Received: 10 May 2020 | Revised: 16 June 2020 | Accepted: 23 June 2020

DOI: 10.1002/jms.4628

SPECIAL ISSUE - RESEARCH ARTICLE

Journal of MASS SPECTROMETRY WILEY

Coulometry-assisted quantitation in spray ionization mass spectrometry

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Funding information
Scientific Research Startup Foundation, Grant/Award Number: IDH1615113; National Natural Science Foundation of China, Grant/Award Number: 81903575; China Postdoctoral Science Foundation, Grant/Award Number: 2019M651337

Abstract

The concentration of target analyte in a mixture can be quantified by combining coulometric measurements with spray ionization mass spectrometry. A three-electrode system screen printed on the polymer support acts both as the coulometry platform for electrochemical oxidation and the sample loading tip for spray ionization. After loading a droplet of the analyte solution onto the tip, two steps were taken to implement quantitation. First, the electrochemical oxidation potential was optimized with cyclic voltammetry followed by coulometric measurements to calculate the amount of oxidized analyte under a constant low voltage within a fixed period of time (5 s). Then, a high voltage (+4.5 kV) was applied to the tip to trigger spray ionization for measuring the oxidation yield from the native analyte ion and its oxidized product ion intensities by mass spectrometry. The analyte's native concentration is quantified by dividing the oxidized product's concentration (based on Coulomb's law) and the oxidation yield (estimated from mass spectrometry [MS] assuming that the parent and oxidation product have nearly the same ionization efficiencies). The workflow has an advantage in being free of any standard for constructing the quantitation curve. Several model compounds (tyrosine, dopamine, and angiotensin II) were selected for method validation. It was demonstrated that this strategy was feasible with an accuracy of ~15% for a wide coverage of different species including endogenous metabolites and peptides. As an example of its possible practical use, it was initially employed to make a bilirubin assay in urine.

KEYWORDS
bilirubin, conductive polymer, coulometry, quantitation, spray ionization

1 | INTRODUCTION

Ambient ionization mass spectrometry (AIMS) refers to the direct mass spectrometric analysis of the components from a complex matrix under atmospheric conditions. Because of its simplification in sample process, AIMS shows its advantage in scenarios that need on-site feedback such as clinical screening,[1] point-of-care test,[2] public security,[3] forensics,[4] and therapeutic drug monitoring.[5,6] It has become one of the evolving fields in mass spectrometry in the past decade.

AIMS has been widely reported with paper spray ionization as the most representative one, which integrates sample storage, analyte extraction, and ionization into a cheap porous medium.[7–9] Other cost-effective porous media also can be used as an alternative for sampling and ionization tips including wooden tip,[10] sponge,[11] and medical swab.[12] Various hydrophobic materials, such as wax,[13] carbon nanotubes (CNTs),[14] polymer particles,[15] and silica,[16] were also introduced to tune the surface porosity and hydrophilicity with the aim of increasing the sensitivity for hydrophilic species. Finally, thorough replacement of porous medium with polymer materials like organosiloxane,[17] Teflon,[18] and CNT-doped PMMA[19] also becomes a branch that further broadens the coverage of AIMS.

In terms of quantitative AIMS, the strategy of using a calibration curve remains the default choice. In this procedure, one constructs a linear relationship between the amount of the target and the ion intensity of the target.[20] The calibration curve helps to normalize differences both in compound-dependent ion efficiency and sample-dependent ion suppression, making the relative concentrations of compounds in various samples more reliable. However, it can only be implemented with the premise of having both an available target standard and a similar compound, often an isotope, to serve as the internal standard (IS). The quantitation progress must be accompanied by the construction of the working curve.

We present here an alternative approach requiring no need to introduce an IS or a target standard. Instead, we combine coulometry with AIMS to achieve standard-free quantitation. Specifically, coulometry first helps to calculate the accurate amount of target analyte being electrochemically oxidized (ECO) according to Faraday's law. Thereafter, conductive polymer spray ionization mass spectrometry (CPSI-MS),[19] as one of the representative AIMS methods, was employed for estimating the ECO yield based on the ratio of the native analyte ion and its oxidized product ion. Given the ECO product's amount and the ECO yield, the accurate quantity or concentration of the target analyte can be thus easily calculated.

In previous studies, this coulometric mass spectrometry strategy has been proven to be successful in quantifying metabolites and peptides using a liquid chromatography–electrochemistry mass spectrometry (LC-EC-MS) platform.[21–23] These works integrate the electrochemical cell between the LC and MS, mainly showing its strategic advantage over the traditional LC–MS quantitation, which relies on construction of a standard curve. In this work, we are aiming to apply this coulometry-assisted standard-free quantitation strategy in an even simpler way by directly using a three-electrode screen printed on the polymer both for electrochemical oxidation and spray ionization for AIMS analysis. However, it needs to be recognized from the start that this approach is not universally applicable. First, it requires the presence of an analyte that can be ECO. Second, it is only applicable to these organic molecules, which do not exhibit a substantial change in ionization efficiency after electrochemical oxidation.

2 | EXPERIMENTAL

2.1 | Reagents and materials

Methanol, ultrapure water, formic acid, and ammonium acetate were purchased from Fisher chemical. The dopamine, tyrosine, bilirubin, angiotensin II, and Nafion perfluorinated resin solution (20 wt.% in lower aliphatic alcohols and water) were purchased from Sigma-Aldrich. Dioctadecyl dimethyl ammonium chloride (DODMAC) and carboxylic multi-walled carbon nanotubes (MWCNTs, ID 2–5 nm, OD < 8 nm, length 10–30 μm) were purchased from Adamas and J&K Scientific, respectively. Urine samples (for urine bilirubin test) were kindly provided by the National Center of Gerontology, Beijing Hospital. The urine collection was approved by the medical ethics committee of the Beijing Hospital. All patients were informed and signed consent forms.

2.2 | SPE fabrication

Polyethylene terephthalate (PET) was used as the substrate. The working electrode (WE) and the counter electrode (CE) were made of carbon. The reference electrode (RE) was made of silver/silver chloride (Ag/AgCl). Silver was screen printed on the PET surface as the conducting wires connected to WE, RE, and CE. The screen-printed electrode (SPE) polymer was fabricated by the Xenslet studio office (SPEnsor) with slight modification based on the custom design, which also printed the carbon ink both onto the triangular tip and the outer ring CE. Thus, this SPE polymer tip with a conducting layer can serve as both the WE and the high voltage triggered spray ionization tip. For the electrochemical oxidation of tyrosine, dopamine, and angiotensin II, the WE was modified with MWCNT (50 mg/mL)/Nafion solution by direct deposition. As for the bilirubin test, the WE was modified with MWCNT (10 mg/mL)/DODMAC (1.0 mg/mL).

2.3 | Instruments and setup

The LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA) was employed for recording mass spectra. The CHI660E potentiostat (Beijing Chinese Science Days Technology Co., Ltd) was employed to record the current response signal and implement the electrochemical experiment including chronoamperometry (CA), cyclic voltammetry (CV), and differential pulse voltammetry (DPV). A high voltage supply (BOHER HV, Genvolt, UK) was used for generating the constant high voltage applied on the SPE polymer tip for triggering the spray ionization. A home-built system connects and switches between the potentiostat, the high voltage supply, and the SPE tip (shown in Figure 1A).

2.4 | Solution preparation

Stock solutions were prepared by dissolving tyrosine, dopamine, and angiotensin II in a concentration of 300 μM in 50 mM ammonium acetate (pH 7.0). To validate the method accuracy and precision, these stock solutions were diluted to three different levels with the concentration of 100 μM (dopamine, DA), 20 μM (tyrosine, Tyr), and 30 μM (Angiotensin II, Ang), respectively. For quantitative comparison between ECO-CPSI-MS and LC–MS quantitation, a series of bilirubin solutions were prepared by dilution with ammonium bicarbonate-ammonia water (pH 8.5). Then they were 10-fold diluted by spiking 20-μL standard solutions into 180-μL urine as mimetic biological samples. Final urine bilirubin concentrations were 20 μM, 100 μM, 200 μM, 400 μM, 600 μM, and 800 μM.

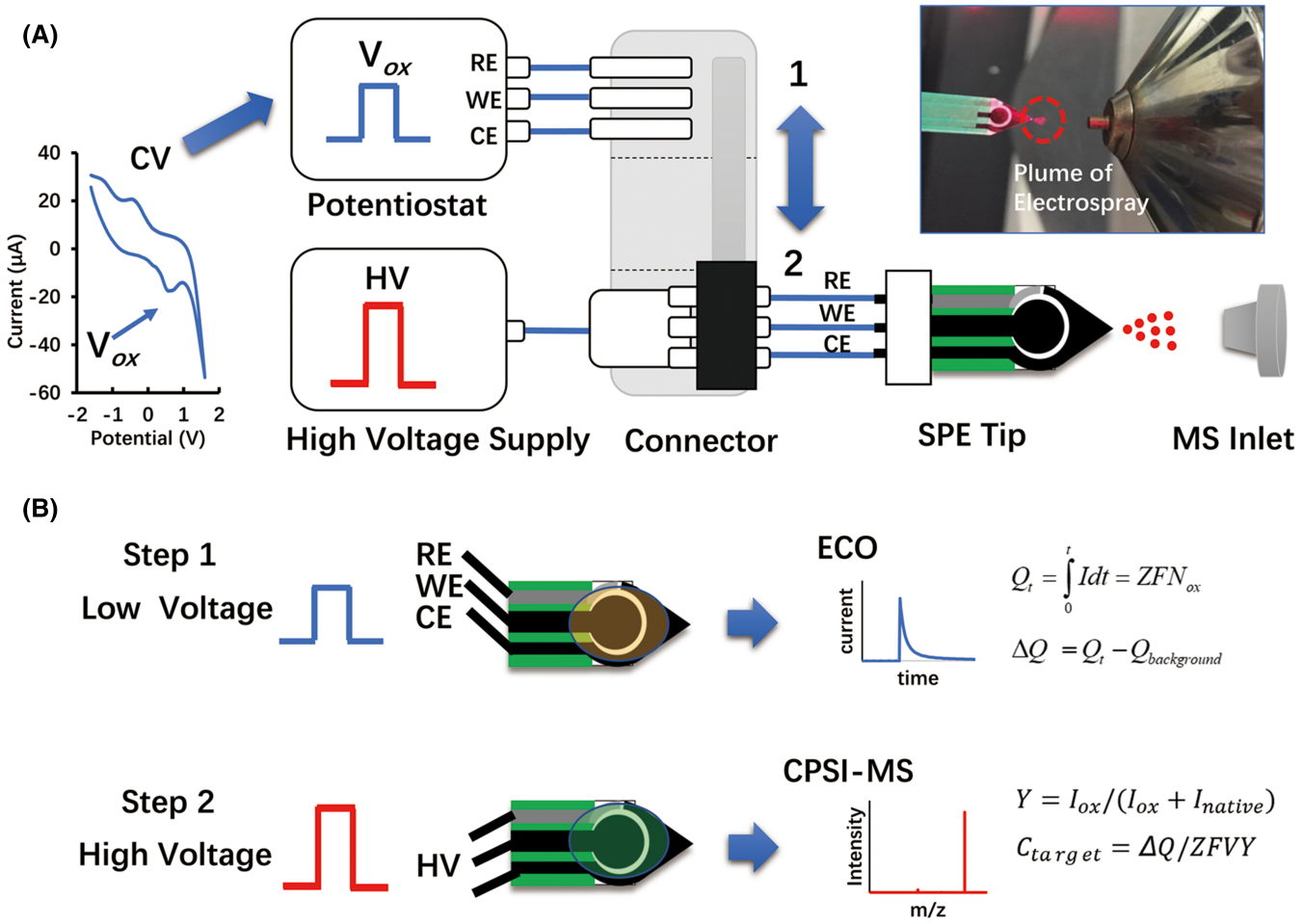

FIGURE 1 Graphic illustration of the in situ quantitation by EC coupled with CPSI-MS. (A) The general workflow of the EC-CPSIMS quantitation strategy. There are two gears on the connector to switch between potentiostat (Position 1) and high-voltage supply (Position 2); (B) The simple customized EC-CPSI-MS setup. The inset photo displays the generation of the electrospray jetting process captured by a beam from a laser pointer

2.5 | ECO-CPSI-MS measurements

The ECO-CPSI-MS procedure includes two steps after loading the 10-μL pure solution or biological fluid onto the SPE polymer tip. First, CA is implemented under a constant oxidation potential, which was confirmed from the CV curve. Typical oxidation time is 5 s. Then the potentiostat records the current change versus the time from which we calculate the molar amount of the oxidized analyte according to Faraday's law. In the second step, a similar procedure is carried out based on previous reports.[21–23] When high voltage (+4.5 kV) is applied onto the SPE polymer tip, the charged microdroplets are generated for spraying into the mass spectrometer. The specific MS scan window was set to monitor the ratio of the analyte ion and its oxidative product ion. The MS capillary temperature was set at 275°C with the tube lens voltage set at 60 V. The automatic gain control was set at 3×10^6 with the maximum injection time set at 400 ms. More specific details for the analyte quantification are illustrated in the next section.

2.6 | ECO-CPSI-MS calculation

During the first step of coulometry, a proportion of native analyte (M) lost two protons (H^+) and electrons (e^-) to generate the electrochemical oxidation product (M − 2H). Based on Faraday's law, the amount of analyte's oxidation product (N_{ox}, mol) can be accurately calculated by Equation 1. Here, F is the Faraday's constant (96 485 C/mol), Z is the number of charges transferred (Z = 2 is the default value), and ΔQ is the total amount of electric charge, which can be calculated based on the trapezoid integration of the current peak area and background peak subtraction (Equations 1 and 2). In terms of the second step, CPSI-MS can provide the intensity ratio between the native analyte (I_{native}) and its oxidized product (I_{ox}). Because there is only a slight difference in the chemical structure, this intensity ratio is presumed to represent the oxidation yield, denoted as Y as shown in Equation 3. Therefore, given the yield, product amount, and the loading volume, V, the accurate concentration C_{target} of the target analyte

can be calculated from Equation 4. The general workflow is shown in Figure 1B.

$$Q_t = \int_0^t Idt = ZFN_{ox}, \quad (1)$$

$$\Delta Q = Q_t - Q_{background}, \quad (2)$$

$$Y = N_{ox}/N_{total} = N_{ox}/(N_{ox} + N_{native}) = I_{ox}/(I_{ox} + I_{native}), \quad (3)$$

$$C_{target} = N_{ox}/VY = \Delta Q/ZFVY. \quad (4)$$

2.7 | LC–MS for cross validation

UHPLC system (1200 Series, Agilent Technologies) coupled with Q-TOF mass spectrometer (G6230B, Agilent Technologies) was employed to monitor the analyte, bilirubin, and zinc protoporphyrin (IS) in the urine samples. Dispersive liquid–liquid microextraction (DLLME) was used for the all-in-one pretreatment including extraction, enrichment, and purification. Each urine sample (1.0 mL) was collected into the conical tub. It was first acidified with 1-μL formic acid and then followed with spiking 20-μL dichloromethane and 50-μL acetonitrile. After vortexing for 3 min and centrifuging at 10 000 rpm for 5 min, the bottom layer of extractant was transferred, quickly dried under vacuum centrifugation, and reconstituted by adding 20-μL ACN. The injection volume is 5 μL for LC–MS analysis. The solvent system was composed of acetonitrile (ACN) and water with the flow rate set at 1000 μL/min (4:1 post-column split ratio). The gradient elution was programmed as follows: (1) 0–2.5 min, 10% ACN; (2) 2.5–7.5 min, 10 → 100% ACN; (3) 7.5–10.0 min, 100 → 10% ACN. The key MS parameters were as follows: gas flow, 10 L/min; nebulizer, 45 psi; cap voltage, 3 kV; and source temperature, 325°C. Selected ion monitoring (SIM) was used for target monitoring with the SIM channels set as bilirubin at *m/z* 585.27 $[M + H]^+$ and IS at *m/z* 627.19 $[M + H]^+$.

3 | RESULTS AND DISCUSSION

Three model compounds, tyrosine, dopamine, and angiotensin II, were selected to investigate the feasibility of the proposed standard-free quantitation. The CV was first implemented to get the optimal WE potential versus RE for achieving the high ECO efficiency. It was found that no obvious oxidation peaks were observed in the CV curves performed by the bare WE. In contrast, the MWCNT/Nafion-

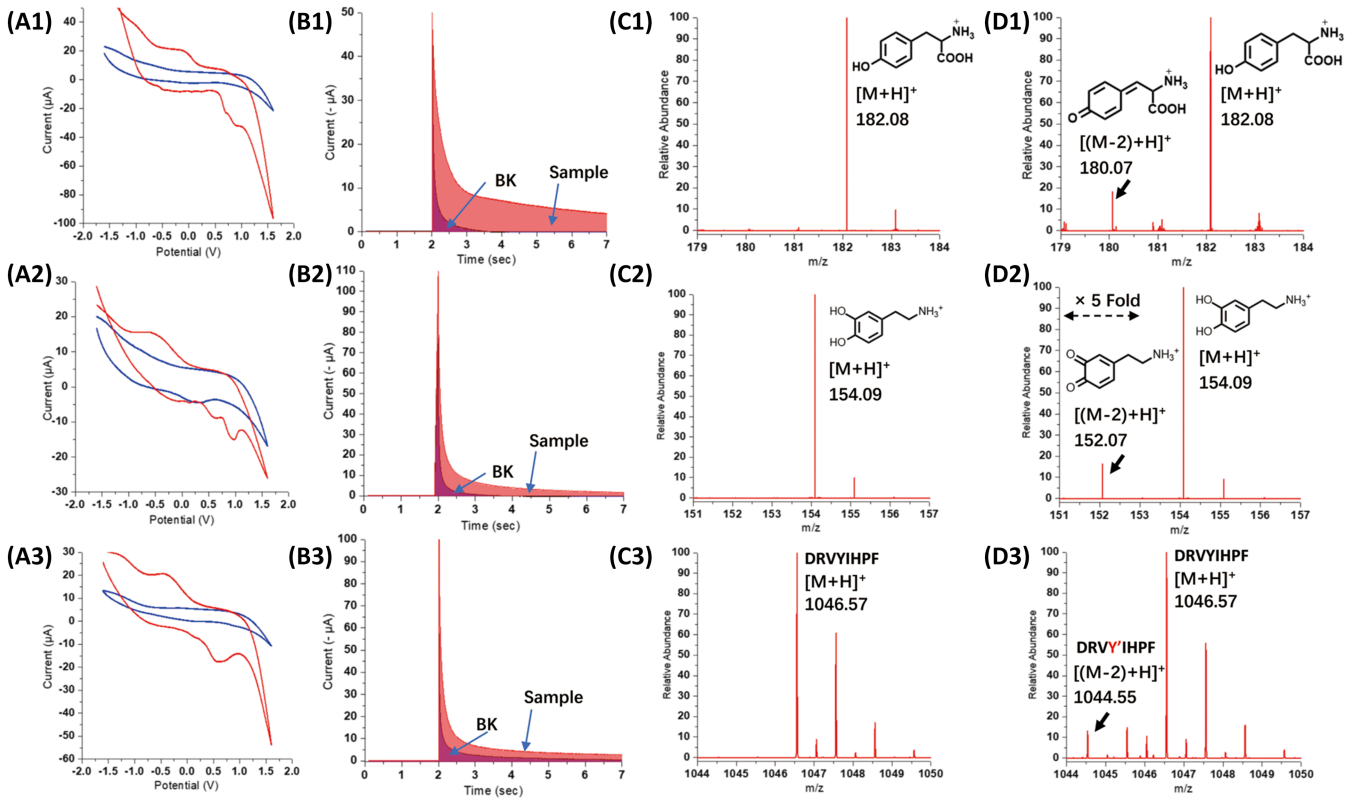

FIGURE 2 The ECO-CPSI-MS analysis results for three model compounds. (A) The cyclic voltammograms of tyrosine, dopamine, and angiotensin II on the Nafion-modified screen-printed WE (red line) and the bare WE (blue line); (B) the current responses versus with the electrochemical oxidation time for analyte solution (sample) and blank solvent (BK); (C,D) mass spectra of dopamine, tyrosine, and angiotensin II acquired before and after electrochemical oxidation

modified WE significantly improved the electrochemical response at approximate +550 mV (Figure 2A). This revealed that the WE modification was necessary for improving the ECO process. The stability of the WE modification by deposition was also investigated to confirm how many times one modified SPE tip can be used. CV experiment was repeatedly implemented for one Ang II solution on the same modified WE. After each round of CV test, the SPE tip was fully washed with deionized water. Based on the CV curve shapes, it was found that modified WE remained relatively stable at least twice. As for the current and oxidation potential, they can remain stable for at least four times with the currents' RSD at 11.7% and potential's RSD at 5.7% (Figure S1).

For each model compound, the negative contrast solvent and the sample solution were tested by the CA experiment, which applied the corresponding optimal potential on the WE. The representative current curves were displayed in Figure 2B, and the area under the curve (AUC) was integrated by the trapezoid method to represent the gross (Q_x) and background (Q_0) transfer charge amounts. By AUC subtraction and Faraday's law (Equations 1 and 2), the charge amount (ΔQ) and the electrolytic product amount (N_{ox}) were calculated (Table 1). Comparing the mass spectra of the target analyte before and after oxidation, the $[(M - 2) + H]^+$ ion was found to be significantly increased (Figure 2C,D). The oxidation peaks and the oxidation site can be further validated from the CID-MS/MS spectra (Figures S2–S4). Based on the intensity ratio of oxidation product and native analyte ions (I_{ox}/I_{native}), the average yield was estimated at 14% for tyrosine, 3% for dopamine, and 10% for angiotensin II (Table 1). Given the oxidized product amount and the oxidation yield, the initial concentration of the three analytes was calculated based on Equation 4. Three aliquots of solutions were tested for each compound to evaluate the accuracy and precision. As results, the mean relative error (RE) was +2% (tyrosine), +7% (dopamine), and +11% (angiotensin II). The relative standard deviation (RSD) was 8% (tyrosine), 6% (dopamine), and 4% (angiotensin II).

Dopamine and angiotensin II were selected as the model compounds for giving a better comparison in computing the quantitation error with the previously reported works.[21,22] As is shown in Table 1, the RE for dopamine is 7% compared with only 0.5% in Xu et al.[22] RE for angiotensin II is 11% compared with −5.5% in Zhao et al.[21] The accuracy does not become improved because the presented method is directly coupled with the ambient ionization MS instead of LC. Unlike the LC system that effectively separates each component from the complex matrix before EC-MS quantitation, the ECO-coupled ambient ionization MS directly quantifies the analyte within the sample. It will inevitably include unknown background components. Although the accuracy is not as good as that in previous reports, it is still within the range of ±15%, which is the basic requirement in accuracy for method validation of biological sample analysis.

It is worth noting that the WE potential was a key parameter to control precisely the electrochemical process. The electrochemical oxidation could fail if the WE potential was set too low. However, if the WE potential was set too high, the over oxidation would generate more than one oxidation product, which complicated the calculation. Taking the tyrosine and dopamine as examples, when the potential applied on the WE reached to +900 mV, there were other new oxidation peaks at the position of $[(M - 4) + H]^+$ (Figures S5 and S6). For angiotensin II, the monitoring result illustrated that the ratio between the native and oxidized peptide ions reached the maximum at +600 mV followed by a decrease with further raising the WE potential (Figure S7). Therefore, the potential control becomes more critical in terms of electrochemically oxidizing the peptide that contains more than one oxidation site. The ECO priority of different amino acid residues was also investigated by implementing the coulometry experiment on the mixture solution of 20 amino acids with same molar concentration. It was shown that tyrosine and tryptophan tend to be oxidized much more easily than the other amino acids (Figure S8).

After proving the feasibility of the proposed ECO-CPSI-MS method, we applied it to test for bilirubin in urine. Bilirubin is an important metabolite marker for indicating various liver and gallbladder diseases such as cirrhosis, hepatitis, jaundice, and biliary tract disease. A urine bilirubin test helps clinical practitioners to diagnose and evaluate the seriousness of the disease. Normally, bilirubin is not

TABLE 1 The quantitation results for three model compounds (n = 3)

Analyte	Theor. conc. (μM)	Charge (Q_x, μC)	Charge (Q_0, μC)	Charge (Q_{neat}, μC)	N_{ox} (pmol)	Yield (%)	Cal. conc. (μM)	Mean RE %	Mean RSD %
Tyr	20	6.66	1.26	5.40	27.98	14.2	19.76	1.73	7.77
		6.12	1.19	4.93	25.55	13.3	19.14		
		7.63	1.33	6.30	32.65	14.8	22.14		
DA	100	7.77	1.49	6.28	32.54	3.23	100.69	6.60	5.54
		8.09	1.42	6.67	34.56	3.07	112.49		
		7.56	1.51	6.05	31.35	2.94	106.61		
Ang II	30	7.24	1.16	6.08	31.51	9.21	34.19	10.62	4.10
		7.88	1.32	6.56	33.99	10.1	33.73		
		8.29	1.55	6.74	34.93	11.0	31.64		

Note: The "Cal. conc." denotes the calculated concentration (C_{native}) according to the Formula 5. The number of charge transfer (Z) is 2. The Faraday constant (F) is 96 485 C/mol. The loading volume (V) is 10 μL.

Abbreviations: RE, relative error; RSD, relative standard deviation.

found in urine. In this research, we first constructed positive urine samples by spiking urine into a series of bilirubin standard solutions for investigating the linearity and accuracy of the method. Urine (10 μL) was spread onto the SPE polymer tip to form a dried urine spot followed by the implementation of the ECO-CPSI-MS measurement. Figure 3A,B displays the mass spectra of bilirubin ions ($[M + H]^+$, *m/z* 585.27) before and after the ECO process. We can see that there is an obvious new peak at *m/z* 583.27 after oxidation. This can be precisely identified as the bilirubin's oxidation product, biliverdin, by CID-MS/MS (Figure 3C,D).

Apart from bilirubin, there were interfering components that may also contribute to the current response. Therefore, a nonenzymatic modification strategy was adopted to make the ECO process specific to bilirubin. The mixture of MWCNT and DODMAC was uniformly spread and deposited onto the WE surface where the oxidation takes place. This modification choice is based on the hydrophobic interaction between the MWCNT and porphyrin, as well as the ionic bond between the DODMAC's quaternary ammonium and bilirubin's two carboxyl groups (Figure 4A). To investigate the selectivity of this DODMAC/MWCNT modification, the CV curves of the bilirubin-spiked urine on the bare WE (carbon) and modified WE were compared. It was shown that the CV curve acquired from the bare WE is smooth and low in current response whereas an oxidation peak was observed at the potential around +230 mV (vs. Ag/AgCl) applied on the modified WE (Figure 4B). DPV revealed that the peak current around +230 mV increased with the urine bilirubin concentration (Figure 4C). In contrast, there was no oxidation peak observed in the blank urine sample. The stable current performed an ideal linearity relationship within the range of 2–80 μM (Figure 4D). It was demonstrated that the DODMAC/MWCNT-modified WE can selectively oxidize the target analyte in the biological fluid, guaranteeing the accurate calculation of the amount of oxidation product.

The LC–MS method was employed to validate the reliability of the ECO-CPSI-MS quantitation result. Meanwhile, the Harrison assay[24] was also employed to give semi-quantitative reference based on the color change (Figure S9). All unknown urine samples collected from the 12 volunteers as well as the constructed mimetic urines were divided into three shares for LC–MS, ECO-CPSI-MS, and Harrison assay, respectively. The representative extracted ion chromatograms for bilirubin and the IS are displayed in Figure 5A,B. As results showed, the average RE (RE%) between the ECO-CPSI-MS and LC–MS was within −3% for the mimetic urine samples and −4% for the

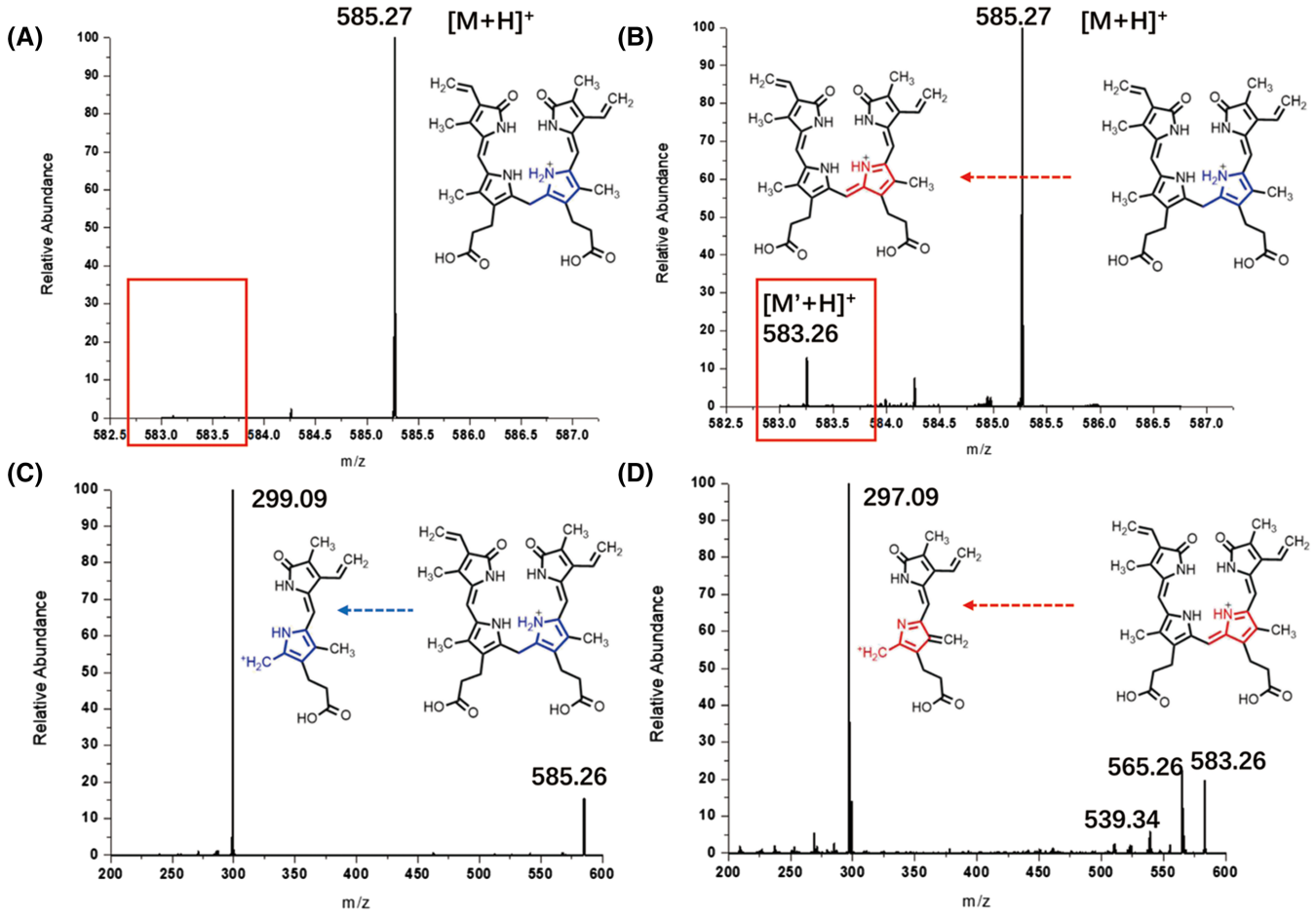

FIGURE 3 Mass spectra of the bilirubin in urine. (A) The mass spectrum of bilirubin before electrochemical oxidation; (B) the mass spectrum of bilirubin after electrochemical oxidation, bilirubin ion [M + H] + *m/z* 585.27, its oxidation product biliverdin ion as [(M − 2) + H] + *m/z* 583.27; (C) the CID-MS/MS spectrum of bilirubin ion; (D) the oxidation product identification by CID-MS/MS

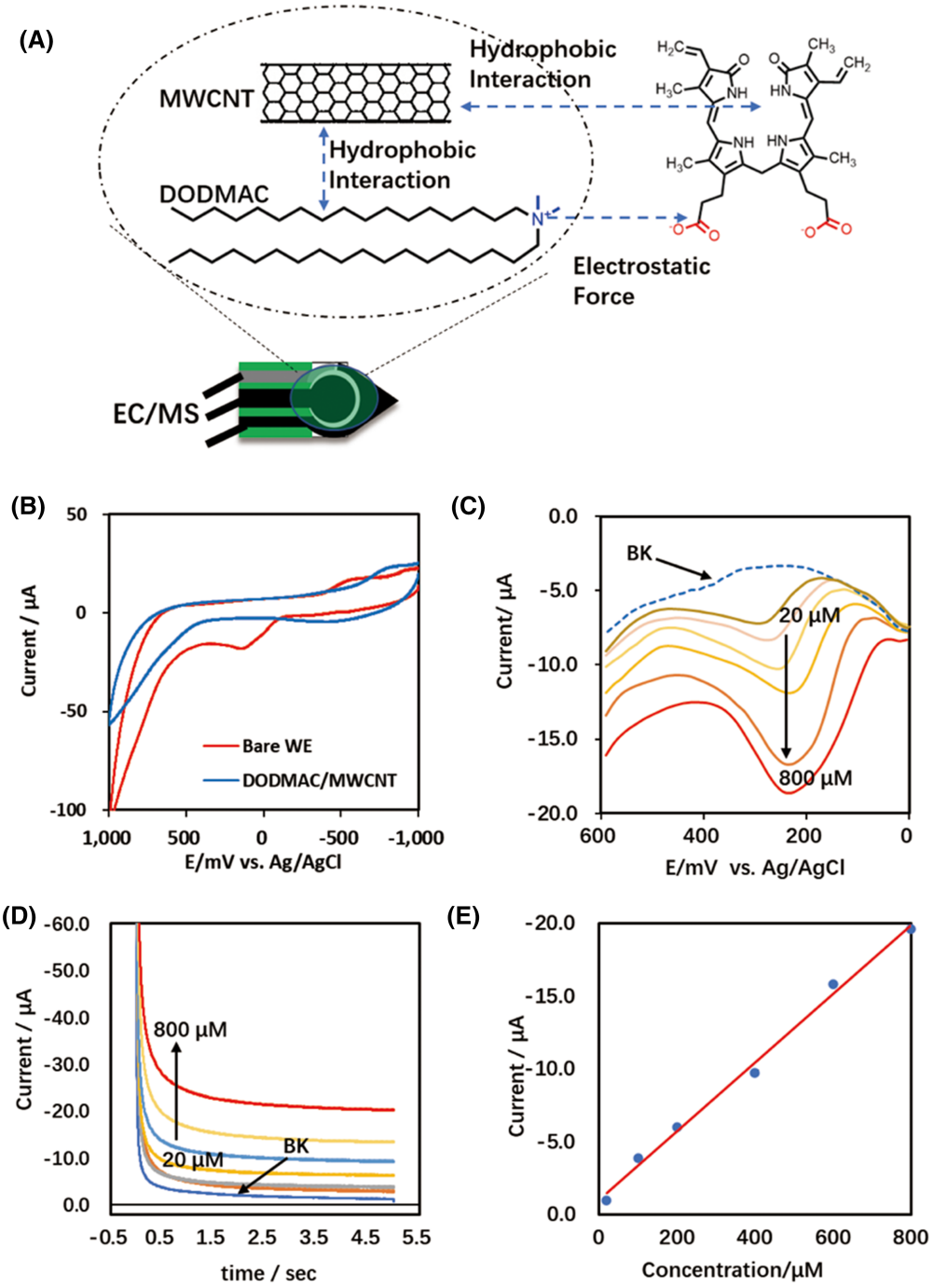

FIGURE 4 Specific response and linearity of the DODMAC/WMCNT-modified working electrode. (A) The illustration of the non-covalent interaction between the non-enzymatically modified materials and bilirubin; (B) the improvement of electrochemical oxidation peak in the CV curve of the modified WE (red line) compared with that on the bare WE (blue); (C) the oxidation peak current increased with the bilirubin concentration in the DPV curves; (D) the stable currents increased with the bilirubin concentration in the chronoamperometry. The inset graph plotted the ideal linearity between the stable currents and the bilirubin concentration; (E) the linear correlation between the stable currents and the bilirubin concentration

collected real patients' urine samples. The correlation of the two quantitation methods was characterized by the Pearson correlation coefficient, which was 0.95 (Figure 5C). In contrast, the Harrison assay only gave a semi-quantitative result, which indicates that all these unknown samples fall into the one "+" range (5–100 μM, Figure S9). These results indicated that the proposed standard-free quantitation strategy can achieve concentration values comparable with the traditional LC–MS and calibration curve-based quantitation method.

The EC-MS has been reported in simulating drug metabolism,[25] capturing reaction intermediate,[26] monitoring polymerization progress,[27] disulfide bond reduction, and protein digestion.[28] The coulometric mass spectrometry has also proved to be an accurate quantitation method in proteomics and metabolomics application scenarios using the LC-EC-MS platform. In this research, coulometry-based quantitation strategy was successfully transferred to the AIMS platform, which further simplified the on-site quantitation of the target analyte. The coulometry provides an accurate oxidized product amount. AIMS quickly provides the yield behind the EC oxidation. Given the complementary advantages of the EC and AIMS, the label-free, absolute quantitation can be implemented in an easier manner.

Nevertheless, we must admit that there is still room for further methodologic improvement. Unlike the LC–MS system, which

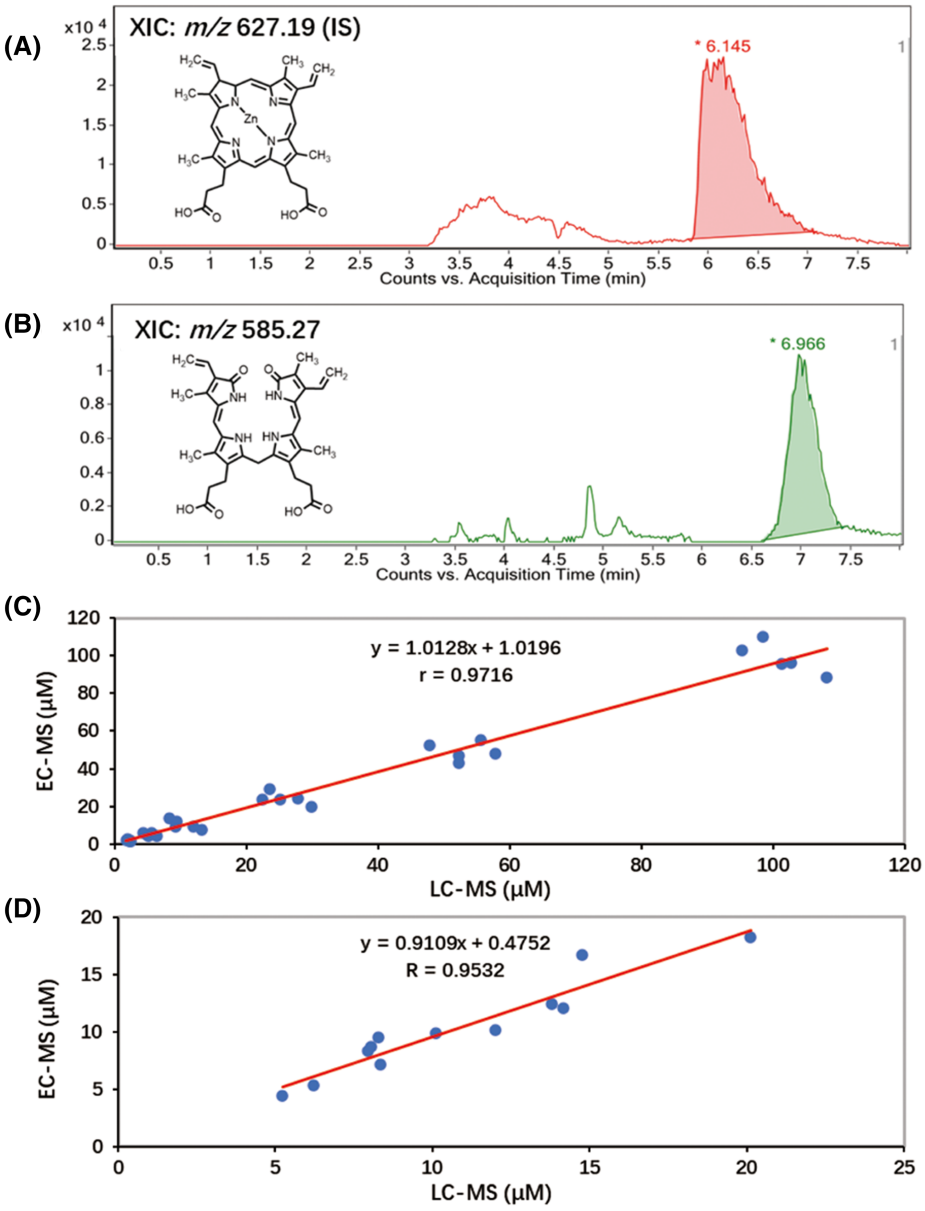

FIGURE 5 Cross validation of the developed EC-CPSI-MS method with the traditional LC–MS quantitation method. (A) The extracted ion chromatography of bilirubin; (B) the extracted ion chromatography of the internal standard, zinc protoporphyrin; (C) the correlation of EC-CPSI-MS and LC–MS quantitation method evaluated with the dilution series of bilirubin-spiked urine samples. (D) The correlation of EC-CPSI-MS and LC–MS quantitation method evaluated with the unknown urine samples

separates the complex components in advance, the nontarget compounds also contribute to the current response, which needs to be subtracted by the control sample. In the study, we not only employed control sample to subtract the background charge but also introduced non-enzyme modification to successfully satisfy the urine bilirubin quantitation with the basic requirement of bioanalysis method, which sets RE at ±15%. We also note that simple pretreatment steps, such as DLLME before LC–MS analysis of urine bilirubin, can be considered if the impurities' interference is too severe to lie within the ±15% range. Alternatively, those frequently used electrochemical modifications on the WE could be recommended for introducing target-specific elements such as enzymes, antibodies, aptamers, lectins, or molecularly imprinted polymer cavities.[29] It would increase not only the selectivity of the EC oxidation but also the accuracy of ECO-CPSI-MS quantitation.

4 | CONCLUSIONS

The three-electrode system screen printed on the polymer substrate can both act as the coulometry platform and the spray ionization probe. Thus, coulometric measurements and mass spectrometric analysis can be easily integrated to implement the proposed standard-free quantitation strategy.

Coulometry was demonstrated to be feasible for calculating the quantity of the ECO analyte product. With the oxidation yield measured by the following spray ionization MS characterization, the analyte's concentration can be accurately calculated with the RE within ±15%, which is an acceptable criterion in the biological sample analysis for metabolites, peptides, or drugs. This coulometry-assisted strategy can simplify the quantitation process in the AIMS field compared with using a calibration based on an added IS. It is believed that

this method represents a promising strategy for AIMS in an on-site testing scenario.

ACKNOWLEDGEMENTS

X.S. thanks the China Postdoctoral Science Foundation (No. 2019M651337) and the National Natural Science Foundation of China (No. 81903575) for support of this study. This work was also supported by the Scientific Research Startup Foundation (IDH1615113) from Fudan University. The authors also thank Dr. Chao Li (Beijing Hospital) for coordinating the urine collection.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Song X, Chen H, Zare RN. Coulometry-assisted quantitation in spray ionization mass spectrometry. *J Mass Spectrom*. 2021;56:e4628. https://doi.org/10.1002/jms.4628