d.) was threaded through a 2.5 cm sleeve of 1/16-inch polyetheretherketone tubing (330 µm i.d.) at each end. It was then connected between the injection needle and the injection loop of the autosampler using a standard 1/16-inch stainless steel nut, ferrule, and connector. The injection loop was retained in the system to avoid fouling of the metering pump. CP-Sil 5CB (100% polydimethylsiloxane, film thickness 5 µm), CP-Sil 19CB (14% cyanopropyl phenyl methylsilicone, film thickness 1.2 µm), CP-Wax 52CB (polyethylene glycol, film thickness 1.2 µm) (Varian Inc., Lake Forest, CA, USA), Carboxen 1006 PLOT (Carboxen molecular sieves, film thickness 15 µm), and Supel-Q PLOT (divinylbenzene polymer, film thickness 17 µm) (Supelco, Bellefonte, PA, USA) were used to compare extraction efficiencies.

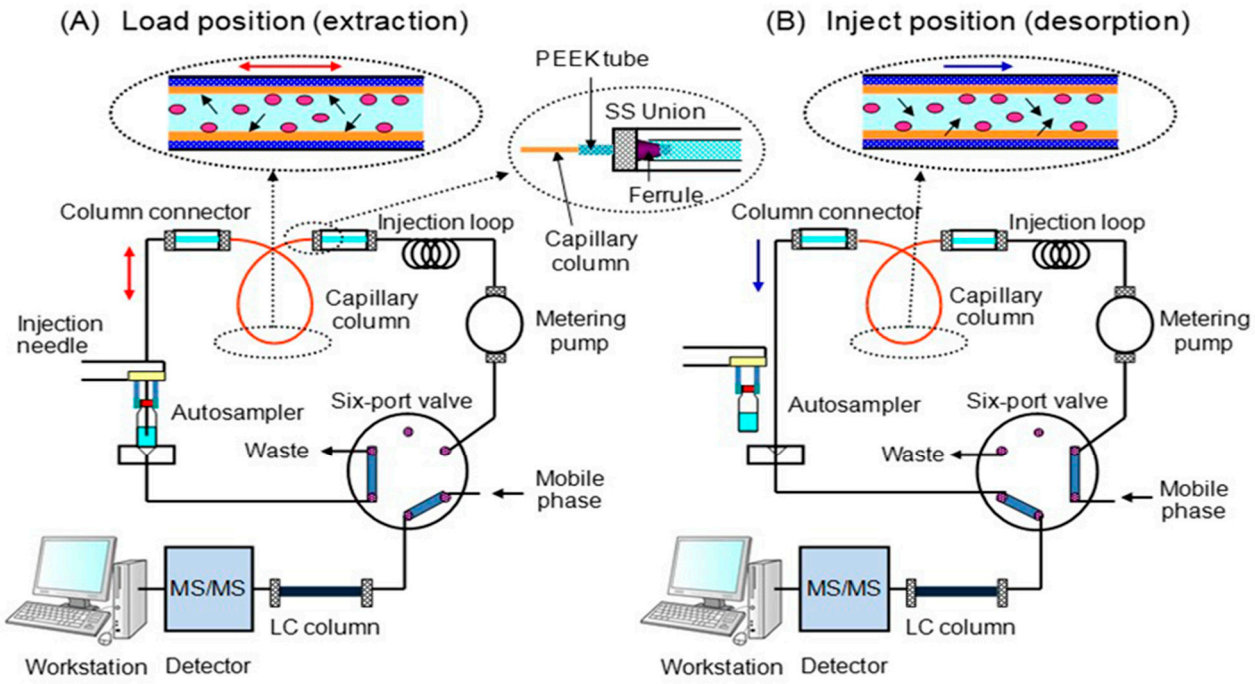

Figure 2. Schematic diagram of the on-line IT-SPME LC–MS/MS system. Analytes in the sample solution are extracted into the capillary column in the Load position (**A**), and after switching the six-port valve in the Injection position (**B**), the mobile phase flows into the capillary to desorb the analytes and inject them directly into the HPLC. The red two-way arrows indicate repeated draw/eject of sample solution, and the blue arrows indicate mobile phase flow.

The autosampler software of Analyst version 1.6.2 was programmed to control the extraction and desorption via IT-SPME and injection into the LC. A 2 mL screw cap autosampler vial with silicone/PTFE septa was filled with 1 mL of sample solution and placed in the autosampler sample tray. In addition, three autosampler vials (1.5 mL methanol, 1.5 mL water, and another blank) were set in the autosampler. Prior to sample extraction, the needle and the inside of the capillary tube were cleaned by two repeated draw/eject cycles of 40 µL of methanol at a flow rate of 200 µL/min, 50 µL of air was aspirated from the blank vial, and then it was conditioned by two repeated draw/eject cycles of 40 µL of water at a flow rate of 200 µL/min with the six-port valve in the LOAD position, Figure 2A. During these operations, the presence of an air gap was necessary to prevent the mixing of the mobile phase and sample. Additionally, it facilitated the desorption of the analyte from the capillary coating by the mobile phase after the extraction step. Steroid hormones were then extracted onto the capillary coating by 25 repeated draw/eject cycles of 40 µL of sample at a flow rate of 200 µL/min with the six-port valve in the LOAD position, Figure 2A. After extraction, the tip of the injection needle was washed with one aspiration/drain cycle of 2 µL of methanol from another autosampler vial.

The extracted compounds were desorbed from the capillary coating with the mobile phase by switching the 6-port valve to the INJECT position, Figure 2B, transported to the LC column, and detected via the MS/MS system. During analysis, the SPME capillary was washed with the mobile phase and conditioned for the next extraction. The extracted compounds were desorbed from the capillary coating with the mobile phase by switching the 6-port valve to the INJECT position, were transported to the LC column, and were detected via the MS/MS system. These steps were fully automated by the autosampler software.

2.4. Method Validation Study

The linearity, limit of detection (LOD), and precision of the developed analytical methods were validated according to the criteria recommended by the ICH guidelines [65].

Since the detection sensitivity of steroid hormones varies among the compounds, the linearities for Group 1 (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 ng/mL), Group 2 (0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 ng/mL), and Group 3 (0.2, 0.4, 1, 2, 4, 10, 20 and 40 ng/mL) were validated by triplicate analyses of standard mixtures in eight concentration ranges, in the presence of 0.5, 2.5, and 10 ng/mL of IS for Groups 1, 2, and 3, respectively. Calibration curves were constructed from the peak height ratios of each compound to their IS at each concentration. The LOD and LOQ were calculated from the signal-to-noise ratio (S/N) of three and ten, respectively. The precision, expressed as relative standard deviation (RSD, %), was validated by six independent analyses on the same day (intra-day precision) and six analyses on six different days (inter-day precision), using mixed standard solutions containing Group 1 (0.05, 0.2, and 1 ng/mL), Group 2 (0.25, 1, and 5 ng/mL), and Group 3 (1, 4, and 20 ng/mL).

2.5. Sampling and Preparation of Saliva Samples

Human saliva samples were provided by five healthy men and five healthy women. The experiment protocol was approved by Shujitsu University ethics committee, and all volunteers provided written informed consent. Saliva samples were collected in Salisoft® consisting of a tube and polypropylene-polyethylene swab (Assist, Tokyo, Japan). The tubes were centrifuged at 2500× g for 1 min to elute the saliva solution. To 0.05 mL of each saliva sample, 0.1 mL of mixed IS solution and distilled water were added to make 0.5 mL, and ultrafiltered using Amicon Ultra® 0.5-mL 3K (Millipore, Tullagreen, Ireland), regenerated cellulose 3000 molecular weight cut-off centrifugal filter device, at 15,000 rpm for 20 min. Each filtrate was pipetted into a 2.0 mL autosampler vial with septa, made up to a total volume of 1.0 mL with distilled water, and used as a sample for IT-SPME LC–MS/MS analysis. The concentrations of the steroid hormones in saliva were calculated using calibration curves constructed from the peak height ratios of each steroid hormone to their IS compounds.

3. Results

3.1. LC–MS/MS Analysis of Steroid Hormones and Their Stable Isotope-Labeled Compounds

The nine steroid hormones and their stable isotope-labeled compounds showed good sensitivity in the ESI-positive ionization mode. The protonated ions $[M+H]^+$ (Q1 mass) for each compound and their fragment ions (Q3 mass) with the highest intensity produced by cleavage of $[M+H]^+$ were selected as precursor and product ion, respectively. The MS/MS operating parameters including CUR, CAD, ion source temperature, ion spray voltage, and declustering, focusing, and collision potentials (DP, EP, CE and CXP) were optimized with API 4000 tuning software of Analyst version 1.6.2. The parameters and MRM transitions set for each compound are shown in Table 1. The findings concurred with previously reported results [18,22–25,27,30,39–42,51,54,55,59].

The nine steroid hormones and their stable isotope-labeled compounds were well separated by LC using a Discovery HS F5 column. Chromatographic conditions were optimized by focusing on short retention times, paying special attention to matrix effects as well as peak shapes. Optimal separation was achieved using 0.2% formic acid/acetonitrile (45/55, v/v), at a flow rate of 0.2 mL/min resulting in good peak shapes and selective detection in MRM mode with a runtime of 6 min, Figure 3. The analysis time per sample was about 24 min, allowing automated analysis of about 60 samples per day by operating overnight.

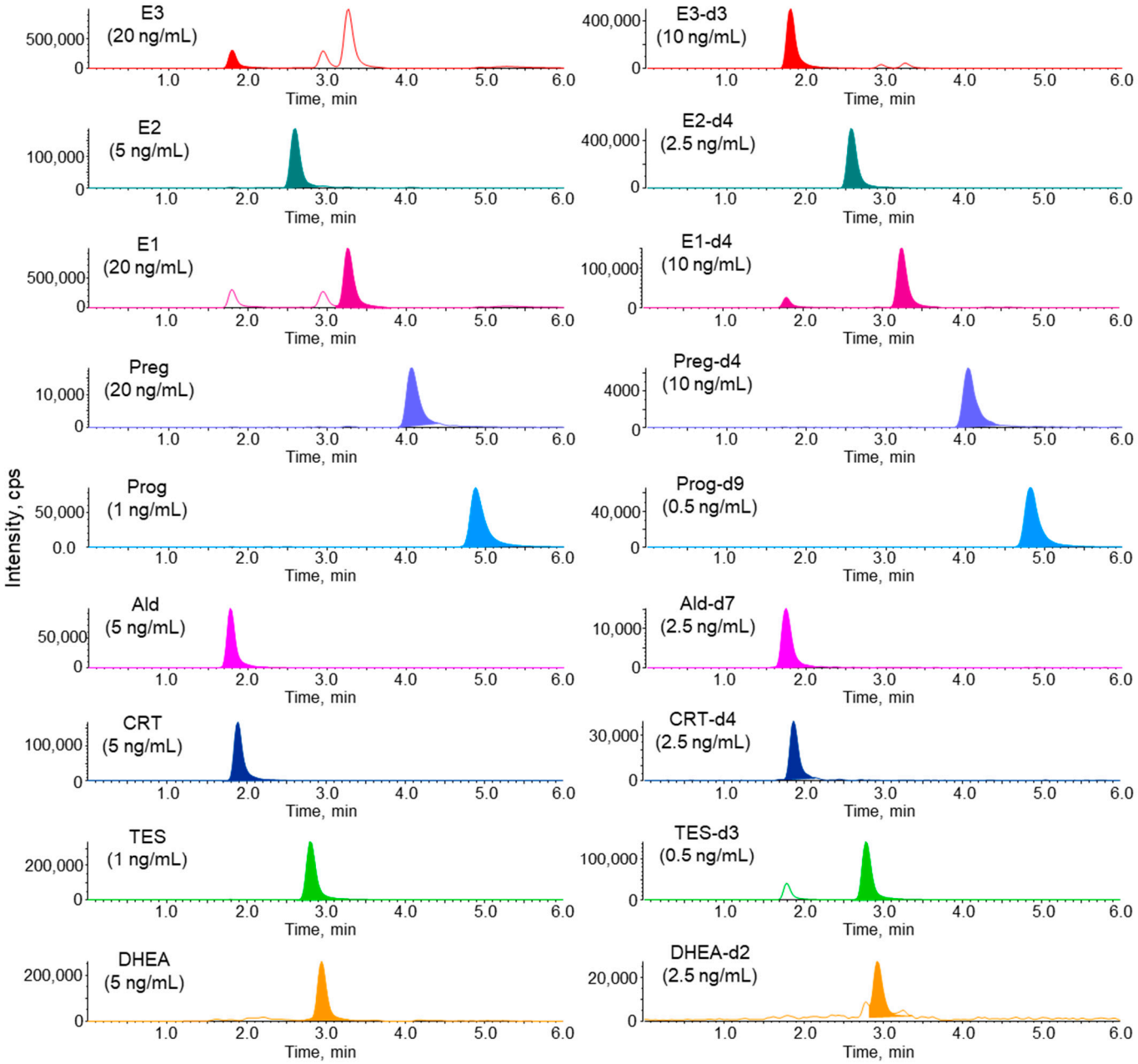

Figure 3. MRM chromatograms obtained from standard solution by IT-SPME LC–MS/MS in the positive ion mode. IT-SPME LC–MS/MS conditions are described in the Experiment section.

3.2. Optimization of IT-SPME and Desorption of Steroid Hormones

In IT-SPME systems that use capillary tubes as extraction devices for target compounds, extraction efficiency is mainly affected by the type of capillary coating, the number and flow rate of draw/eject cycles, and the sample pH. These IT-SPME conditions were optimized at 1, 5, and 20 ng/mL of each compound for groups 1, 2, and 3, respectively.

Since the IT-SPME extraction method relies on the distribution of compounds between the mobile and stationary phases, it is important to use a capillary with a high partitioning efficiency into the stationary phase. Among the GC capillaries examined, the Supel-Q PLOT column was found to be superior, Figure 4. The PLOT-type column has a larger adsorption surface area and thicker film, providing more extraction than liquid phase type columns. The capillary length depends on draw/eject sample volumes and is an important factor affecting extraction efficiency and time. However, if the capillary is too long and the

sample volume too large, it will increase bandwidth and take more time. A capillary 60 cm long and 0.32 mm i.d. proved optimal for a draw/eject volume of 40 µL of sample.

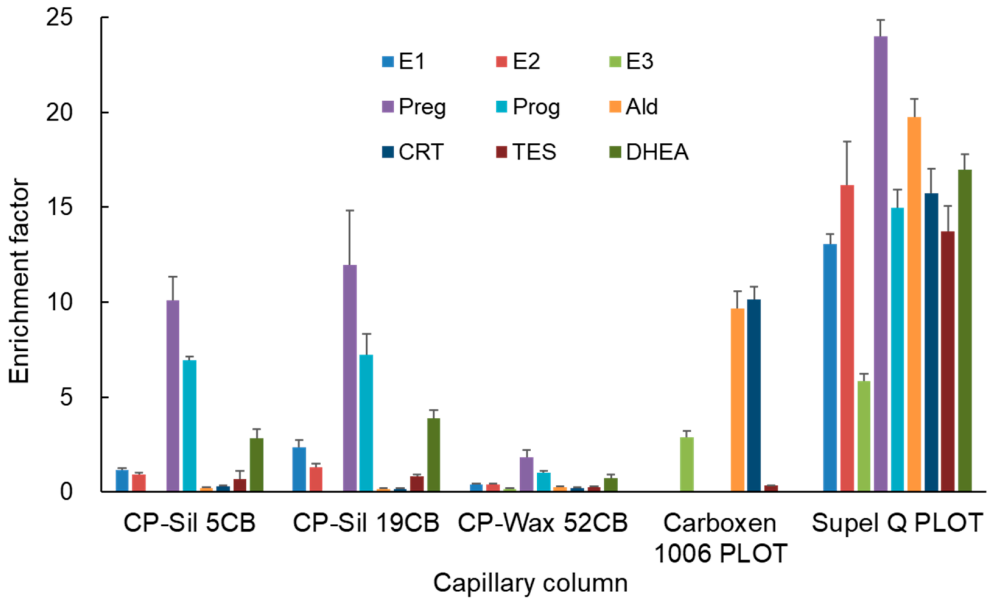

Figure 4. Effects of capillary coatings on IT-SPME of steroid hormones. The three compounds were extracted by 25 draw/eject cycles of 40 µL of standard solution.

In addition, the number of draw/eject cycles, flow rate, and sample pH affect the amount of compounds extracted and the extraction rate. As shown in Figure 5, all nine steroid hormones were efficiently extracted into a Supel-Q PLOT capillary by more than 25 repeated draw/eject cycles of 40 µL samples at a flow rate of 0.2 mL/min. If the flow rate was too slow, extraction took too long, while if it was too fast, bubbles formed in the capillary and extraction efficiency decreased. Adjusting sample pH with a buffer solution was no more effective than extraction with an aqueous solution due to increased salt concentration.

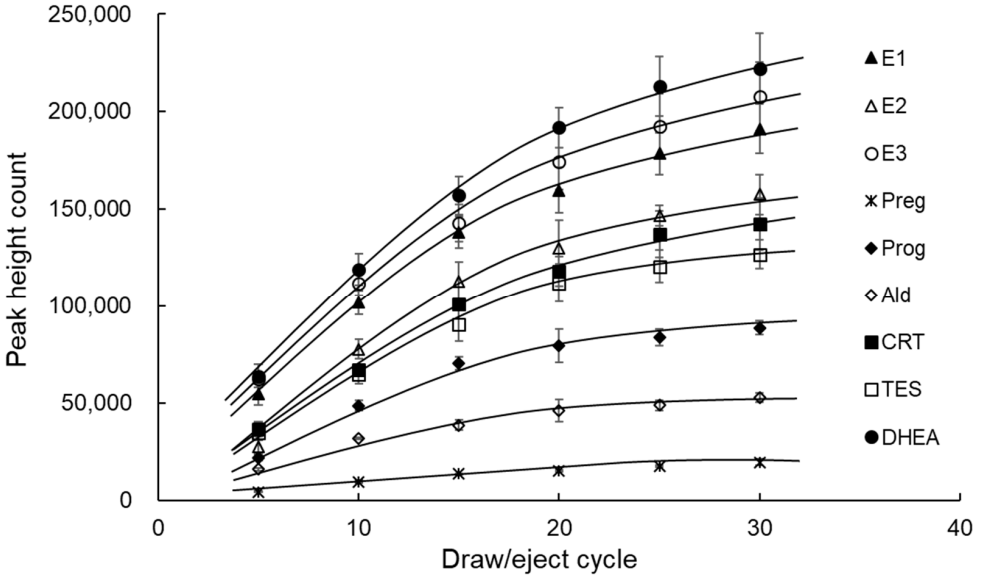

Figure 5. Effects of draw/eject cycles on IT-SPME of steroid hormones. These compounds were extracted with Supel-Q PLOT capillary by draw/eject cycles of 40 µL of standard solution.

The absolute extractable amounts of steroid hormones into the capillary tube by IT-SPME were calculated by comparing peak area counts obtained by direct injection of a known amount of standard solution. Using 1 mL standard solution containing 1, 5, and 20 ng/mL of each compound for groups 1, 2, and 3, respectively, the extraction yields of each compound into the Supel-Q PLOT capillary column were 12–37%. Although these yields were relatively low, their reproducibility was good due to the use of an autosampler.

The steroid hormones extracted into the capillary tube were dynamically desorbed and introduced directly into the LC column by online mobile phase flow using a column switching system, Figure 2B. During analysis, the mobile phase flows through the capillary tube and conditioned with methanol and distilled water flow just prior to the next analysis. This process allows for repeated use of the capillary tube without a carryover and is programmed and automated to save on labor costs through unmanned nighttime operation.

3.3. Linearity, Detection Limits, and Precisions of Steroid Hormones

The performance of the developed IT-SPME LC–MS/MS method was validated by measuring analytical parameters, such as linearity, LOD, and precision. As shown in Table 2, the calibration curves of steroid hormones, constructed by comparing peak height ratio with each IS, showed a linear relationship for each compound, with all correlation coefficients above 0.9990 (n = 24). The relative standard deviations (RSDs) of the peak height ratios at each concentration ranged from 0.2% to 9.6% (n = 3). The LODs (S/N = 3) for each compound ranged from 0.7 to 21 pg/mL. The IT-SPME method demonstrated a 17- to 40-fold increase in sensitivity compared to the direct injection method (10 µL injections). The intra- and inter-day precisions (RSD, %) obtained with three concentrations were 0.4–8.1% and 3.1–15%, respectively, with acceptable precision for quantitative analysis (Table 3).

Table 2. Linearity and sensitivity of the IT-SPME LC–MS/MS method for steroid hormones.

Compound	Linearity		LOD [2] (pg/mL)		LOQ [3] (pg/mL)
	Range (ng/mL)	CC [1]	Direct Injection	IT-SPME	IT-SPME
E1	0.2-40	0.9990	270	8.9	295
E2	0.05-10	0.9992	63	2.2	73
E3	0.2-40	0.9993	560	21	680
Preg	0.2-40	0.9992	289	9.2	303
Prog	0.01-2	0.9990	60	2.3	77
Ald	0.05-10	0.9998	119	7.0	233
CRT	0.05-10	0.9998	83	4.3	142
TES	0.01-2	0.9993	21	0.7	24
DHEA	0.05-10	0.9997	320	8.1	268

[1] Correlation coefficient (n = 24). [2] Limits of detection: pg/mL sample solution (signal-to-noise ratio, 3). [3] Limits of quantification: pg/mL saliva sample (signal-to-noise ratio, 10).

Table 3. Precision of the IT-SPME LC–MS/MS method for steroid hormones.

Compound	Concentration (ng/mL)	Precision (RSD [1] %), (n = 6)	
		Intra-Day	Inter-Day
E1	1.0	8.1	11
	4.0	3.4	6.8
	20	5.9	11
E2	0.25	3.5	5.6
	1.0	4.4	7.9
	5.0	0.4	9.2

Table 3. *Cont.*

Compound	Concentration (ng/mL)	Precision (RSD [1] %), (n = 6)	
		Intra-Day	Inter-Day
E3	1.0	5.5	11
	4.0	4.3	8.2
	20	4.0	13
Preg	1.0	6.1	7.4
	4.0	7.5	11
	20	5.7	10
Prog	0.05	1.9	4.0
	0.2	2.1	3.1
	1.0	4.8	4.5
Ald	0.25	4.0	8.1
	1.0	2.2	4.4
	5.0	2.6	4.4
CRT	0.25	0.8	6.3
	1.0	1.8	3.5
	5.0	2.8	7.1
TES	0.05	6.5	9.7
	0.2	1.6	15
	1.0	4.2	9.9
DHEA	0.25	3.0	4.5
	1.0	1.5	10
	5.0	2.2	3.9

[1] RSD, relative standard deviation.

3.4. Application to the Analysis of Saliva Samples

Saliva is an excellent sample because it can be obtained noninvasively, and the concentration of steroid hormones in saliva mirrors that of the free form concentration found in serum and plasma [4,8,9,25,27,34–39]. Since steroid hormones are affected by circadian rhythms [2,27,30–32], saliva samples were collected between 2:00 p.m. and 4:00 p.m., when concentration changes are relatively small [25]. They were collected using Salisoft® containing a polypropylene-polyethylene swab and ultracentrifuged with Amicon Ultra® to remove the macromolecular matrix components such as mucin and coexisting proteins, which may interfere with extraction into capillary tubes. Steroid hormones in the ultrafiltrate could be stored stably in a −20 °C freezer if not analyzed immediately.

Stable isotope-labeled compounds as IS were added to saliva samples prior to extraction to correct the influence of matrix effects on the analysis of steroid hormones in the samples. As shown in Figure 6, the saliva samples were successfully analyzed without interference peaks via the IT-SPME LC–MS/MS method with MRM mode detection. The LOQ (S/N = 10) of the nine steroid hormones calculated according to the criteria recommended by the ICH guidelines [65] ranged from 24 to 680 pg/mL saliva (Table 2). To confirm the validity and accuracy of the method, known amounts of the steroid hormones were spiked into pooled saliva samples, and their recoveries were calculated. The overall recoveries of these compounds ranged from 82 to 114% with RSDs of 0.9–16% (Table 4). The results show the IT-SPME LC–MS/MS method has good accuracy and precision in analyzing saliva samples. Prog, CRT, TES, and DHEA were detected in most samples, while E1 and Ald were detected in trace amounts only in a few samples, mostly below LOQ (Table 5).

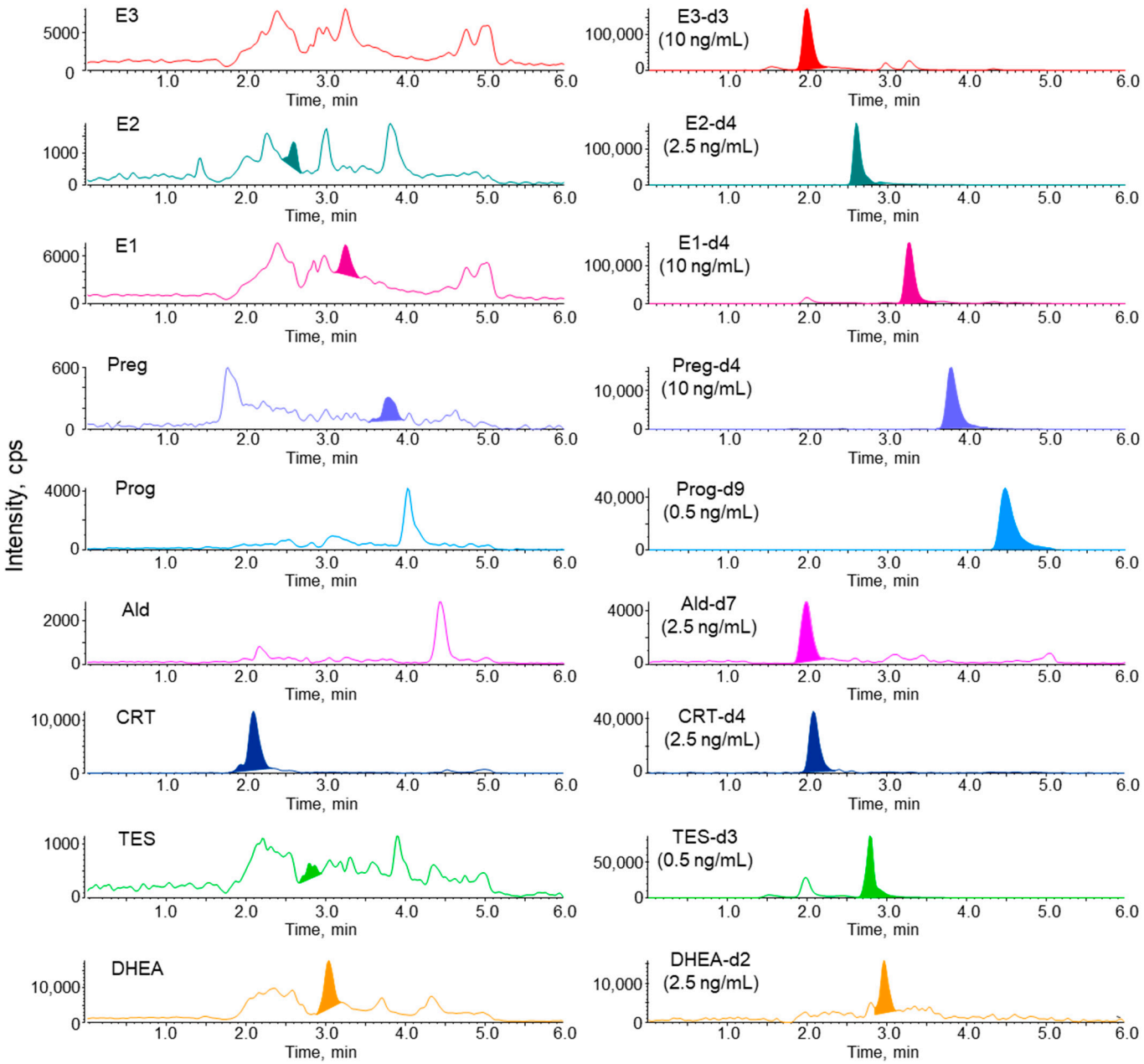

Figure 6. MRM chromatograms obtained from 0.05 mL of saliva sample via IT-SPME LC–MS/MS in positive ion mode. IT-SPME LC–MS/MS conditions are described in the Experiment section.

Table 4. Recoveries of steroid hormones spiked into saliva samples.

Compound	Spiked (ng/mL Saliva)	Recovery ± SD (%), (*n* = 3)
E1	10	97 ± 7
	40	100 ± 8
	200	89 ± 11
E2	2.5	105 ± 12
	10	96 ± 3
	50	94 ± 7
E3	10	106 ± 7
	40	114 ± 1
	200	101 ± 10

Table 4. *Cont.*

Compound	Spiked (ng/mL Saliva)	Recovery ± SD (%), (*n* = 3)
Preg	10	92 ± 12
	40	100 ± 4
	200	97 ± 8
Prog	0.5	82 ± 2
	2	85 ± 3
	10	82 ± 4
Ald	2.5	103 ± 2
	10	104 ± 7
	50	102 ± 5
CRT	2.5	88 ± 5
	10	93 ± 5
	50	91 ± 2
TES	0.5	97 ± 13
	2	93 ± 15
	10	102 ± 11
DHEA	2.5	89 ± 6
	10	87 ± 8
	50	101 ± 14

Table 5. Contents of steroid hormones in saliva samples.

Subject			Content [1] (ng/mL Saliva)								
No	Sex [2]	Age	E1	E2	E3	Preg	Prog	Ald	CRT	TES	DHEA
1	M	25	<LOQ [3]	<LOQ	<LOQ	<LOQ	0.48	<LOQ	0.19	0.27	<LOQ
2	M	26	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.25	2.42	0.09	0.43
3	M	27	<LOQ	<LOQ	<LOQ	<LOQ	0.10	<LOQ	2.98	0.09	<LOQ
4	M	36	<LOQ	<LOQ	<LOQ	0.85	0.58	<LOQ	0.31	0.14	0.83
5	M	65	<LOQ	<LOQ	<LOQ	1.1	2.5	<LOQ	<LOQ	0.06	<LOQ
6	F	23	<LOQ	0.22	0.75	0.77	<LOQ	<LOQ	4.15	0.10	0.53
7	F	24	<LOQ	0.26	0.68	2.3	3.6	<LOQ	0.30	0.07	0.82
8	F	25	1.1	0.22	0.79	1.1	0.11	0.86	4.83	0.12	1.06
9	F	34	<LOQ	0.25	0.68	<LOQ	0.66	<LOQ	0.29	<LOQ	0.68
10	F	63	<LOQ	<LOQ	1.1	1.1	2.1	<LOQ	0.31	0.05	0.99

[1] The content in saliva represents the average of three independent measurements. [2] M, male; F, female. [3] LOQ, limit of quantification.

3.5. Comparison with Previously Reported LC–MS/MS Methods for Salivary Steroid Hormones

Previously reported LC–MS/MS methods to determine salivary steroid hormones are summarized in Table 6 giving sample preparation and detection sensitivity. Commercially available Salivette® is often used for saliva collection, but cotton swabs interfere with steroid hormone analysis due to adsorption [18,66]. Polymeric saliva collectors like Salisoft® used in this study have a fluid volume recovery rate of 98%, and significant positive correlations have been found between Salisoft® and steroid hormone concentrations in saliva samples collected via the passive drooling method [67]. Although LC–MS/MS is a specific and sensitive method, sample pretreatment, including extraction, enrichment, and derivatization, is essential before analysis, since steroid concentrations in saliva are very low. Although derivatization of steroid hormones with O-ethylhydroxylamine hydrochloride and 2-hydrazino-1-methylpyridine improves sensitivity by enhancing ionization efficiency [25,39,41,55], it is complicated and time-consuming due to the need for pretreatment such as LLE. The most common method for extracting steroid hormones from saliva samples is LLE [39–41,55] or its alternative SLE [21,30]. The SPE method is also used as an efficient method [18,22,25,27]. Though these pretreatments reduce matrix

effects and allow for sample enrichment, LLE and SLE require relatively large volumes of organic solvent, and strong solvents such as dichloromethane and ethyl acetate are used. SPE can be extracted with small volumes of solvent, but is often used in combination with deproteinization or LLE. Relatively high sensitivity at the pg level has been obtained using these pretreatment methods. In contrast, IT-SPME [51,59,62,63] requires almost no organic solvent and can be used directly as a sample for LC–MS/MS analysis by ultrafiltration alone. Furthermore, online coupling of IT-SPME and LC–MS/MS enables full automation. Although the sensitivity was equal to or slightly less than that of the conventional method, it was fully applicable to saliva sample analysis. The slightly lower sensitivity compared to our previous method is due to the increase in the number of target compounds, the dependence on MS scan speed, and channel limitations for the software [4]. Since many compounds were detected in saliva samples at the trace level but below the LOQ, future work is needed to increase the sensitivity of compounds with low sensitivity and to further improve the enrichment effect of compounds with low content. Furthermore, there are several issues that need to be resolved in the use of saliva samples, such as the correlation between salivary and blood concentrations of various steroid hormones, the effect of saliva sample collection time on circadian rhythm, and concentration correction for changes in salivary secretion.

Table 6. Main LC–MS/MS methods for the determination of salivary steroid hormones.

Compound	Sampling and Sample Preparation	Salivary Content	Sensitivity	Ref.
TES	Passive drool using Salimetrics®, OASIS MAXµElution Plate	2–59 pg/mL in healthy adults	LOD: 2 pg/mL; LOQ: 6 pg/mL	[18]
TES, androstanedione (AN)	Passive drool, Isolute SLE + 400 plate, XBridge C18 cartridges	TES: 13 pmol/mL, AN: 143 pmol/mL	LOQ: 5–6.25 pmol/mL	[21]
CRT, cortisone (CRN)	Salivette® (cotton swab), Oasis® HLB SPE cartridges (online SPE)	CRT: 3–21 nmol/mL, CRN: 10–42 nmol/mL	LOD: 0.2–0.3 nmol/mL; LOQ: 0.51–0.55 nmol/mL	[22]
TES, DHEA	Passive drool, acetonitrile pretreatment, Strata-X cartridge, derivatization	46–131 pg/mL	LOQ: 10 pg/mL	[25]
10 Steroid hormones [1]	Passive drool, SPE plate using a Positive Pressure-96 Processor	0.01–21 ng/mL	LOD: 0.8–14 pg/mL; 4.8–24 pg/mL	[27]
19 Steroid and metabolites [2]	Salivette® (cotton swab), Isolute SLE + 400 96-well plates for extraction	Detected but no data listed	LLOQ: 0.05–1.25 ng/mL	[30]
CRT, CRN	Salivette® (polyester wool swab), LLE with ethyl acetate, derivatization	Detected but no data listed	LLOD: 2–5 pg/mL; LLOQ: 5–10 pg/mL	[39]
TES	Direct spitting or drool, LLE with methyl *tert*-butyl ether	Male: 93–378 pg/mL; female: 5–46 pg/mL	LLOQ: 5 pmol/mL	[40]
CRT, CRN, TES, DHEA, Prog, 17α-OH-Prog	Expectoration via polypropylene straw, LLE with methyl *tert*-butyl ether, derivatization	Morning: <57 nM; night: <18	LOD: 0.011–7 pg/mL; LOQ: 0.02–20 ng/mL	[41]
TES, DHEA, AN, Prog, 17α-OH-Prog, Preg, 17α-OH-Preg	Direct spitting, LLE with methyl *tert*-butyl ether, derivatization	Detected but no data listed	LOD: 0.05–1 pg/mL; LOQ: 0.15–3 pg/mL	[55]
CRT, TES, DHEA	Salisoft® (polypropylene–polyethylene swab), ultrafiltration, IT-SPME	0.032–1.07 ng/mL	LOD: 0.3–8.9 pg/mL; LOQ: 0.01–0.29 ng/mL	[51,59]

Table 6. *Cont.*

Compound	Sampling and Sample Preparation	Salivary Content	Sensitivity	Ref.
Sulfates of E2, Preg, CRT and DHEA	Salisoft® (polypropylene–polyethylene swab), ultrafiltration, IT-SPME	<11.9 ng/mL	LOD: 0.3–3.2 pg/mL; LOQ: 0.016–0.172 ng/mL	[62]
CRT, TES, DHEA, DHEA-sulfate	Salisoft® (polypropylene–polyethylene swab), ultrafiltration, IT-SPME	<7.27 ng/mL	LOD: 0.4–8.5 pg/mL; LLOQ: 0.036–0.768 ng/mL	[63]
E1, E2, E3, Preg, Prog, Ald, CRT, TES, DHEA	Salisoft® (polypropylene–polyethylene swab), ultrafiltration, IT-SPME	<4.83 ng/mL	LOD: 0.7–20.5 pg/mL; LOQ: 24–680 pg/mL	This method

[1] TES, AN, Prog, 17α-OH-Prog, Ald, CRT, CRN, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone. [2] CRT, CRN, corticosterone, 11-deoxycortisol, 21-deoxycortisol, 11-deoxycorticosterone, 11-dehydrocorticosterone, TES, AN, 5α-dihydrotestosterone, DHEA, Prog, 17α-OH-Prog, Preg, 17α-OH-Preg, Ald, E1, E2, E3.

4. Conclusions

The IT-SPME LC–MS/MS method developed in this study was able to simultaneously analyze nine steroid hormones with high sensitivity and selectivity by simply ultrafiltrating a small amount of saliva. This method is a non-invasive, environmentally friendly method that does not use organic solvents, and can be fully automated from extraction and enrichment of sample solution to separation analysis and data analysis by online coupling of IT-SPME and LC–MS/MS, allowing unattended overnight operation and saving labor costs. In particular, this method is significant in that it enables simultaneous analysis of different types of steroid hormones, whereas our previous methods were limited to the analysis of specific compounds, and will lead to comprehensive analysis via metabolomics in the future, which will provide useful information. Therefore, this method is expected to be a useful tool for analyzing the regulation of steroid metabolism and diagnosing related diseases.

Author Contributions: Conceptualization, H.K.; methodology, T.H. and H.K.; software, T.H. and H.K.; validation, T.H. and H.K.; formal analysis, T.H. and H.K.; investigation, T.H. and H.K.; resources, T.H. and H.K.; data curation, T.H. and H.K.; writing—original draft preparation, H.K.; writing—review and editing, H.K.; visualization, T.H. and H.K.; supervision, H.K.; project administration, H.K.; funding acquisition, H.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by JSPS KAKENHI Grant Number JP20K07007 and Smoking Research Foundation 2023.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the ethics committee of Shujitsu University (approval code 207; 14 October 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors acknowledge the anonymous volunteers that agreed to participate in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

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