Talanta 86 (2011) 415–420

Contents lists available at SciVerse ScienceDirect

Talanta

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

Steroid hormones analysis with surface-assisted laser desorption/ionization mass spectrometry using catechin-modified titanium dioxide nanoparticles

Tai-Chia Chiu*

Department of Applied Science, National Taitung University, 684, Section 1, Chunghua Road, Taitung, 95002, Taiwan

ARTICLE INFO

Article history:
Received 27 July 2011
Received in revised form
20 September 2011
Accepted 21 September 2011
Available online 29 September 2011

Keywords:
Catechin
Steroid hormones
Surface-assisted laser
desoprtion/ionization mass spectrometry
Titanium dioxide nanoparticles

ABSTRACT

This paper describes the application of catechin-modified titanium dioxide nanoparticles (TiO_2 NPs) as matrices to analyze four steroid hormones by surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS). The catechin-modified TiO_2 NPs have high absorbance at 337 nm and are effective SALDI matrices when using a nitrogen laser. Four test steroid hormones—cortisone, hydrocortisone, progesterone, and testosterone—were directly analyzed by SALDI-MS. The limits of detection at a signal-to-noise ratio of 3 for cortisone, hydrocortisone, progesterone, and testosterone were 1.62, 0.70, 0.66, and 0.23 μM, respectively. This approach provides good quantitative linearity for the four analytes ($R^2 > 0.986$) with good reproducibility (the shot-to-shot and batch-to-batch variations for the four analytes were less than 10% and 15%, respectively). We validated the practicality of this approach—considering its advantages in sensitivity, repeatability, rapidity, and simplicity—through the analysis of testosterone in a urine sample.

1. Introduction

Steroids are compounds that contain cyclopental[a]-phenalthrene ring systems. Substitutions in this ring system form diverse classes of steroids that have distinct physiological actions that serve important biological functions such as producing hormones during chemical signaling (e.g., testosterone) and forming structural compounds in cell membranes (e.g., cholesterol) [1]. Steroid derivatives are also used widely as drugs for the treatment of a variety of disorders, as well as substances of abuse (e.g., anabolic steroids) in sports medicine [1]. These compounds promote the development of secondary male sexual characteristics (androgenic effects) and accelerate muscle growth (anabolic effects). The determination of testosterone levels in body fluids such as urine and plasma are of great importance for the medical diagnosis of hirsutism, polycystic ovary disease, and virilization [1,2].

Since it was first introduced by Karas et al. in 1988, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has become a powerful analytical tool for bioanalysis [3,4]. As a result of rapid energy transfer from UV-absorbing matrices, analytes undergo soft and efficient desorption/ionization with a minimum degree of fragmentation. Although MALDI-MS is successfully used for the analyses of a variety of molecules, especially peptides and proteins, it has not been employed extensively for the characterization of low-molecular weight compounds (<500 Da) [5]. This is partly because of the presence of a variety of abundant matrix-related ions in the low-mass region of the MS spectra, and partly because the inhomogeneous co-crystallization of analytes with traditional organic matrices such as 2,5-dihydroxybenzoic acid and sinapinic acid usually leads to the existence of "sweet spots" on the sample probe, resulting in quantitative errors and irreproducibility. These disadvantages have hindered efforts to utilize the full power of MALDI-MS for the high-throughput analyses of drugs and their metabolites in complex mixtures containing high concentrations of salts. To overcome these problems, surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS) has been implemented for eliminating matrix-ion interferences and for improving sample homogeneity. Useful SALDI matrices include gold nanoparticles (NPs) [6–8], silver NPs [9–11], silicon oxide NPs [12], titanium dioxide (TiO_2) NPs [13–16], zinc oxide NPs [17] and nanowire [18], zinc sulfide NPs [19], zinc selenium quantum dots [20], iron (III) oxide NPs [21,22], platinum NPs [23] and nanoflowers [24,25], and HgTe nanostructures [26].

Although SALDI-MS was developed nearly two decades ago, it was not as widely used as MALDI-MS for the analysis of biomolecules, mainly because its accessible mass range is limited to ca. 20 kDa and its mass resolution is limited to around a few hundred or less [24]. However, owing to the recent increased interest in nanoscience, SALDI-MS has become a popular technique. Monodisperse nanomaterials have been prepared as efficient SALDI-MS matrices through several synthetic routes. Compared with conventional micro-nanosized particles and/or organic matrices, monodisperse nanomaterials offer several

* Tel.: +886 89 353840; fax: +886 89 342539.
E-mail address: tcchiu@nttu.edu.tw

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.09.038

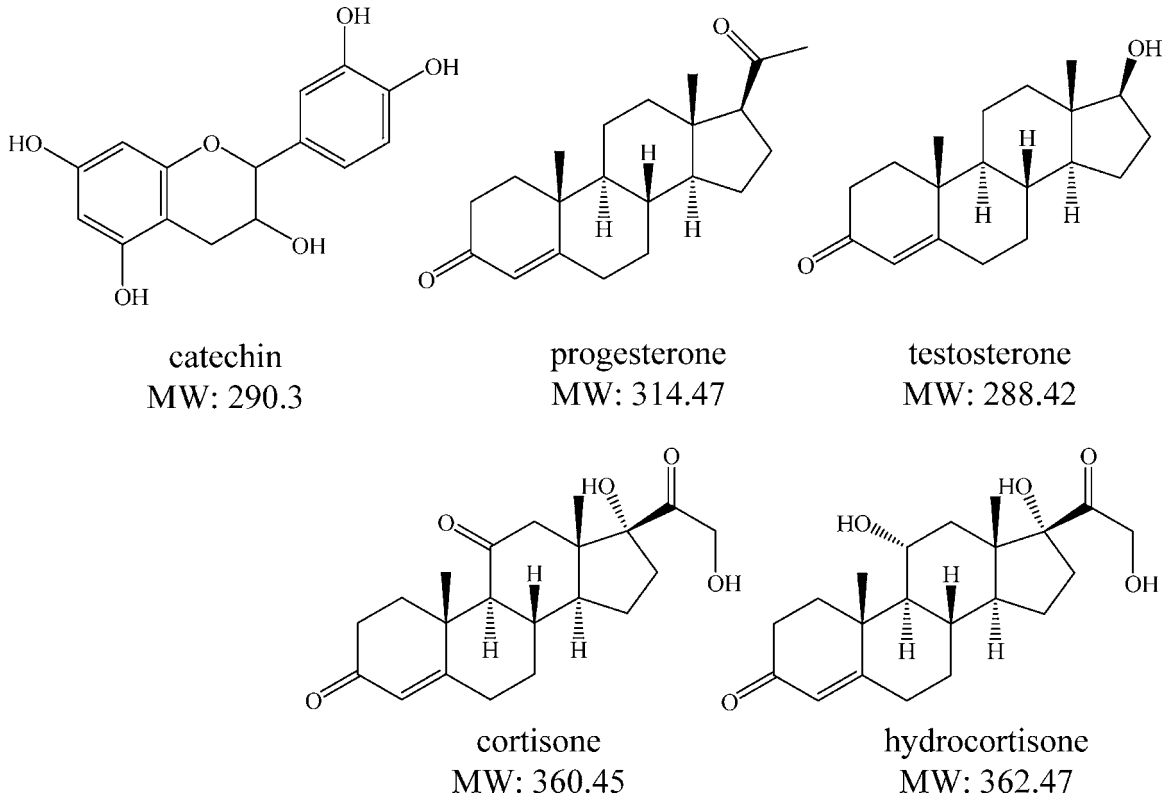


Fig. 1. Chemical structures of catechin and four steroid hormones.

advantages including simple and facile surface modification, large surface-area-to-volume ratios, flexibility of sample deposition under specific conditions, and high extinction coefficients in the UV range. Several bioconjugated nanomaterials such as gold [8] and titanium dioxide [13] serve not only as efficient matrices but also as effective media for the required concentration of analytes and for minimizing sample-matrix interferences.

In the UV region, TiO_2 NPs exhibit strong absorption characteristics (band gap of bulk anatase TiO_2: 3.2 eV) that mainly depend on their size, shape, and composition. TiO_2 NPs are usually prepared through sol–gel reactions and by carefully controlling parameters such as the reaction solution pH, water/alkoxide molar ratios, reaction temperature, and the nature of the solvents and additives—the size and shape of TiO_2 NPs can be fine tuned. In this study, catechin-modified TiO_2 NPs are used as matrices for the analysis of steroid hormones and are analyzed by SALDI-MS, which provides advantages such as easy and quick sample preparation and high repeatability.

2. Experimental

2.1. Chemicals

(+)-Catechin hydrate, citric acid, cortisone, β-glucuronidase/sulfatase from *Helix pomatia* (Type H-2), hydrocortisone, progesterone, sodium acetate, testosterone, and methanol were purchased from Sigma (St. Louis, MO, USA). (+)-L-ascorbic acid, dichloromethane, and nitric acid were purchased from Acros Organics (Geel, Belgium). The structures of catechin and the four steroid hormones are shown in Fig. 1. TiO_2 NPs (ca. 5 ± 1 nm) solution was received as kindly gifts from Professor Chang [13]. According to their synthetical procedures, TiO_2 NPs were prepared through a sol–gel reaction by hydrolysis of titanium isopropoxide (12.5 mL, 97%, density: 0.96 g/mL) in 0.1 M nitric acid (75 mL), the sizes and distributions were confirmed by transmission electron microscopy. Owing to assume that the titanium isopropoxide reacted completely to form TiO_2 NPs (density: 4.23 g/cm^3), the concentration of as-prepared TiO_2 NPs was estimated be 240 μM (2×10^{17} particles/mL). Thus, the calculated molecular weight of a 5-nm-TiO_2-NP is about 156 kDa.

The UV–Vis reflectance spectrum of catechin powder (5 mg) and UV–Vis absorbance spectrum of catechin (1–50 μM) and catechin (1–100 μM) modified TiO_2 NPs in 10 mM nitric acid was measured with a Varian Cary 100 UV–Vis spectrophotometer (Mulgrave, Victoria, Australia).

2.2. Preparation of standard solutions

For stock solutions (10 mM) of cortisone, hydrocortisone, progesterone, and testosterone were separately prepared in methanol. The TiO_2 NPs (240 μM) were diluted to various concentration (0.24 nM–2.4 μM) using 0.01 M nitric acid solution. Catechin (10 μL; 1–100 μM) were then added separately to the various concentration of the TiO_2 NP solutions and equilibrate for 30 min in the dark. A mixture (1 mL) containing 10 mM HNO_3 (pH 2.0), 10 μL catechin-modified TiO_2 NPs, and 10 μM analytes was equilibrated for 30 min. Each solution (1 μL) was pipetted into a stainless steel 384-well MALDI target and dried in air at room temperature before SALDI-MS measurements.

We have tested and transferred the TiO_2 NPs in different buffers (e.g. ammonium citrate or phosphate buffers), but they precipitated easily at pH 5.0 and higher pH. Therefore, the as-prepared TiO_2 NPs were only stable in 1–100 mM nitric acid.

2.3. Analysis of urine samples

A urine sample was collected from a healthy man (age 33) and until analysis stored at −20 °C in a 500 mL bottle containing

0.5 g of ascorbic acid. Because endogenous testosterone and their metabolites excreted in urine are present as glucuronidated (major) and sulfated (minor) forms, a hydrolysis step was performed prior to the SALDI-MS sample preparation. To a 50 mL aliquot of urine, 0.02 g sodium acetate and 0.25 g L-ascorbic acid were added followed by 10 μL β-glucuronidase/sulfatase from *Helix pomatia* (Type H-2). The samples were incubated overnight at 37 °C. After hydrolysis, 10-mL urine samples were subjected to slow inverse extraction using 20 mL dichloromethane under centrifugation at 10 rpm (Sigma 3K30 centrifuge, Sigma Laborzentrifugen GmbH, Postfach, Germany) for 30 min. After extraction, the samples in the organic phase were collected and then subjected to evaporation under vacuum.

To the dried samples, 987.5 μL of 10 mM HNO_3 (pH 2.0) and 12.5 μL of 240 nM catechin-modified TiO_2 NPs solution were added and equilibrated for 30 min. Before SALDI-MS measurements, 1.0 μL of the solution was pipetted into a stainless steel 384-well MALDI target and was dried in air at room temperature.

2.4. SALDI-MS measurements

MS experiments were performed in the positive-ion mode on a reflectron-type time-of-flight (TOF) mass spectrometer (Biflex III, Bruker Daltonics, Bremen, Germary) equipped with a 1.25 m flight tube. The samples were irradiated with a 337 nm nitrogen laser (50 μm diameter) at 10 Hz (pulse duration: 4 ns). Ions produced by laser desorption were energetically stabilized during a delayed extraction period of 200 ns and then accelerated through the linear TOF reflectron before entering the mass analyzer. The available accelerating voltages are in the range +20 to −20 kV. To obtain good resolution and signal-to-noise (S/N) ratios, the laser fluence was adjusted to slightly higher than the threshold, and each mass spectrum was generated by averaging 250 laser shots.

3. Results and discussions

3.1. Catechin modified TiO_2 NPs as matrices for SALDI-MS

The use of TiO_2 NPs as selective probes and matrices for the determination of enediol compounds by SALDI-MS has been demonstrated previously [13]. A number of advantages over conventional organic matrices are provided by using TiO_2 NP matrices including ease of sample preparation, less background noise in the low-mass region, and high repeatability. However, one drawback of using TiO_2 NPs as SALDI matrices is their weak absorption at ca. 337 nm, which is the emission wavelength of most common used nitrogen lasers. The UV–Vis absorption spectrum of 1–100 μM catechin in 10 mM nitric acid and the UV–Vis reflectance spectrum of catechin powder (5 mg) were shown in Fig. S1. The spectra indicated that the characteristic peak of catechin in solution and catechin powder were at 278 nm and 305 nm, respectively. Thus, these two matrices would not be suitable for MALDI. When adding small amount of catechin in TiO_2 NPs solutions (10 mM nitric acid), the absorption at 337 nm became stronger (Fig. 2). The modified mechanism of catechin on TiO_2 NPs was shown in Scheme 1. TiO_2 NPs with diameters smaller than 20 nm interacted with enediol compounds such as catechin. Catechin was found to undergo unique binding at the TiO_2 NP surface, resulting in new hybrid

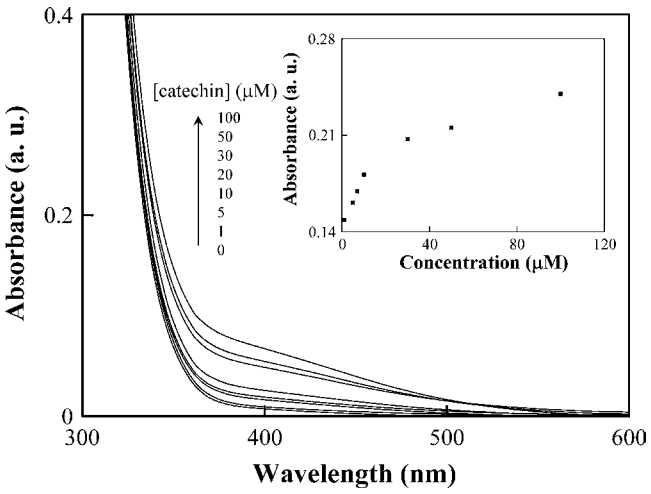
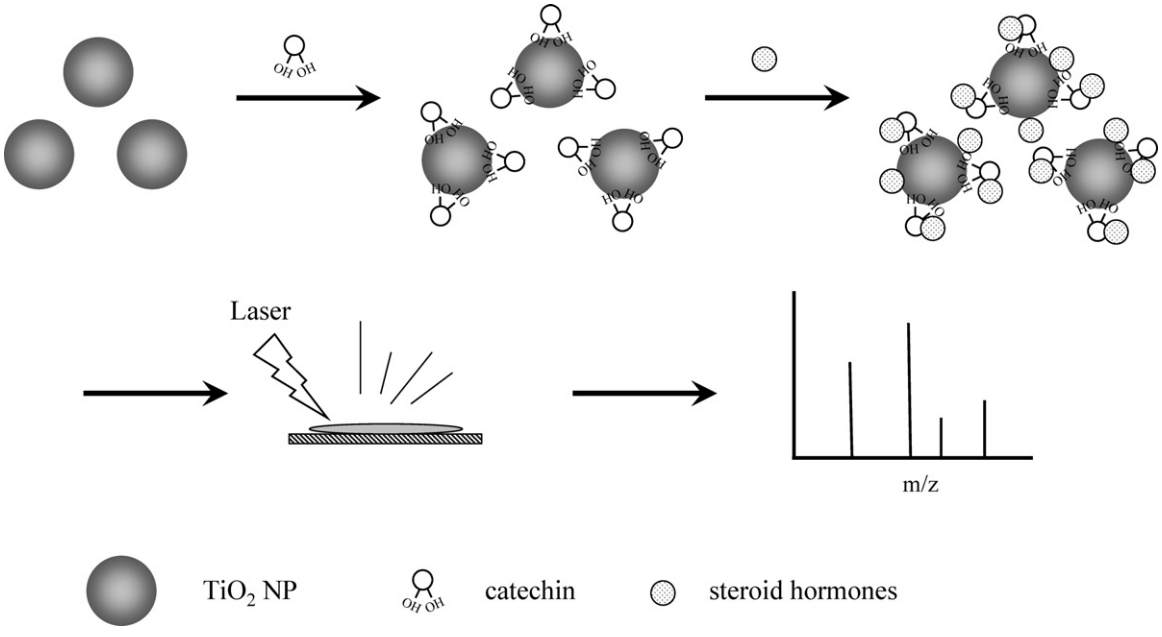


Fig. 2. Absorbance spectra of catechin-modified TiO_2 NPs in 10 mM nitric acid. The inset displayed the plots of the signal intensities for catechin-modified TiO_2 NPs at 337 nm.



Scheme 1. Illustration of the modified mechanism of catechin on TiO_2 NPs.

Table 1
Impacts of TiO_2 NPs concentration, laser fluence and catechin concentration on the S/N ratio of testosterone.

	S/N ratios of testosterone peak (a. u.)				
[TiO_2 NP] (nM)[a]	0.24	2.4	24	240	2400
	4(5%)[b]	12(11%)	26(11%)	103(25%)	41(37%)
Laser fluence (μJ)[c]	102.9	103.8	104.7	105.6	106.5
	0(0%)	11(7%)	103(25%)	31(25%)	15(31%)
[catechin] (μM)[d]	1.0	5.0	10	50	100
	52(27%)	83(19%)	110(20%)	103(25%)	80(32%)

[a] pH = 2.0, laser fluence = 104.7 μJ and [catechin] = 10 μM.
[b] RSD, n = 10.
[c] pH = 2.0, [TiO_2 NPs] = 240 nM and [catechin] = 10 μM.
[d] pH = 2.0, [TiO_2 NPs] = 240 nM and laser fluence = 104.7 μJ.

properties of the surface modified NPs [27]. These hybrid properties arise from the ligand-to-metal charge transfer interaction between the ligand and surface metal atoms. As a result, the absorption at 337 nm was increased, as compared to that for unmodified TiO_2 NPs. Therefore, we expected that SALDI-MS with catechin-modified TiO_2 NPs as assisted matrices would be useful for increasing the desorption and ionization efficiency and for improving the sensitivity of the analysis of analytes. The absorbance at 337 nm increased from 0.14 to 0.23 units (Fig. 2) when the concentration of catechin increased from 1.0 to 100 μM. Increase in absorption efficiency of laser light increases the desorption and ionization efficiencies of the analytes. The TiO_2 NPs matrices provide a number of advantages over conventional organic matrices including ease of sample preparation, less background noise in the low-mass region, and high repeatability.

3.2. Optimization of SALDI analysis

In previous studies, the concentration of NPs played a significant role in determining the sensitivity of the system. The capture capability is high at high NP concentrations, while the number of adsorbed molecules in each NP is lower if the concentration of the analyte is limited. In addition, high background noise is usually generated in the mass spectrum at high NP concentrations, leading to poor sensitivity. The optimum conditions (Table 1) for the concentration of TiO_2 NPs, laser fluence, and concentration of catechin were 240 nM, 104.7 μJ, and 10 μM, respectively, when using testosterone as the testing compound. The effects of different matrices such as no matrices, catechin solutions, and TiO_2 NPs on the S/N ratios of testosterone were also investigated. The results showed that there was no MS signal of testosterone (10 μM) founded without matrices and with 10 μM catechin as matrices. We also noted that using 240 nM TiO_2 NPs without catechin modification as matrices, the S/N ratio is 90% less when comparing to the optimal conditions (data not shown).

To test the potential of catechin-modified TiO_2 NPs as effective matrices, a simultaneous analysis of testosterone, progesterone, cortisone, and hydrocortisone was conducted as shown in Fig. 3. Under the optimum conditions, we detected the major ion peaks for testosterone, progesterone, cortisone, and hydrocortisone at m/z 311.1, 337.1, 383.1, and 385.1, respectively; these ions are assigned to [testosterone + Na]$^+$, [progesterone + Na]$^+$, [cortisone + Na]$^+$, and [hydrocortisone + Na]$^+$, respectively. The peaks of m/z 332.3, 360.2, and 376.2 are system peaks from the TiO_2 NPs solution. In addition, we also note that SALDI-MS using catechin-modified TiO_2 NPs provides advantages of small shot-to-shot (all less than 10%, n = 10) and batch-to-batch (all less than 15%, n = 7) variations.

3.3. Quantitative analyses of the four steroid hormones

Under the optimal conditions, we obtained a calibration curve of the MS signals for testosterone, progesterone, cortisone, and hydrocortisone against their concentration in the range 1–100 μM (Fig. 4). The limits of detection (LODs) and linearity of the four analytes are summarized in Table 2. The correlation coefficients (R^2) for testosterone, progesterone, cortisone, and hydrocortisone are 0.989, 0.991, 0.989, and 0.989, respectively, over the concentration ranges 1.0–30.0, 5.0–100.0, 7.0–100.0, and 5.0–100.0 μM, respectively. On the basis of the peak heights, we estimated the LOD at S/N = 3 for testosterone, progesterone, cortisone, and hydrocortisone to be 0.23, 0.66, 1.62, and 0.70 μM, respectively.

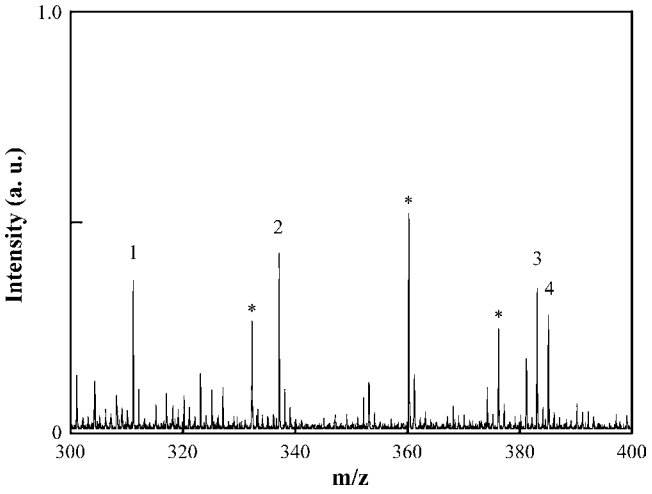


Fig. 3. SALDI-MS of a mixture of testosterone (10 μM), progesterone (10 μM), cortisone (50 μM), and hydrocortisone (50 μM). The ions at m/z (1) 311.1, (2) 337.1, (3) 383.1, and (4) 385.1 were assigned to [testosterone + Na]$^+$, [progesterone + Na]$^+$, [cortisone + Na]$^+$, and [hydrocortisone + Na]$^+$. Peaks from the background are marked as *.

3.4. Determination of testosterone in human urine

To demonstrate the practical ability of the present methodology, its application to a urine sample was evaluated. Fig. 4 indicates that good linearity (R^2 = 0.989) exists in the testerostone concentration range 1.0–30.0 μM. Next, we tested the practicality of the present SALDI-MS technique through the determination of testerostone in the urine samples. Fig. 5 displays a representative mass spectrum of a urine sample. The signals for the ions of testosterone (m/z 311.1) are readily identifiable. By comparing the peak height with

Table 2
Linearities and LODs of the four analytes.

	Linear range (μM)	Linear equation	R^2	LOD (μM)
Testosterone	1–30	$y = 76.7x + 173.5$	0.989	0.23
Progesterone	7–70	$y = 45.2 - 49.0$	0.991	0.66
Cortisone	7–100	$y = 8.7x + 53.7$	0.989	1.62
Hydrocortisone	7–100	$y = 14.1x + 98.1$	0.989	0.70

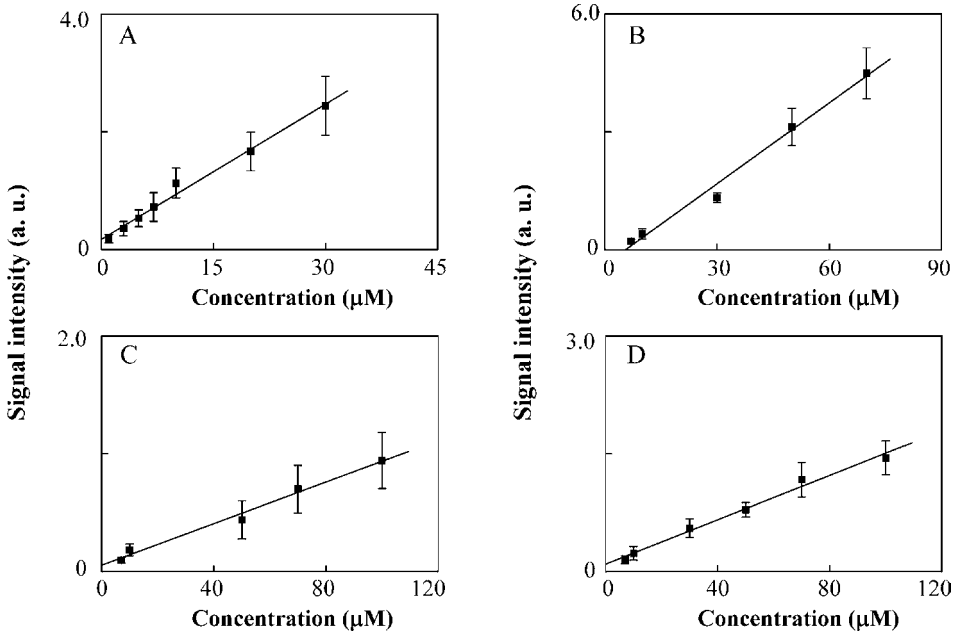


Fig. 4. MS signal intensity of the $[testosterone+Na]^+$, $[progesterone+Na]^+$, $[cortisone+Na]^+$, and $[hydrocortisone+Na]^+$ ions plotted against the concentration of testosterone (1.0–30.0 μM), progesterone (5.0–70.0 μM), cortisone (7.0–100.0 μM), and hydrocortisone (7.0–100.0 μM). All points were measured five repeated times.

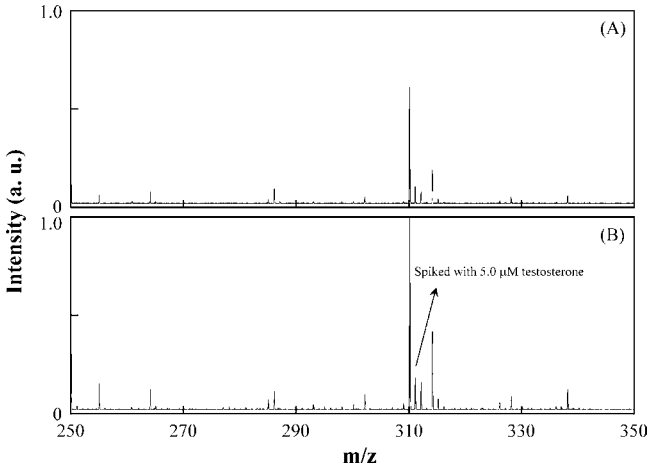


Fig. 5. SALDI-MS of a man's urine: (a) original sample and (b) sample spiked with 5.0 μM testosterone.

the standard calibration curve, we estimated that the concentration of testosterone in the man's urine was 0.34 (±0.09) μM ($n = 3$), which is in good agreement with literature values [28,29]. We note that, because of a serious matrix effect, it was essential to perform liquid–liquid extraction of the urine samples after enzymatic digestion.

4. Conclusions

In this study, we employed catechin-modified 5-nm-TiO_2-NPs as SALDI-MS matrices for the analysis of steroid hormones. When using catechin-modified TiO_2 NPs as matrices, the LODs (S/N = 3) for testosterone, progesterone, cortisone, and hydrocortisone were 0.23, 0.66, 1.62, and 0.70 μM, respectively. This approach is advantageous in terms of simplicity, reproducibility, and sensitivity for determining the level of testosterone in a urine sample. The analyzed samples are important biological compounds, illustrating that this technique holds great potential for applications in life sciences.

Acknowledgement

This work was supported by the National Science Council of Taiwan under contract numbers NSC-99–2113-M-143–001-MY2.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.09.038.

References

[1] O. Nozaki, J. Chromatogr. A 935 (2001) 267–378.
[2] M.M. Kushnir, R. Neilson, W.L. Robert, A.L. Rockwood, Clin. Biochem. 37 (2004) 357–362.
[3] M. Karas, F. Hillenkamp, Anal. Chem. 60 (1988) 2299–2301.
[4] M. Tanaka, H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida, Rapid Commun. Mass Spectrom. 2 (1988) 151–153.
[5] T.-C. Chiu, L.-S. Huang, P.-C. Lin, Y.-C. Chen, Y.-J. Chen, C.-C. Lin, H.-T. Chang, Recent Patents Nanotechnol. 1 (2007) 99–111.
[6] Y.-F. Huang, H.-T. Chang, Anal. Chem. 79 (2007) 4852–4859.
[7] C.-L. Su, W.-L. Tseng, Anal. Chem. 79 (2007) 1626–1633.
[8] N.-C. Chiang, C.-K. Chiang, Z.-H. Lin, T.-C. Chiu, H.-T. Chang, Rapid Commun. Mass Spectrom. 23 (2009) 3063–3068.
[9] T.-C. Chiu, L.-C. Chang, C.-K. Chiang, H.-T. Chang, J. Am. Soc. Mass Spectrom. 19 (2008) 1343–1346.
[10] K. Shirvas, H.-F. Wu, Anal. Chem. 20 (2008) 2583–2589.
[11] S.D. Sherrod, A.J. Diaz, W.K. Russell, P.S. Cremer, D.H. Russell, Anal. Chem. 80 (2008) 6796–6799.
[12] X.J. Wen, S. Dagan, V.H. Wysocki, Anal. Chem. 79 (2007) 434–444.
[13] K.-H. Lee, C.-K. Chiang, Z.-H. Lin, H.-T. Chang, Rapid Commun. Mass Spectrom. 21 (2007) 2023–2030.
[14] A.L. Castro, P.J.A. Madeira, M.R. Nunes, F.M. Costa, M.H. Florencio, Rapid Commun. Mass Spectrom. 22 (2008) 3761–3766.
[15] P. Lorkiewicz, M.C. Yappert, Anal. Chem. 81 (2009) 6596–6603.
[16] T. Watanabe, K. Okumura, H. Kawasaki, R. Arakawa, J. Mass Spectrom. 44 (2009) 1443–1451.
[17] T. Watanabe, H. Kawasaki, T. Yonezawa, R. Arakwa, J. Mass Spectrom. 43 (2008) 1063–1071.
[18] W.J. Shin, J.H. Shin, J.Y. Song, S.Y. Han, J. Mass Spectrom. 21 (2010) 989–992.
[19] S.K. Kailasa, K. Kiran, H.-F. Wu, Anal. Chem. 80 (2008) 9681–9688.
[20] H.-F. Wu, F.-T. Chung, Rapid Commun. Mass Spectrom. 25 (2011) 1779–1786.
[21] W.-Y. Chen, Y.-C. Chen, Anal. Bioanal. Chem. 386 (2006) 699–704.
[22] S. Taira, Y. Sahashi, S. Shimma, T. Hiroki, Y. Ichiyanagi, Anal. Chem. 83 (2011) 1370–1374.

[23] T. Yao, H. Kawasaki, T. Watanabe, R. Arakawa, Int. J. Mass Spectrom. 291 (2010) 145–151.
[24] H. Kawasaki, T. Yonezawa, T. Watanabe, R. Arakwa, J. Phys. Chem. C 111 (2007) 16278–16283.
[25] H. Kawasaki, T. Yao, T. Suganuma, K. Okumura, Y. Iwaki, T. Yonezawa, T. Kikuchi, R. Arakawa, Chem. Eur. J. 16 (2010) 10832–10843.
[26] C.-K. Chiang, Z. Yang, Y.-W. Lin, W.-T. Chen, H.-J. Lin, H.-T. Chang, Anal. Chem. 82 (2010) 4543–4550.
[27] T. Rajh, L.X. Chen, K. Lukas, T. Liu, M.C. Thurnauer, D.M. Tiede, J. Phys. Chem. B 106 (2002) 10543–10552.
[28] M.M. Robbins, N.M. Czekala, Am. J. Primatol. 43 (1997) 51–64.
[29] H. Liu, C. He, D. Wen, H. Liu, F. Liu, K. Li, Anal. Chim. Acta 557 (2006) 329–336.