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Improvements for absolute quantitation using electrochemical mass spectrometry

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ABSTRACT

We recently reported a new quantitation method using mass spectrometry (MS) in combination with electrochemistry (EC, *J. Am. Soc. Mass Spectrom.*, 2019, 30, 685). The strength of this method is that no reference standard or isotope-labeled compound is required for absolute quantitation. The method relies on electrochemical oxidation of an electrochemically active target compound to determine the amount of the oxidized compound using Faraday's Law. On the other hand, the oxidation reaction yield can be determined based on the MS signal change following electrolysis. Therefore, the absolute amount of the analyte can be calculated. Our experiment is carried out using a coupled liquid chromatography/electrochemistry/mass spectrometry (LC/EC/MS) apparatus. In this study, the method is further optimized. First, quantifying the compounds in a mixture is possible after the chromatographic separation. Gradient elution is used for separation and each compound can be quantified using the electrochemical mass spectrometry method. Second, for compounds that are already purified, LC column is not necessary and can be removed (i.e., flow-through analysis), thus shortening the analysis time for each injected sample from 10 min to 2 min. With using an LC auto-sampler, multiple samples can be injected sequentially. All the quantitation errors shown in this study are within 5%, indicating a good accuracy of our method.

1. Introduction

Mass Spectrometry (MS) has become a powerful tool commonly used for identification of a variety of chemical species due to its high sensitivity and capability of providing structural information [1–5]. Nevertheless, accurate quantitation by MS is still challenging due to fluctuation of the MS signals; and the signal intensity in a MS spectrum does not always correlate well with the amount of analytes [6–8]. Therefore, accurate MS quantitation often has to use reference standards for calibration or isotope-labeled compounds as a reference [9,10]. To date, many quantitative MS methods have been developed with great successes, such as isotope-coded affinity tags (ICAT) [11–17], stable isotope labeling with amino acids in cell culture (SILAC) [15,18–21], isobaric tags for relative and absolute quantification (iTRAQ) [22–25], metal element chelated tags (MECT) [26] and isotope-coded protein labeling (ICPL) [27] *etc.* However, the reference standards or isotope-labeled compounds that are required for quantification might not be readily available, and sometimes their sytheses are cost prohibitive and time-consuming [28–31].

To overcome this issue, we recently developed an electrochemistry (EC)-assisted absolute quantitation method using MS without the need for the reference standards or isotope-labeled compounds [32]. In our method, a target analyte, if electrochemically oxidizable, is first introduced to an electrochemical cell for oxidation and followed by MS detection. The integration of the resulting electrochemical current peak over time provides information about the amount of the compound, based on the Faraday's Law:

$$n = \frac{Q}{zF\Delta i} \quad (1)$$

where n is the moles of the analyte, Q is the total charge involved in the oxidation reaction, z is the number of electrons transferred per

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molecule during oxidation, F is the Faraday constant (9.65×10^4 C/mol), and Δi is the redox conversion yield. Meanwhile, this electrochemically active analyte shows a reduced intensity in the acquired MS spectra from electrochemical oxidation. The relative MS intensity change can be used to determine the redox conversion yield (Δi). Thus, the amount of the analyte can be calculated using Eq. (1). Using this approach, various analytes such as dopamine, norepinephrine, and rutin as well as peptide glutathione in low quantity were successfully quantified. However, only pure compound or a single species in a biological matrix (e.g., uric acid in urine) was quantified in this prototype experiment [32].

In this study, we further show that our method can be applied to absolute quantitation of individual compounds in a mixture following chromatographic separation. As a demonstration, dopamine and serotonin (5-HT) were separated using a hydrophilic interaction liquid chromatography (HILIC) column with gradient elution, followed by electrochemical oxidation and MS detection for quantitation.

On the other hand, for absolute quantitation of samples that are already purified, LC column can be removed (i.e., flow-through analysis) so that analysis time for each injected sample can be greatly reduced, from 10 min in our previous study [32] to 2 min. By using an auto-sampler, consecutive injections can be made sequentially, allowing fast analysis of multiple samples. These improvements would help to expand the application of our method for quantitative analysis.

2. Experimental section

2.1. Chemicals

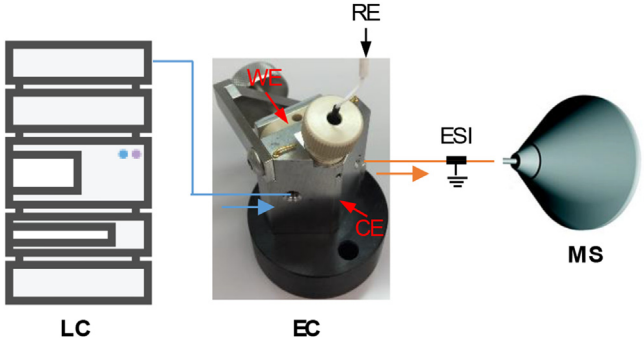
Dopamine (DA) hydrochloride, (−)-norepinephrine (NE), and serotonin (5-HT) hydrochloride were purchased from Sigma Aldrich (St. Louis, MO). Formic acid and acetonitrile were obtained from Fisher Chemical (Fair Lawn, NJ), and deionized water used for sample preparation was obtained using a Millipore Direct-Q5 purification system (Burlington, MA).

2.2. Instrumentation

For the experimental setup (illustrated in Scheme 1), a Waters Ultra-Performance Liquid Chromatography (UPLC, Milford, MA) was coupled with a BASi electrochemical thin-layer flow cell (West Lafayette, IN). The electrochemical cell was equipped with a glassy carbon disc electrode (*i.d.*, 6 mm) as the working electrode (WE). For cleaning process, this glassy carbon electrode can be polished on a polishing pad with the use of alumina polishing solution. A Ag/AgCl (3 M NaCl) electrode was used as the reference electrode (RE) and stainless steel cell body served as a counter electrode (CE). A positive potential of +1.05 V (vs. Ag/AgCl) was applied to the WE electrode for analyte oxidation. The oxidation current response was monitored and recorded by a ROXY™ potentiostat (Antec BV, Netherland). The electric current peak area was integrated by importing the current data to OriginPro 2018b to calculate the total electric charge (Q) involved in the oxidation reaction. The eluate flowing out of the cell was subsequently analyzed using online electrospray ionization mass spectrometry (ESI-MS). MS data were collected using a high-resolution Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA). The sheath gas flow rate was 10 L/h. The spray voltage was +4 kV and the inlet capillary temperature was kept at 250 °C. Extracted ion chromatograms (EIC) of the analytes were acquired by Thermo Xcalibur (3.0.63).

For flow-through analysis of the pure compounds, the LC column was removed. The mobile phase flow rate was kept at 100 μL/min. An auto-sampler was used to inject samples with the injection volume of 6 μL. As illustrated in Fig. 1, an injection sequence consisted of three steps: 1) a blank solvent (ACN/H_2O/FA, 50:50:0.1 by volume) injection to check if there was any sample leftover, 2) an analyte solution injection when the electrochemcial cell was turned off (i.e., the "cell-off "mode), 3) an analyte solution injection when the electrochemcial cell was turned on (i.e., the "cell-on "mode). The running time for each injected samples was 2 min, except that the electrochemical cell was turned on 1.5 min before step 3), so that the charging current could fade away and not interfere with the analyte oxidation current. DA and NE were chosen as two test samples and their injected concentrations were 50 μM.

For the mixture analysis, A Waters XBridge™ Amide column (2.1 mm × 150 mm, 3.5 μm) was selected for HILIC separation. A mixed solution containing DA and 5-HT was tested as a demonstration. The mobile phase flow rate was 200 μL/min. The mobile phase A was 10 mM of NH_4OAc dissolved in ACN/H_2O (90:10) solution, and mobile phase B was 10 mM of NH_4OAc in H_2O. The mobile phase B started from 5% and increased to 15% in 10 min, and then climbed to 35% from 10 min to 20 min. After that the mobile phase B was kept at 35% for 5 min, and then went back to 5% in 1 min. The concentrations of DA and 5-HT were both 50 μM in the mixture solution, and the injection volume was 3 μL.



Scheme 1. Schematic showing the LC/EC/MS apparatus used for absolute quantitation.

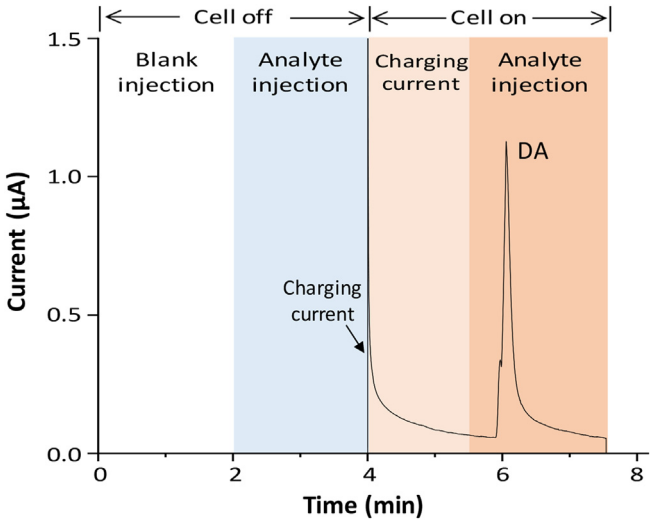


Fig. 1. A schematic showing of the first 7.5 min of the injection sequence, where 0–2 min was a blank solvent injection for cleaning purpose; 2–4 min was an analyte injection in the "cell-off" mode. The electrochemical cell was turned on at 4 min, and a charging current was generated as the potential was applied. Another injection for the analyte solution was injected in the "cell-on" mode, 1.5 min after the cell was turned on. The time period of 0–7.5 min could be considered as a cycle, and the second consecutive cycle started at 7.5 min (see Fig. S1, Supporting Information).

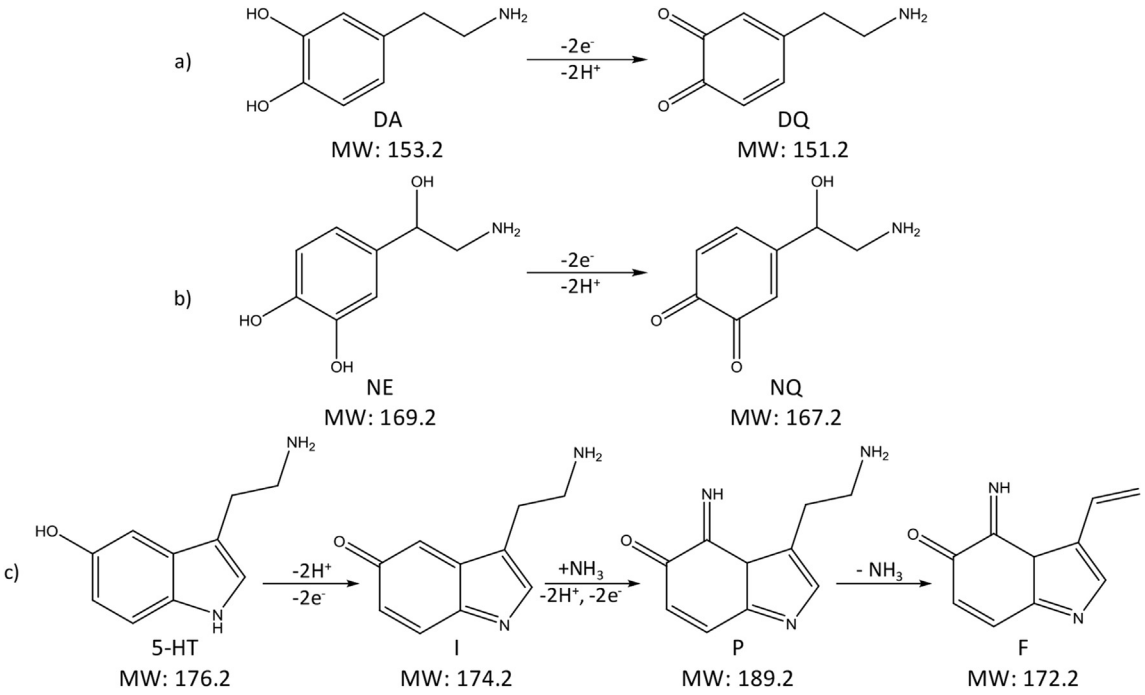
3. Results and discussion

To speed up the LC/EC/MS workflow, we reasoned that it is unnecessary to have LC column used when pure compounds are aimed to be quantified in our approach. In this case, the LC column was removed and flow-through analysis was conducted. In our quantitation experiment, the MS spectra of the analyte before and after the electrode was turned on were collected to determine the conversion yield. Each compound was measured in triplicates. As shown in the first step of the sample injection sequence (Fig. 1), a solvent blank was injected for cleaning purpose to prevent carry-over and cross-contamination, followed by an injection of the analyte in the "cell-off" mode. After that, the cell was turned on and a charging current showed up immediately. The charging current faded away and went back to baseline in around 1.5 min. Then another injection of the analyte was made in the "cell-on" mode and a faradaic current from the oxidation of the target analyte was recorded. Each injection analysis was 2 min, which is only 1/5 of the time in our previous study where a LC column was installed in the LC/EC/MS system [32]. After the first cycle, a second injection sequence could be performed, as illustrated in Fig. S1 (Supporting Information). In this experiment, DA and NE were chosen as two analyte samples and each of them was run in triplicates (Fig. S1, Supporting Information). In this way, 12 injections of both DA and NE samples and 6 injections of blank solvent were done in a total 45 min sequence, whereas 120 min would be needed for 10 min per injection using the previous protocol that we reported [32], excluding extra time needed for LC column equilibration and cleaning. The analysis results of DA and NE from the triplicate measurements are discussed below.

DA, a known neurotransmitter, undergoes electrochemical oxidation via a two-electron transfer reaction to produce dopamine *o*-quinone (DQ, Scheme 2a). Before electrolysis (Figs. S2–a, Supporting Information), the protonated DA was detected at *m/z* 154. After electrolysis (Figs. S2–b), a peak at *m/z* 152 was observed, corresponding to +1 ion of the oxidized DA product. The integrated area for the MS peak of DA was reduced by 8.1% on average upon electrolysis, indicating that the oxidation yield for DA was 8.1% (see data in Table S1, Supporting Information). In the cell-on mode, the DA oxidation current peak was detected, as shown in Figs. S2–d (Fig. S2c shows the background current diagram for blank solvent sample under the same + 1.05 V potential as a contrast). Based on the integration of the current peak area, the amount of the oxidized DA was calculated to be 25.5 pmol on average. Therefore, our measured amount of DA was 314 pmol, which was close to the injected amount of 300 pmol with a measurement error of 4.8% (Table S1, Supporting Information).

Norepinephrine (NE), another neurotransmitter compound, is known to undergo electrochemical oxidation via a two-electron transfer reaction to form norepinephrine *o*-quinone (NQ, Scheme 2b). The averaged oxidation yield was measured to be 2.4% by MS analysis of the NE compound before and after electrolysis (Table S1). Figs. S3–d (Supporting Information) displays the electric current diagram showing a sharp peak resulting from the NE oxidation. Integration of the oxidation peak showed the amount of oxidized NE to be 6.8 pmol (Table S1, Supporting Information). Therefore, the measured amount of NE was 288 pmol. In comparison to the injection amount of 300 pmol (6 μL of 50 μM of NE was injected for analysis), our quantitation measurement error was −4.0%. It can been seen that good quantitation accuracy was obtained, using such an automated analysis sequence with reduced analysis time.

This quantitation approach can also be applied to mixture analysis in combination with chromatographic separation. As a simple demonstration, we tested a mixture of DA and 5-HT by first separating them on a LC column and then electrochemically oxidizing each eluting compound. Due to the high polarity of both DA and 5-HT, a HILIC column was adopted for separation [33]. The mobile phase used was ACN/H_2O with additives of NH_4OAc, which was found to be compatible with the subsequent electrolysis and ESI-MS detection. By using a HILIC column, 5-HT and DA were separated with the retention time at 6.6 min and 9.4 min,



Scheme 2. Equations showing the electrochemical oxidation of a) DA, b) NE, and c) 5-HT.

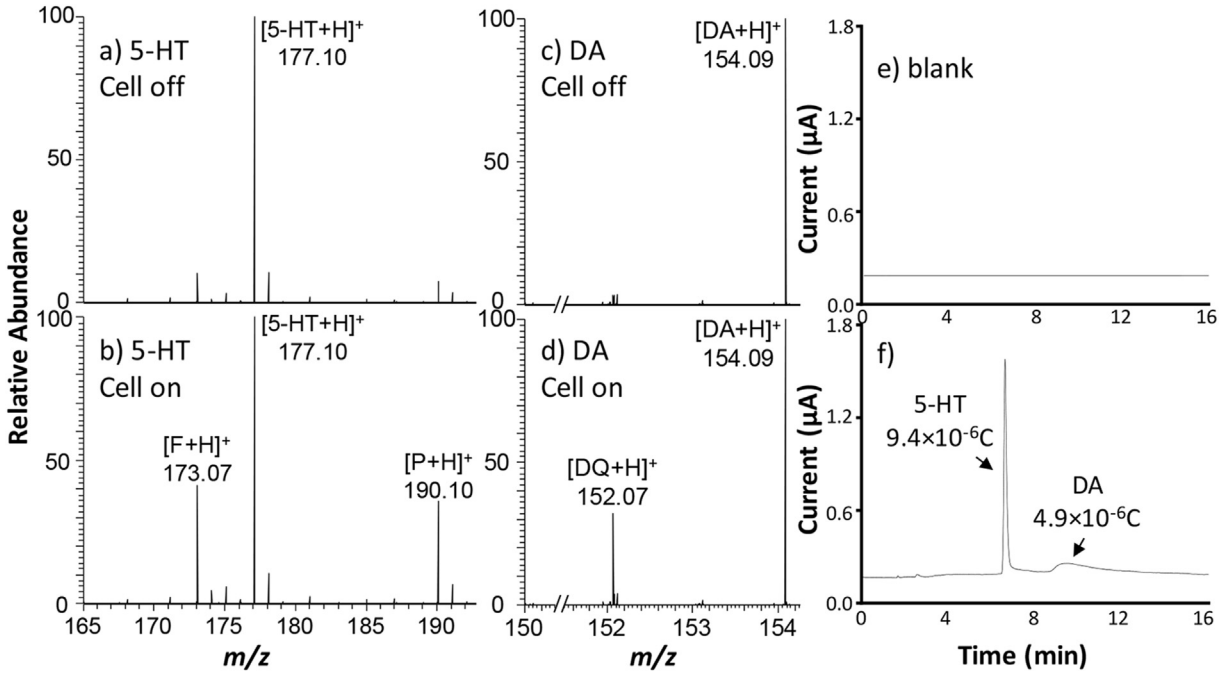


Fig. 2. ESI-MS spectra of 5-HT when the applied potential was a) 0 V and b) +1.05 V. The peak of the oxidized product of 5-HT was seen at *m/z* 190 (+1 ion) in b). ESI-MS spectra of DA when the applied potential was c) 0 V and d) +1.05 V. The peak of the oxidized product of DA was seen at *m/z* 152 (+1 ion) in d). Electric current responses were shown e) from the blank solvent and f) the oxidation of 5-HT and DA.

respectively. Before electrolysis (Fig. 2a), the +1 ion of 5-HT was detected at *m/z* 177. After electrolysis (Fig. 2b), a peak at *m/z* 190 was observed, corresponding to +1 ion of the oxidized 5-HT product [**P** + H]$^+$ (see the proposed reaction mechanism in Scheme 2c). Note that the appearance of peaks at *m/z* 177 and 190 seen in Fig. 2a was probably due to in-source ESI oxidation [34]. During the electrochemical oxidation, 5-HT could first lose two electrons and two protons to become an intermediate structure **I** (MW 174.2 Da). Then addition of ammonia (probably from the mobile phase NH_4OAc) occurred, followed by further oxidation via losses of two electrons and two protons to produce the final product **P** (theoretical mass for the protonated **P**: 190.09749, observed mass: 190.09757, mass error: 0.42 ppm). A similar mechanism for electrochemical oxidation of 5-HT was proposed in literature [35]. A fragment **F** ([P + H−NH_3]$^+$) was observed at 173 (theoretical mass 173.07094, observed mass 173.07111, mass error: 0.98 ppm), presumably from the precursor ion of 190 by loss of NH_3 due to in-source ion dissociation. The integrated area for the 5-HT peak at *m/z* 177 was reduced by 15.7% upon electrolysis (see data in Table S2, Supporting Information). In the cell-on mode, the 5-HT oxidation current peak was detected as shown in Fig. 2f (Fig. 2e shows the background current diagram for blank solvent sample under the same + 1.05 V potential as a contrast). Based on the integration of the current peak area, the amount of the oxidized 5-HT was calculated to be 24.3 pmol on average (z = 4 in this case). Therefore, our measured amount of 5-HT was 155 pmol, which was close to the injection amount of 150 pmol with the measurement error of 3.2%.

In addition, similar to the flow-through analysis of pure DA mentioned above, the +1 ion of DA in the mixture was detected at *m/z* 154 following HILIC separation (Fig. 2a). After electrolysis (Fig. 2b), a peak at *m/z* 152 was observed, corresponding to +1 ion of the oxidized DA product. The integrated area for the DA peak was reduced by 17.8% upon electrolysis (Table S2). The higher oxidation yield in this case than that in the flow-through analysis (8.1%) is probably due to that fact that it is easier to oxidize DA in a neutral media than in an acidic mobile phase. The DA oxidation current peak was also detected, as shown in Fig. 2f. Based on the integration of the current peak area, the amount of the oxidized DA on average was calculated to be 25.6 pmol. Therefore, our measured amount of DA was 144 pmol, which was close to the injection amount of 150 pmol with the measurement error being −3.9%.

4. Conclusions

In this study, absolute quantitation of electrochemically oxidizable compounds using electrochemical mass spectrometry without using reference standards or calibration curves were demonstrated again with improvements in two aspects. For pure compounds, a flow-through analysis sequence was developed using a LC auto-sampler and resulted in a significant decrease in the sample analysis time. This would be of value for high throughput quantitation analysis (e.g., for fast screening and quantifying electroactive compounds in the pharmaceutical ingredients). Furthermore, we also demonstrated the feasibility of applying the EC/MS detection to quantitate individual components in a mixture of 5-HT and DA following HILIC separation. It could be extended and applied to quantify electro-oxidizable compounds such as peptides in biological samples. These improvements would facilitate the implementation of the electrochemical mass spectrometry method for real-world applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijms.2019.05.017.

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