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RESEARCH ARTICLE

# Absolute Quantitation of Oxidizable Peptides by Coulometric Mass Spectrometry

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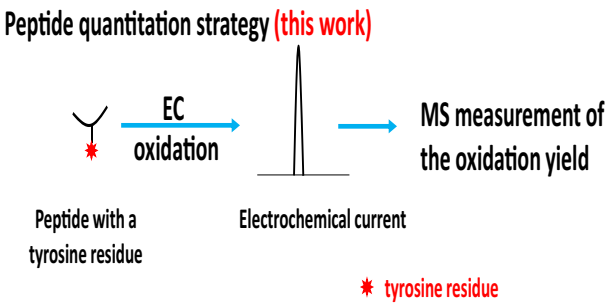


**Abstract.** Quantitation methods for peptides using mass spectrometry have advanced rapidly. These methods rely on using standard and/or isotope-labeled peptides, which might be difficult or expensive to synthesize. To tackle this challenge, we present a new approach for absolute quantitation without the use of standards or calibration curves based on coulometry combined with mass spectrometry (MS). In this approach, which we call coulometric mass spectrometry (CMS), the mass spectrum of a target peptide containing one or more tyrosine residues is recorded before and after undergoing electrochemical oxidation. We record the total integrated oxidation current from the electrochemical measurement, which according to the Faraday's Law of coulometry, provides the number of moles of oxidized peptide. The ion intensity ratio of the target peptide before and after oxidation provides an excellent estimate of the fraction of the peptide that has been oxidized, from which the total amount of peptide is calculated. The striking strength of CMS is that it needs no standard peptide, but CMS does require the peptide to contain a known number of oxidizable groups. To illustrate the power of this method, we analyzed various tyrosine-containing peptides such as GGYR, DRVY, oxytocin, [Arg$^8$]-vasotocin and angiotensinogen 1–14 with a quantification error ranging from – 7.5 to + 2.4%. This approach is also applicable to quantifying phosphopeptides and could be useful in proteomics research.

**Keywords:** Mass spectrometry, Electrochemistry, Coulometry, Quantitation, Peptide

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## Introduction

With rapid advances in instrumentation and technologies, quantitation methods based on mass spectrometry (MS) have gained increasing popularity in proteomics research [1–10]. Relative and absolute quantitation strategies are two categories that all the approaches fall into and protein quantitation is performed, based on the quantification of peptides resulting from protein digestion [11].

Relative quantitation strategies compare the levels of each peptide in a sample to the same peptide in an identical but modified sample. One label-free approach is to analyze samples and compare their MS spectra by determining peptide abundance in one sample relative to another [12, 13]. More commonly, relative quantitation methods use isotopes to label peptides from two samples with heavy and light isotopic atoms. The peak intensities of heavy- and light-labeled peptides are then compared to determine the change in abundance of one sample relative to the other. Methods using this approach include isotope-coded affinity tags (ICAT) [14–17], stable isotope labeling by amino acids in cell culture (SILAC) [18–22], isobaric tags for relative and absolute quantitation (iTRAQ) [23–26], metal element chelated tags (MECT) [27], isotope-coded protein labeling (ICPL) [28], and *N*,*N*-dimethyl leucine (DiLeu) isobaric tag methods [29–32]. In absolute proteomic quantitation

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approaches, a known amount of isotope-labeled target peptide is spiked into an experimental sample and then LC-MS or LC-MS/MS analysis is performed [33]. Unlike relative quantitation, the abundance of the target peptide in the experimental sample is compared to the corresponding labeled peptide and then selected reaction monitoring (SRM) method can be used to construct a standard curve to yield absolute quantitation of the target peptide [34]. It appears that absolute quantitation should be a more advantageous method compared to relative quantitation because absolute amounts of target peptides from different samples can be determined. However, standard or isotope-labeled peptides that are needed for absolute quantitation might not be available at hand and their synthesis can be costly and time-consuming [35, 36]. It would be ideal to have a label-free, standard-free absolute quantitation method available to quantify peptides and proteins for proteomics research.

Herein, we present an electrochemistry (EC)-assisted absolute quantitation method for peptides by MS without the use of standards or isotope-labeled peptides. In our method (illustrated in Scheme 1), a target peptide, if it contains an oxidizable residue like tyrosine (an electrochemically oxidizable amino acid with an oxidation potential of 0.93 V vs. NHE) [37], is first introduced to an electrochemical cell for electrochemical oxidation and followed by MS detection. Electrochemical oxidation reaction results in an electric current response. The integration of the current peak over time provides information about the amount of the oxidized peptide, using the Faraday's Law. According to the Faraday's Law, the total electric charge (Q) involved in the oxidation reaction, which can be measured from the integration of Faradaic current over time, is proportional to quantity of the oxidized peptide: $Q = nzF$, where $n$ is the moles of the oxidized peptide, $z$ is the number of electrons transferred per molecule during the redox reaction, and $F$ is the Faraday constant ($9.65 \times 10^4$ C/mol). Therefore, the moles of the oxidized peptide can be calculated as $n = Q/zF$. Meanwhile, the electrochemically active peptide shows a reduced intensity in the acquired MS spectra from electrochemical oxidation, and the relative MS intensity change upon oxidation, $\Delta i$, reflects the oxidation yield. In a case where the peptide is introduced to the electrochemical cell by liquid chromatography (LC), $\Delta i$ can be calculated by comparing the integrated peak area of the target peptide ion in the extracted ion chromatogram (EIC) before and after electrolysis. Indeed, in a separate experiment, we confirmed that the EIC peak area is proportional to the peptide quantity (see discussion in the Supporting Information Figure S1 and Table S1). Thus, the moles of the oxidized peptide, in combination with the oxidation yield, can be used to calculate the total amount of the peptide analyte. In other words,

$$\begin{aligned}&\text{Total amount of the peptide}\\&= (\text{amount of the oxidized peptide})/(\text{the oxidation yield})\\&= (Q/zF)/\Delta i\\&= Q/(zF\Delta i)\end{aligned} \tag{1}$$

Previously, we have shown that this strategy can be used for accurately quantifying organic molecules such as neurotransmitters (e.g., dopamine and norepinephrine), flavonoid (e.g., rutin), and urea [38, 39]. Peptide glutathione can be also measured by this method, based on the cysteine oxidation to form a disulfide bond. In this work, the method is extended to the quantitation of peptides containing tyrosine, which is more abundant in proteins than the cysteine residue [40]. It was reported that the occurrence frequencies per residue in proteins for tyrosine and cysteine are 2.86% and 1.16%, respectively (Table 1) [40].

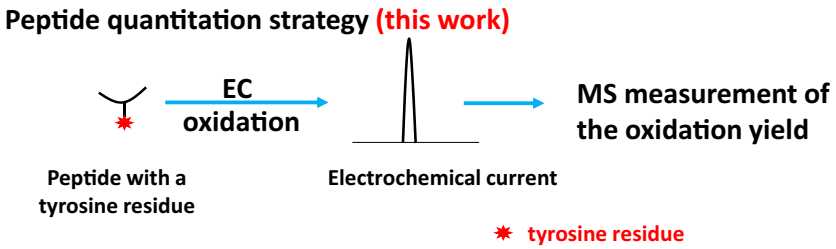
## Experimental Section

### *Chemicals*

Gly-Gly-Tyr-Arg (GGYR, HPLC grade), Met-Gly (MG, HPLC grade), Lys-Arg-Thr-Leu-Arg-Arg (KRTLRR, HPLC grade), Arg-Gly-Asp (RGD, HPLC grade), Ser-Leu-Ile-Gly-Lys-Val-amide (SLIGKV-amide, HPLC grade), Arg-Lys-Arg-Ser-Arg-Ala-Glu (RKRSRAE, HPLC grade), Ser-Phe-Leu-Leu-Arg-Asn-amide (SFLLRN-amide, HPLC grade), Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide (FLFQPQRF-amide, HPLC grade), oxytocin (CYIQNCPLG, disulfide bridge 1–6, HPLC grade), alkaline phosphatase, and bradykinin acetate salt (RPPGFSPFR, HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO). Asp-Arg-Val-Tyr (DRVY, HPLC grade) and [Arg8]-vasotocin (CYIQNCPRG, disulfide bridge 1–6) were purchased from American Peptide (Sunnyvale, CA). Gly-His-Gly (GHG) was obtained from MP Biomedicals (Santa Ana, CA). Tyrosine phosphopeptide (RRLIEDAEpYAARG, HPLC grade) was purchased from EMD Millipore (Temecula, CA). Phosphorylated UOM9 (KRPpSQRHGSKY, HPLC grade) was purchased from AnaSpec (Fremont, CA).

### *Instrumentation*

For the setup (illustrated in Scheme 2), a Waters ultra-performance liquid chromatography setup (UPLC, Milford, MA) was coupled with an Antec electrochemical thin-layer flow cell (Antec BV, The Netherlands) or a BASi electrochemical flow cell (West Lafayette, IN). The Antec electrochemical cell was equipped with a Magic Diamond (boron-doped diamond) disc electrode (*i.d.*, 8 mm) as the working electrode (WE) used for peptide oxidation. A HyREF™ electrode was used as the reference electrode (RF) and carbon-loaded PTFE was used as a counter electrode (CE). The BASi electrochemical cell was equipped with a 6-mm *i.d.* glassy carbon WE and a Ag/AgCl RE. A BEH C18 reversed phase column (2.1 mm × 50 mm, 1.7 μm) was installed for the UPLC separation. A positive potential of + 1.0–1.1 V was applied to the WE electrode for oxidation of LC-separated peptides. The oxidation current response was monitored and recorded by using a ROXY™ potentiostat (Antec BV, The Netherlands). The electric current peak area was integrated by importing the current data point to software 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




**Scheme 1.** Our approach for quantitation of tyrosine-containing peptides. Note that, currently this strategy is applicable to quantitation of a peptide carrying one oxidizable residue like tyrosine

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 capillary temperature was kept at 250 °C. Extracted ion chromatograms (EIC) of peptides were acquired by Thermo Xcalibur (3.0.63).

Peptides that contain one tyrosine residue, including GGYR, DRVY, oxytocin, [$Arg^8$]-vasotocin, and phosphorylated UOM9, were analyzed using the online LC/EC/MS apparatus, and the mobile phase flow rate was set as 0.1 mL/min. An isocratic elution program using 80% A (mobile phase A: water with 0.1% acetic acid and mobile phase B: acetonitrile with 0.1% acetic acid) for 5 min was used for all of compounds. The peptide concentration used was 50 μM and the injection volume was 3–6 μL.

Peptides containing no tyrosine residue such as GHG, MG, KRTLRR, RGD, SLIGKV-amide, RKRSRAE-amide, SFLLRN-amide, FLFQPQRF-amide, and bradykinin were also analyzed using LC/EC/MS apparatus as controls. An isocratic elution program was used with 80% A for 10 min with the mobile phase flow rate of 0.1 mL/min. The peptide injection concentration and volume were 50 μM and 6 μL, respectively. The potential applied for oxidation was + 1.1 V.

Angiotensinogen 1–14 (also called tetradecapeptide TDP), containing two tyrosine residues, was also tested. The mobile phase flow rate was set as 0.3 mL/min. An isocratic elution program using 80% A (mobile phase A: water with 0.1% acetic acid and mobile phase B: acetonitrile with 0.1% acetic acid) for 5 min was used. The sample concentration used was 50 μM and the injection volume was 6 μL.

Electrodes after use can be cleaned using alternating positive and negative potentials.

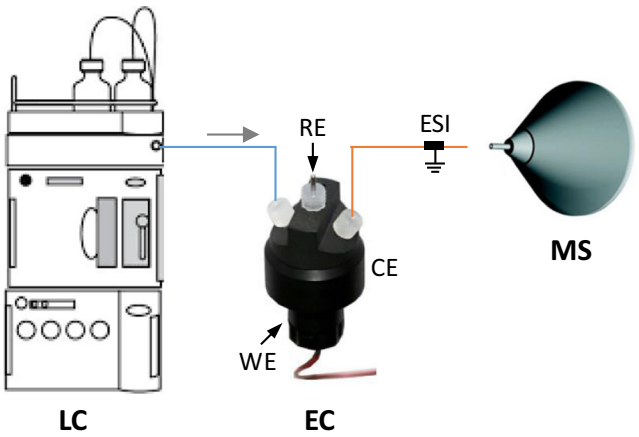
## Results and Discussion

Among the 20 naturally occurring amino acids, four are oxidizable: cysteine (C), tyrosine (Y), tryptophan (W), and methionine (M), given in the order of increasing oxidation potential. Table 1 lists values for their respective oxidation potentials and occurrence frequencies per residue in proteins. In particular, tyrosine has a relatively low oxidation potential and relatively high abundance in proteins. The combination of MS and EC [43–52] has been our research interest, and previously, we have shown that the combined EC/MS technique can be used to detect transient electrochemical reaction intermediates [53–56] and to assist protein sequencing and protein conformational structure elucidation by using fast electrochemical reduction of disulfides [57–63]. In consideration of the fact that tyrosine is electroactive, our goal in this study was to develop a direct quantitation method for peptides based on tyrosine residue oxidation, without using any standards or isotope-labeled peptides. Under an appropriate oxidation potential, the phenol side chain of a peptide tyrosine residue can be oxidized into a semi-quinone by loss of two electrons and two protons (Scheme 3), in which a mass shift of 2 Da occurs, which can be readily monitored by MS analysis. It has been reported that peptides and proteins carrying tyrosine (or tryptophan) can be electrochemically oxidized and analyzed by MS [64–66]. However, peptide quantitation based on tyrosine oxidation has not been explored. Several peptides were tested and quantified in this work, based on the electrochemical oxidation of their tyrosine residues (see summarized results in Table 2).

GGYR was first chosen as a test example. GGYR is a Tyr-containing peptide and it can be oxidized by losing two electrons. Indeed, before electrolysis (Figure 1a), the protonated GGYR was detected at *m/z* 452. After electrolysis (Figure 1b), a peak at *m/z* 450 was observed, corresponding to + 1 ion of the oxidized GGYR product, GGY′R (Y′ represents the oxidized tyrosine residue). Figure 1c and d show the EIC (*m/z* 452, the protonated GGYR) of 50 μM GGYR with an injection volume of 6 μL (injected amount 300 pmol) with the applied potential of 0 V and + 1.0 V, respectively. The integrated area for the peak shown in Figure 1d was smaller by 5.2%, in comparison with that of the peak shown in Figure 1c, indicating that the oxidation yield for GGYR was 5.2% (averaged value from a

**Table 1.** Amino Acids Known to be Oxidizable

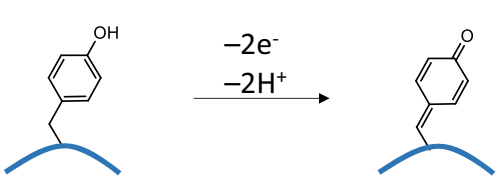
| Electroactive amino acids | Tyrosine (Y) | Tryptophan (W) | Cysteine (C) | Methionine (M) |
|---|---|---|---|---|
| Oxidation potential (vs. NHE) (V) | 0.93 [37] | 1.01 [37] | 0.23 [41] | 1.51 [42] |
| Occurrence frequency per residue in proteins [40] (%) | 2.86 | 1.50 | 1.16 | 2.86 |




**Scheme 2.** Schematic showing our LC/EC/MS apparatus for peptide quantitation

triplicate measurement). On the other hand, a sharp GGYR oxidation current peak was detected, as shown in Figure 1f. Figure 1e shows the background current diagram from a blank solvent injection under the same oxidation condition as a control. In contrast, no electric current peak was observed for the solvent, suggesting that the electric current detected in Figure 1f is caused by the oxidation of GGRY. In addition, a series of peptides containing no tyrosine residues such as GHG, MG, bradykinin (RPPGFSPFR), KRTLRR, RGD, SLIGKV-amide, RKRSRAE-amide, SFLLRN-amide, and FLFQPQRF-amide were tested as control experiments and no oxidation electric current was recorded (Figure S2, Supporting Information), indicating that the oxidation might occur to the tyrosine residue in the case of GGRY. Based on the integration of the current peak area (Figure 1f), the amount of the oxidized GGYR on average was calculated to be 14.9 pmol. Therefore, our measured amount of GGYR was 285 pmol, which was close to the injection amount of 300 pmol with the measurement error of −5.0% (see data in Table S2, Supporting Information). In this study, we call our method coulometric mass spectrometry (CMS) due to the use of Faraday's Law, although the oxidation yield is not 100%.

To confirm that the oxidized product (*m/z* 450) was truly produced from electrochemical oxidation of tyrosine in the GGYR, MS/MS analysis of *m/z* 450 was performed. In this experiment, the GGYR sample was injected into the electrochemical cell by a syringe pump for oxidation at +1.0 V potential. The resulted product was collected and re-ionized




**Scheme 3.** Equation showing electrochemical oxidation of a peptide carrying an oxidizable residue like tyrosine. Note that, if other oxidizable residues co-exist with the tyrosine, the oxidation reaction could be more complicated

by nano-electrospray ionization (nESI), in which the oxidation product GGY′R (Y′ represents the oxidized tyrosine) was observed at *m/z* 450. Collision-induced dissociation (CID) data of *m/z* 450 was recorded, to compare with that of the intact peptide GGYR ion of *m/z* 452. As shown in Figure 2, upon CID, the oxidized peptide ion (*m/z* 450) gave rise to fragment ions $y_1$ (*m/z* 175) and $y_2'$ (*m/z* 336) while the intact peptide (*m/z* 450) produced $y_1$ (*m/z* 175) and $y_2$ (*m/z* 338), confirming that the oxidation occurrence to the 3rd tyrosine residue of GGYR, in which two hydrogen losses took place, resulting in 2-Da mass shift for the $y_2$ ion. Furthermore, we observed the fragment ion of *m/z* 344 resulting from dissociation of *m/z* 450 by loss of the oxidized tyrosine side chain $O{=}C_6H_4{=}CH_2$ as shown in Figure 2a, providing evidence that oxidation indeed occurs to the side chain of tyrosine of this peptide.

In this experiment, it is critical to control both oxidation potential and oxidation time. To ensure that the recorded current is Faradic current from the oxidation of peptide resides, not from solvent, it is important to control the applied oxidation potential to avoid solvent oxidation (e.g., water oxidation). In our study, we found that a low potential like +1.0 V allows for selective oxidation of peptide (see Figure 1e, f). It is also known that peptides with tyrosine (and also tryptophan) could undergo secondary electrochemical oxidation and eventually lead to peptide cleavage upon electrolysis [64–66]. This would cause it to be difficult to apply the Faraday's Law for quantitation, as the value of *n* in Eq. 1 is not clear. Our strategy to achieve selective primary oxidation (as shown in Scheme 3) and to avoid these side reactions is to control the electrooxidation time. When the peptide is introduced by LC into a thin-layer electrochemical cell, the small dead volume of the cell (0.7 μL) and a relatively high mobile phase flow rate (100 μL/min) led to the oxidation of the peptide in less than 500 ms. Indeed, we observed no side oxidation reactions including peptide cleavage and secondary oxidation of tyrosine for GGYR under these conditions.

DRVY, another tetrapeptide that contains Tyr, was also analyzed by the same approach. Before electrolysis (Figure S3a, Supporting Information), the protonated DRVY was detected at *m/z* 552. After electrolysis (Figure S3b), a peak at *m/z* 550 was observed, corresponding to +1 ion of oxidized DRVY product, DRVY′. Figure S3c and d showed the EIC (*m/z* 552, the protonated DRVY) of 50 μM DRVY with an injection volume of 6 μL (injected amount 300 pmol) with the applied potential of 0 V and +1.0 V, respectively. The integrated area for the peak shown in Figure S3d was smaller by 3.1%, in comparison with that of the peak shown in Figure S3c, indicating that the oxidation yield for DRVY was 3.1% (see data in Table S3, Supporting Information). On the other hand, the DRVY oxidation current peak was detected, as shown in Figure S3f (Figure S3e shows the background current diagram for blank solvent sample under the same +1.0 V potential as a contrast). Based on the integration of the current peak area, the amount of the oxidized DRVY on average was calculated to be 8.5 pmol. Therefore, our measured amount of DRVY was 277 pmol, which was close to the injection amount of 300 pmol

**Table 2.** List of Peptides Quantified by CMS

| # | Name | Peptide sequence | Molecular weight (Da) | Electric current from oxidation (Y/N) | # of Y residues | Quantitation measurement error |
|---|---|---|---|---|---|---|
| 1 | Gly-His-Gly | GHG | 269.3 | N | 0 | – |
| 2 | Met-Gly | MG | 206.3 | N | 0 | – |
| 3 | Lys-Arg-Thr-Leu-Arg-Arg | KRTLRR | 829.0 | N | 0 | – |
| 4 | Arg-Gly-Asp | RGD | 346.3 | N | 0 | – |
| 5 | Ser-Leu-Ile-Gly-Lys-Val-amide | SLIGKV-$NH_2$ | 614.8 | N | 0 | – |
| 6 | Arg-Lys-Arg-Ser-Arg-Glu-amide | RKRSRAE-$NH_2$ | 901.0 | N | 0 | – |
| 7 | Ser-Phe-Leu-Leu-Arg-Asn-amide | SFLLRN-$NH_2$ | 747.9 | N | 0 | – |
| 8 | Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide | FLFQPQRF-$NH_2$ | 1081.3 | N | 0 | – |
| 9 | Bradykinin | RPPGFSPFR | 1060.2 | N | 0 | – |
| 10 | Tyrosine phosphopeptide | RRLIEDAEpYAARG | 1598.7 | N | 1 | – |
| 11 | Gly-Gly-Tyr-Arg | GGYR | 451.5 | Y | 1 | −5.0% |
| 12 | Asp-Arg-Val-Tyr | DRVY | 551.6 | Y | 1 | −7.5% |
| 13 | Oxytocin | CYIQNCPLG, disulfide bond 1–6 | 1007.2 | Y | 1 | 2.4% |
| 14 | [$Arg^8$]-vasotocin | CYIQNCPRG, disulfide bond 1–6 | 1050.2 | Y | 1 | 0.8% |
| 15 | Angiotensinogen (1–14) | DRVYIHPFHLLVYS | 1760.1 | Y | 2 | −5.5% |
| 16 | Phosphorylated UOM9 | KRPpSQRHGSKY | 1422.5 | Y | 1 | −5.8% |

with a measurement error of −7.5% (Table S3, Supporting Information).

We also tested oxytocin (CYIQNCPLG, disulfide bridge 1–6), a larger Tyr-containing peptide than GGYR and DRVY. Before electrolysis (Figure 3a), the +2 charged oxytocin ion was detected at *m/z* 504. After electrolysis (Figure 3b), a peak at *m/z* 503 was observed, corresponding to +2 ion of the oxidized oxytocin product (2-Da mass shift corresponding to losses of two hydrogens). Figure 3c and dshow the EIC (*m/z* 504, the +2 charged oxytocin ion) of 50 μM oxytocin with an injection volume of 6 μL (injected amount 300 pmol) with the applied potential of 0 V and +1.0 V, respectively. The integrated area for the peak shown in Figure 3d was smaller by 4.7%, in comparison with that of the peak shown in Figure 3c, indicating that the oxidation yield for oxytocin was 4.7% (see data in Table S4, Supporting Information). On the other hand, the oxytocin oxidation current peak was detected, as shown in Figure 3f (Figure 3e shows the background current diagram for blank solvent sample under the same +1.0 V potential as a contrast). Based on the integration of the current peak area, the amount of the oxidized oxytocin on average was calculated to be 14.4 pmol. Therefore, our measured amount of oxytocin was 307 pmol, which was close to the injection amount of 300 pmol with the measurement error being 2.4%. We also successfully quantified another neurohypophyseal peptide, [$Arg^8$]-vasotocin, which shared structural similarity with oxytocin. Using the same approach, the measurement error was found to be 0.8% (Figure S4 and Table S5 in Supporting Information).

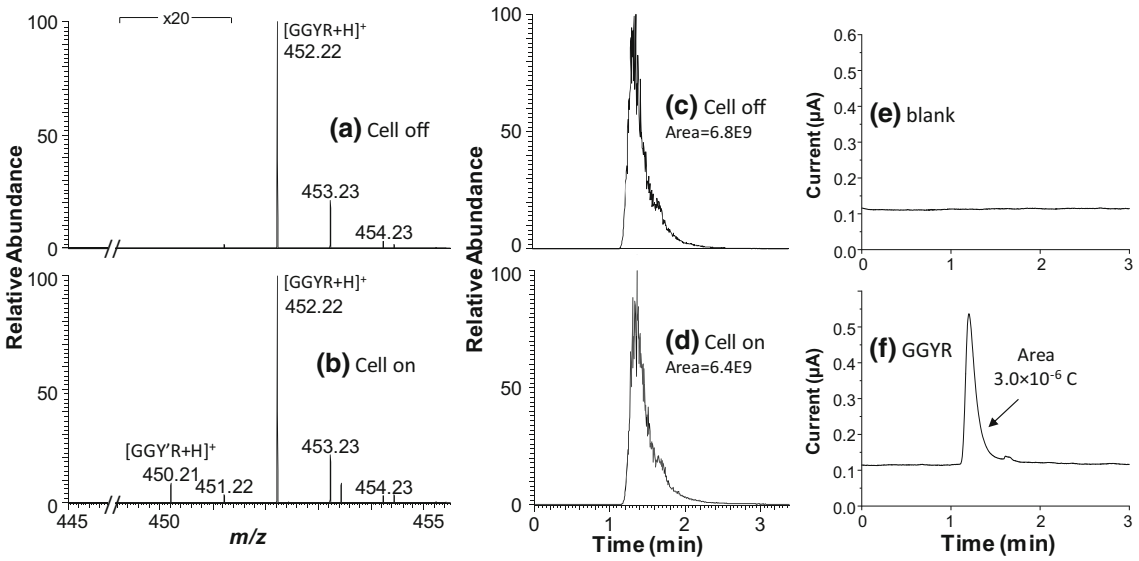



**Figure 1.** ESI-MS spectra of GGYR when the applied potential was (**a**) 0 V and (**b**) + 1.0 V. The peak of the oxidized product GGY'R was clearly seen at *m/z* 450 in **b**. EIC of GGYR was recorded when the applied potential was (**c**) 0 V and (**d**) + 1.0 V. Electric current responses were shown due to the oxidation of (**e**) a blank solvent and (**f**) GGYR peptide

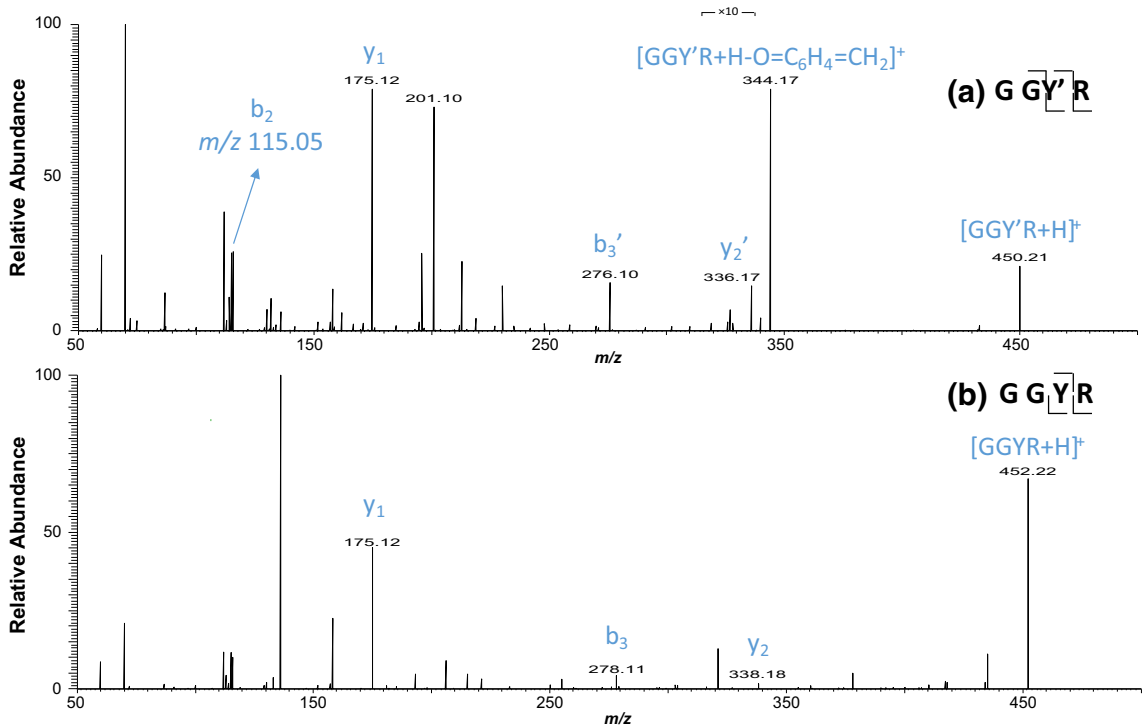



**Figure 2.** CID MS/MS spectra of (**a**) the oxidized peptide ion $[GGY'R+H]^+$ (*m/z* 450) and (**b**) the intact peptide ion $[GGYR+H]^+$ (*m/z* 452). Fragment ion of *m/z* 344 in **a** resulted from the loss of the oxidized tyrosine side chain from *m/z* 450

So far, we have only considered peptides having one tyrosine residue. But, CMS can also be applied to peptides containing more than one tyrosine residue. In that case, ideally, experimental condition can be chosen so that we oxidize only one of the tyrosine residues. As an example, we examined angiotensinogen (1–14), a known tetradecapeptide (TDP) having the sequence DRVYIHPFHLLVYS. This is a peptide precursor of angiotensin I, which is a peptide hormone leading to vasoconstriction and blood pressure increase [67]. To avoid simultaneous oxidation of two tyrosine residues of TDP, we reasoned that a high sample flow rate (in other words, a short residence time in the electrochemical cell) could help. To confirm this hypothesis, we introduced TDP into the electrochemical cell by LC at different mobile phase flow rates (0.1–0.3 mL/min) for oxidation. Indeed, upon

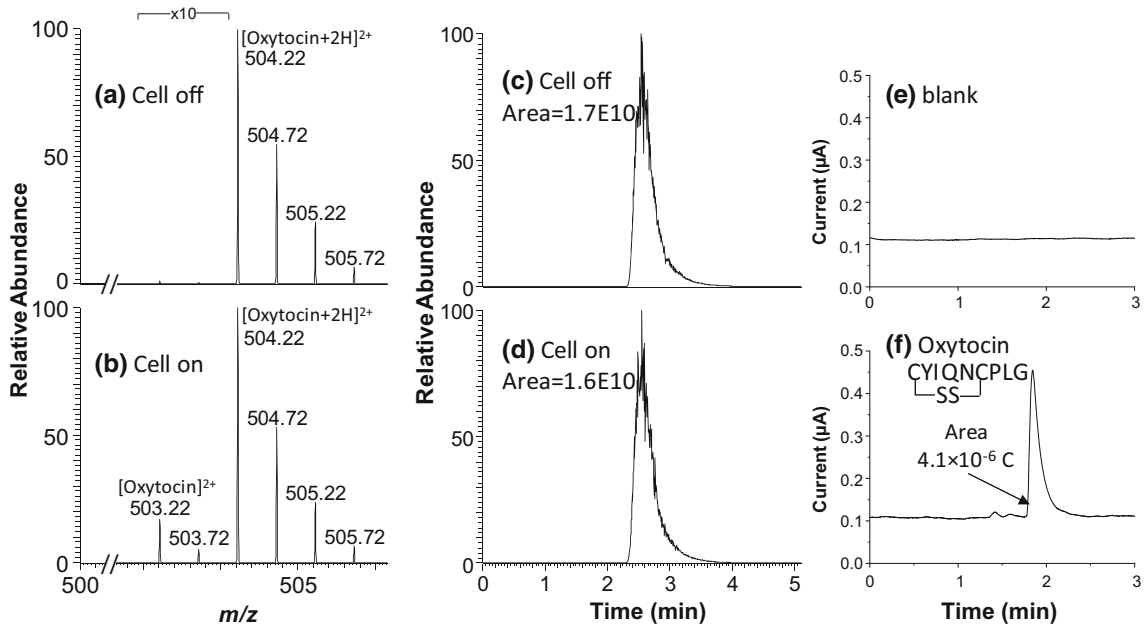



**Figure 3.** ESI-MS spectra of oxytocin when the applied potential was (**a**) 0 V and (**b**) + 1.0 V. The peak of the oxidized product of oxytocin was seen at *m/z* 503 (+ 2 charged) in **b**. EIC of the + 2 charged oxytocin ion was recorded when the applied potential was (**c**) 0 V and (**d**) + 1.0 V. Electric current responses were shown (**e**) from the blank solvent and (**f**) the oxidation of oxytocin peptide

oxidation, two +4 ions of products appeared at *m/z* 439.98 and 439.48, corresponding to the oxidation products resulting from one tyrosine oxidation and from two tyrosine oxidations, respectively. When the sample introduction flow rate was increased from 0.1 to 0.3 mL/min, the intensity ratio of *m/z* 439.48 to *m/z* 439.98 decreased from 39.9 to 4.2% (Table S6, Supporting Information), supporting our hypothesis that less oxidation time allows oxidation to occur predominantly at only one of the tyrosine residues of the peptide.

To better tolerate a relatively high flow rate of 0.3 mL/min and the pumping pressure, we used a BASi stainless electrochemical flow cell (West Lafayette, IN) equipped with a 6-mm *i.d.* glassy carbon WE and a Ag/AgCl RE. Before electrolysis (Figure 4a), the +4 charged TDP ion was detected at *m/z* 440.49. After electrolysis (Figure 4b), a peak at *m/z* 439.98 was observed, corresponding to the +4 ion of oxidized TDP product resulting from oxidation of one of the tyrosines. Another smaller peak at *m/z* 439.48 resulting from the oxidation of both tyrosines was also seen (Figure 4b) with the intensity ratio of *m/z* 439.48 to *m/z* 439.98 being 0.12:1. Figure 4c and d show the EIC of *m/z* 440.49, the +4 charged TDP ion, from the injection of 6 µL of 50 µM TDP (total amount 300.0 pmol) with the applied potential of 0 V and + 1.05 V (vs. Ag/AgCl), respectively. The integrated area for the peak shown in Figure 4dwas smaller by 2.6% than that of the peak shown in Figure 4c, indicating that the oxidation yield for TDP was 2.6% (see data in Table S7, Supporting Information). On the other hand, the TDP oxidation current peak was detected, as shown in Figure 4f (Figure 4eshows the background current diagram for blank solvent sample under the same + 1.05 V potential as a contrast). By integration of the current peak area, the total electric charge Q involved in the TDP oxidation, leading to the formation of *m/z* 439.98 via one tyrosine oxidation (2 $e^-$ per mole peptide) and the formation of *m/z* 439.48 via two tyrosine oxidation (4 $e^-$ per mole peptide), could be calculated. In consideration of the intensity ratio of *m/z* 439.48 to *m/z* 439.98 of 0.12:1 and the similarity of the two structures of the oxidation products, the molar ratio of the two oxidation products would be approximately 0.12:1. Thus, we calculate the total amount of the oxidized peptide to be 7.2 pmol (an averaged value from triplicate measurements). Considering the oxidation yield of 2.6%, our measured amount of TDP was therefore 283 pmol. In comparison to the injection amount of 300 pmol, we find the quantitation error to be −5.5% (see Table 2 and Table S7). This result illustrates one approach that is able to successfully quantitate peptides containing more than one tyrosine residue. This approach might be possible to apply to quantify a peptide with both tyrosine and tryptophan residues, provided that all the oxidation products have similar ionization efficiencies.

It may not be possible, however, to find suitable conditions in which only one of the tyrosine residues is oxidized. In such cases, we might need to invoke the assumption, based on the similarity of structures, that the intensities of the different mass

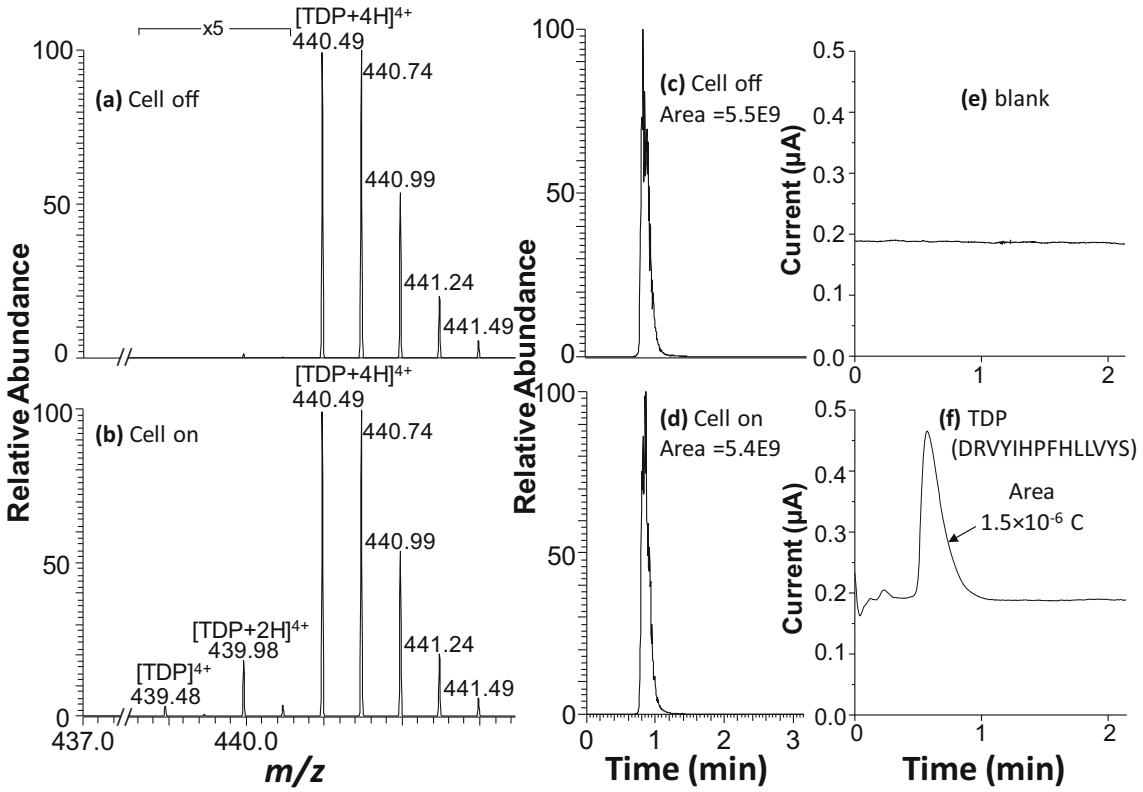



**Figure 4.** ESI-MS spectra of TDP when the applied potential was (**a**) 0 V and (**b**) + 1.05 V. The major peak of the oxidized product of TDP was seen at *m/z* 439.98 (+ 4 charged) in **b**. EIC of the + 4 charged TDP ion was recorded when the applied potential was (**c**) 0 V and (**d**) + 1.05 V. Electric current responses were shown (**e**) due to the blank solvent and (**f**) the oxidation of TDP peptide

peaks of the oxidized products represent to good approximation the concentrations of the different oxidized forms, allowing us nevertheless to determine the amount of the original peptide from CMS.

We also tested the phosphopeptide RRLIEDAEpYAARG, whose tyrosine reside is phosphorylated. Interestingly, such a peptide does not display an oxidation current upon oxidation (Figure S2-k, Supporting Information). This result is in agreement with previously reported electrochemical studies of phosphopeptides [68, 69] and provides another way to judge whether or not a peptide tyrosine residue is phosphorylated [70, 71].

Furthermore, our method can be used to directly quantify phosphopeptides containing a free oxidizable residue such as tyrosine. Absolute quantitation of phosphopeptides typically needs multiple-step synthesis of isotope-labeled phosphopeptide standards [72]. In our approach, phosphorylated UOM9 (sequence: KRPpSQRHGSKY), a kinase substrate peptide originating from myelin basic protein (MBP) [73], was chosen as a test sample. The peptide is phosphorylated in its serine residue and contains one free tyrosine residue. Before electrolysis (Figure 5a), the + 5 ion of the peptide was detected at *m/z* 285.35. After electrolysis (Figure 5b), a peak at *m/z* 284.94 was observed, corresponding to + 5 ion of the oxidation product. Figure 5c and d show the EIC (*m/z* 285.35) of 50 μM phosphorylated UOM9 with an injection volume of 6 μL (injected amount 300 pmol) with the applied potential of 0 V and + 1.05 V (vs. Ag/AgCl), respectively. The integrated area for the peak shown in Figure 5d was smaller by 2.9%, in comparison with that of the peak shown in Figure 5c, indicating that the oxidation yield for this peptide was 2.9% (see data in Table S8, Supporting Information). On the other hand, the peptide oxidation current peak was detected, as shown in Figure 5f (Figure 5e 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 peptide on average was calculated to be 8.1 pmol. Therefore, our measured amount of phosphorylated UOM9 was 283 pmol, which was close to the injection amount of 300 pmol with a measurement error of − 5.8% (Table S8, Supporting Information). To the best of our knowledge, this data represents absolute quantitation of phosphopeptide without using any standards, for the first time.

The sensitivity of the method was also evaluated, using a low quantity of peptide DRVY. In our experiment, a BASi stainless electrochemical flow cell (West Lafayette, IN) equipped with a 6-mm *i.d.* glassy carbon WE and a Ag/AgCl RE was used. 3 μL of 0.1 μM DRVY (injection amount 300 fmol) was injected into LC/EC/MS for quantitation and the measured peptide quantity was 319 fmol (6.2% quantitation error, see detailed results in Table S9 in Supporting Information), indicating the reasonably good sensitivity and accuracy of our method. Further improvements in sensitivity are possible if background noise in electric current measurement could be reduced, for example, using a newer potentiostat with a Faraday cage. In addition, DRVY at other different concentrations ranging from 1, 10, 50 μM (3 μL injection volume) were also successfully quantified and the results are shown in Table S10 (Supporting Information), indicating that the dynamic range of our method with the current setup is 0.1–50 μM.

Furthermore, as the oxidation potential of tyrosine in a peptide could be affected by the peptide sequence and the

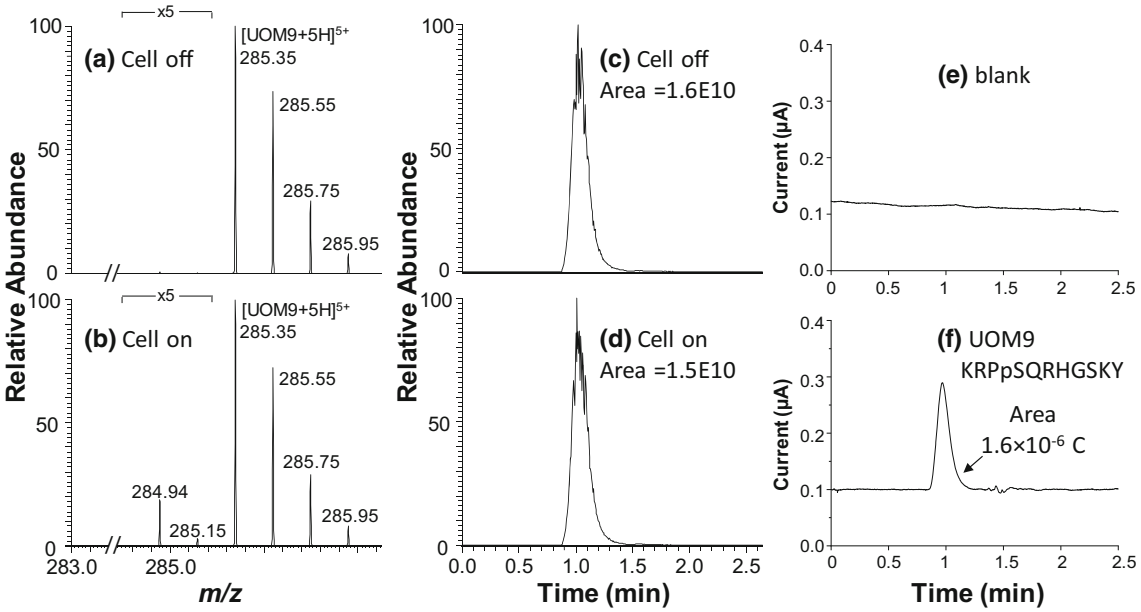



**Figure 5.** ESI-MS spectra of phosphorylated UOM9 when the applied potential was (**a**) 0 V and (**b**) + 1.05 V (vs. Ag/AgCl). The peak of the oxidized peptide product was seen at *m/z* 284.94 (+ 5 ion) in **b**. EIC of the + 5 ion of phosphorylated UOM9 was recorded when the applied potential was (**c**) 0 V and (**d**) + 1.05 V. Electric current responses were shown (**e**) from the blank solvent and (**f**) the oxidation of the phosphorylated UOM9 peptide

surroundings of the tyrosine residue, it is suggested that a cyclic voltammetry (CV) should be run to find out an optimal potential for a target tyrosine-containing peptide, prior to LC/EC/MS quantitation.

## Conclusions

In this study, several tyrosine-containing peptides including phosphopeptides were successfully quantified using coulometric mass spectrometry. The striking strength of this method is that it requires no standard/isotope-labeled peptides for absolute quantification. In addition, the use of MS provides a means to identify the electrochemical reaction products, which is difficult to achieve by using electrochemical technologies alone. The results demonstrate the validity of this method in which we achieve absolute quantitation of peptides by electrochemical oxidation of tyrosine. It is most likely that this approach can be applicable to quantify peptides containing other oxidizable residues such as tryptophan, methionine, and cysteine. Future work will be focused on the method sensitivity improvement and the application of this method to quantitation of peptides from protein digestion, which may provide a new way to quantify proteins. In addition, this CMS method is also expected to be potentially useful in proteomics including quantitation of hormone, antibacterial, and neurotransmitter peptides [74–76].

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