Development of Coulometric Mass Spectrometry for Absolute Chemical Quantitation

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Prepared for *Encyclopedia of Analytical Chemistry*

Abstract

In the past decades, mass spectrometry (MS)-based absolute quantitation has become a powerful tool to facilitate advancement in both biomedical and clinical fields. The main advantage of MS-based quantitation is the accurate and simultaneous quantitation of multiple analytes with high sensitivity. However, a reference material (e.g., authentic target analytes, or isotope-labeled peptide and protein standards) is indispensable for those quantitation methods to build a calibration curve for each analyte, which might be not available or difficult to synthesize especially for some drug molecules with complicated structure or proteins/peptides with post-translational modifications (PTMs). To tackle this issue, our group recently developed a standard-free absolute quantitation approach based on the combination of electrochemistry (EC) and MS, involving the coulometric measurement of analyte oxidation/reduction current as well as mass spectrometric measurement of the redox reaction yield. We named the method as coulometric mass spectrometry (CMS). Although the combined EC/MS techniques is a quickly growing research field in analytical chemistry with numerous distinct applications, the quantitation centered application has not been systematically reported. In this review, the principle, apparatus, and various applications of this emerging EC/MS hybrid quantitation method are presented and discussed in detail. An overview of the capabilities of CMS for absolute quantitation across a variety of modalities including small molecule metabolites, peptides, proteins, large molecule biotherapeutics, and PTMs as well as the role of CMS for drug impurity quantitation is provided. Simultaneously, the limitations of the method, which need further optimization and improvement, as well as promising future applications in drug development and proteomics are presented and evaluated.

Keywords: Mass spectrometry; electrochemistry; chromatography; absolute quantitation; proteins; biotherapeutics; drug impurity.

1. Introduction

Quantification of analytes is extremely important in analytical chemistry. Numerous analytes are quantified on a day-to-day basis in various industry settings to answer questions regarding the analytes in the samples. For instance, in the pharmaceutical industry, complex drug molecules are synthesized from simple starting materials in multiple steps, and quantitation of the drug analytes is required to monitor each step in the in-process testing and the final purity of the drug material.[1] In the biotechnology industry, a plethora of pharmaceutically active proteins[2] and peptides[3] and their impurities are made, and quantification is needed to monitor their production. In hospitals and clinical laboratories, body fluids are analyzed to diagnose disease severity and patients' conditions.[4]

For quantitation, commercially available standards or in-house synthesized standards are traditionally compared to the analyte in the sample using various analytical instruments. In the pharmaceutical industry, the common techniques such as high performance liquid chromatography (HPLC) with ultraviolet visible (UV-Vis) light detector,[5] charged aerosol detector (CAD),[6] evaporative light scattering detector (ELSD),[7] or refractory index detector (RI),[8] and gas chromatography (GC)[9] have been widely used. In the biotechnology industry, enzyme-linked immunosorbent assay (ELISA),[10] Western blotting,[11] gel electrophoresis,[12, 13] and size exclusion chromatography[14] are popular, whereas immunoassay-based instruments[15] and spectrophotometers[16] are commonly used in the hospital and clinical settings. Although these instruments are user friendly, relatively inexpensive, and are routinely used for quantitation, limitations do exist. For example, the UV-Vis detector can only detect molecules with chromophores. Other detectors such as CAD, ELSD, and RI can analyze molecules without chromophores but ultimately lack sensitivity.[17]

Nowadays MS has emerged as a leading technique for accurate and sensitive analyte quantification and characterization. The *m/z* value, MS/MS capabilities,[18] and high resolution of mass spectrometers[19] help in the identification and confirmation of the target compound structure with great selectivity and sensitivity. MS is capable of analyzing compounds with a wide range of molecular weights such as small drug molecules, peptides,[20] proteins,[20] lipids,[21] polymers,[22] DNA,[23] and RNA.[24] A variety of mass spectrometers with different types of ionization sources and mass analyzers are used based on the requirements of the analysis.

The MS-based quantification of proteins can broadly be categorized into two categories: absolute quantification and relative quantification. Both isotope labelling strategies and label free strategies[25-30] are mainly used. Relative quantitation methods provide information regarding the protein abundance ratio or relative change between two or more samples by comparing the amount of a surrogate peptide from a single protein with an isotope labeled standard peptide. Usually, relative quantitation methods in proteomics are used to help to understand the dynamics of the whole proteome. In discovery-based proteomics, for example, a broad analysis of the proteome is carried out to look for quantitative differences in proteins using relative quantitation.[31] In contrast, absolute quantitation methods provide the actual concentration of a specific peptide or a protein in a sample, proteins and post translationally modified proteins, [32-34] and proteins within protein complexes can be accurately quantified using absolute quantification technique.[35] Comparison of concentrations between laboratories is possible using absolute quantification technique.[36]

In relative quantitation using isotope-labelled strategies, a stable isotope-labeled peptide that is chemically identical to the target peptide in the sample is used. Therefore, the two peptides behave identically during liquid chromatography/mass spectrometry (LC/MS) analysis. Given that MS can recognize the mass difference between the labeled and unlabeled forms of a peptide, quantification is achieved by comparing their respective ion signal intensities.[37, 38] Various methods of isotope labelling are available. Some of the most popular methods include isotope-coded affinity tags (ICAT),[39] stable isotope labeling by amino acids in cell culture (SILAC),[40] isobaric tags for relative and absolute quantitation (iTRAQ),[41] metal element chelated tags (MECT),[42] and isotope-coded protein labeling (ICPL)[43]. Because differentially labelled peptides can be resolved with mass spectrometry and the labelled samples are pooled before LC/MS, simultaneous analysis of multiple samples can be performed in one run by a multiplexing strategy, and the differences due to sample losses during sample preparation and changing ionization efficiency between injections can be circumvented. Although multiple samples can be compared in a single run using isotope labelled strategy, labelling procedures are often time-consuming, and the cost of labelling reagents are high.[44] Label-free methods for relative quantitation are available. Each sample is separately prepared and then subjected to individual LC-MS/MS.[45] Protein quantification is generally based on ion intensity changes or the spectral counting of identified proteins after MS/MS analysis.[46] Although a relative quantification method allows for the quantitative profiling of tens of thousands of peptides from thousands of proteins within a single experiment and provides a broad scope of the proteome, obtaining an absolute concentration via absolute quantification is often desired. Absolute quantification provides a far more precise description of molecular events than relative quantification.

In absolute quantitation using isotope labelled strategies, the isotope labelled standards exhibit similar chromatographic behavior but exhibit differences in mass and possibly isotopic behavior. The extracted peak areas or peak heights from the isotope labelled peptide and peptide from the sample are compared for absolute quantitation.[47] Three types of isotope labelled strategies are used for quantitation in the bottom-up methodology: absolute quantification (AQUA), quantification conCATamer (QconCAT) and protein standard absolute quantification (PSAQ). AQUA is a variation of the isotope dilution mass spectrometry strategy used for quantifying small molecules.[48] The AQUA peptides are added in a known quantity to the sample preparation right before LC/MS analysis. Both the AQUA peptide and the native peptide are measured by LC/MS. The analyte and the standard are identical except for the masses. The ratio of the peak intensities is used to measure the absolute amount of the analyte peptide.[49] The AQUA peptide strategy is attractive. However, custom synthesis of peptides might be required to quantify atypical proteins.[47] Typically, one surrogate peptide in a protein is used to quantify a protein of interest using AQUA strategy as quantifying multiple surrogate peptides from a protein would require purchasing multiple peptides.[50] The QconCAT method can address this issue of the need to purchase multiple peptides to quantify multiple peptides in a protein. QconCATs are proteins encoded by synthetic genes that are concatamers of peptide internal standards with usually more than one target peptide per protein. Multiple peptides in a protein can be quantified using peptide standards resulting from digestion of a single QconCAT protein standard. The native proteins and QconCAT proteins are trypsin digested and the digested native and QconCAT peptides are compared for quantification.[51] The QconCAT strategy also circumvents the protein digestion efficiency issue with the AQUA peptides strategy. In the AQUA peptide strategy, since the AQUA peptides are added after sample protein digestion, the digestion efficiency of the sample protein for incomplete digestion must be monitored closely. However, with the QconCAT strategy, the QconCAT proteins and sample proteins are digested simultaneously and the digestion efficiency of the QconCAT proteins and the sample proteins is similar.[47] The QconCAT method can circumvent the two aforementioned issues with AQUA strategy, which are protein digestion efficiency and the use of multiple peptides for quantitation. Protein standard absolute quantification (PSAQ) methodology is used in situations where the sample preparation is extensive. In PSAQ methodology, the DNA sequence to code for the PSAQ protein is cloned into a plasmid. The protein is expressed in an expression medium such as *E. coli*.. Usually, arginine and lysine residues are labelled so that the tryptic digested peptides show a consistent mass difference compared to the native peptide. This method eliminates the differences between the sample and the standard in all the prefractionation steps.[52]

For traditional absolute quantitation, a calibration curve using isotope-labeled standard or peptide standard is necessary.[53] Commercially available standards can be expensive. In some cases, some peptides and small molecules are not commercially available and in-house synthesis can be time-consuming or complicated. The most common way to obtain peptides is solid phase chemical synthesis. It requires covalently attaching the first amino acid to an insoluble support. Then, subsequent amino acids are added by a series of coupling and deprotection steps.[3] This kind of synthesis is inconvenient and time consuming. In all of the aforementioned techniques, there is no single strategy or technique that can absolutely quantify analytes without the use of a calibration curve or standards. A technique which can quantify without standards will be ideal and useful. Herein, we review a standard and label-free strategy "Coulometric Mass Spectrometry" (CMS), for the absolute quantification of analytes wherein LC/MS is combined with electrochemistry (EC). This technique uses the electroactive property of the analyte to quantify the analyte of interest. Using this technique, a wide variety of molecules were quantified, including small molecules with electroactive functional groups such as dopamine (DA) and norepinephrine (NE).[54, 55] Small molecules metabolites with electroactive functional groups have been quantified[56]. Additionally, peptides and proteins with electroactive amino acid residues such as tyrosine (Y)[57, 58] and tryptophan (W)[59] were quantified and demonstrated for high throughput analysis. Post-translationally modified peptides such as phosphorylated peptides were quantified.[58] Proteins are digested and the surrogate peptides containing electroactive amino acids are measured to obtain the concentration of the precursor protein.[57] Drug impurities such as nitrosamines or host cell proteins (HCPs) were also quantified.[60]

2. CMS principle and instrumentation

2.1. Principle of CMS

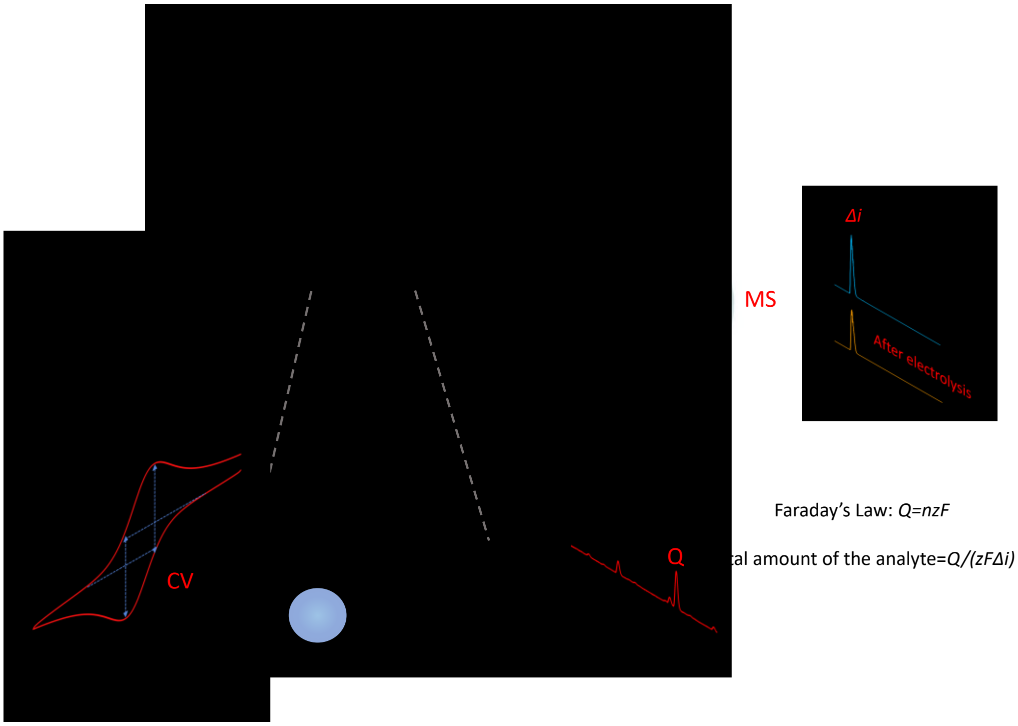
The combined technique of EC with MS, EC/MS, has many applications, including drug metabolism study, protein structural analysis, and electrochemical reaction mechanism elucidation.[61-67] The method can also be used to observe elusive reaction intermediates[68-77] and for electrosynthetic reaction screening.[78-80] As a new application of EC/MS techniques, the unprecedented advantage of CMS is the capability of absolute quantitation without using any standards or establishing a calibration curve. Although the traditional coulometric approach could also realize standard free quantitation based on Faraday's law, the complete electrochemical oxidation or reduction conversion is often realistically difficult to achieve. Since MS is integrated after EC, it enables the measurement of the oxidation or reduction conversion yield and is ideal for monitoring the reaction products to further confirm and validate the reaction mechanism and electron transfer. Theoretically, CMS quantitation can be applicabe for analytes which contain an electroactive functional group with known electrochemical activities. Before the coulometric measurement, the redox potential can be optimized with cyclic voltammetry (CV) and selected carefully to improve the electrochemical response and avoid over-oxidation/reduction. The applied working electrode (WE) potential needs to be optimized and controlled for the best sensitivity and selectivity in terms of electric current signal, since over-oxidation/reduction may give rise to a competitive side reaction. This could impact the accuracy of CMS quantitation if the number of electrons per molecule involved in the side reaction is different from that of the expected oxidation (or reduction) reaction. Once the electrochemical reaction is triggered, the total electric charge responsible for oxidizing/reducing analyte substance in coulombs in the redox reaction, Q, is directly proportional to the quantity of the oxidized/reduced substance: Q=nzF, where n is the moles of analyte, Z is the number of electrons transferred per molecule for the redox reaction, and F is the Faraday constant (9.65×10^4 C/mol). Q can be directly measured from the integration of Faradaic current over time. The moles of the analyte that is oxidized or reduced can be calculated as n=Q/zF. On the other hand, electroactive species shows reduced intensities in the acquired MS spectra upon oxidation/reduction, and the relative MS intensity change upon redox reaction, Δi, can reflect the redox conversion yield. From the acquired MS spectra before and after electrolysis, Δi can be measured in two ways: the relative change of the target analyte peak intensity (relative to a reference peak) or the relative change of the target analyte peak area in the extracted ion chromatogram (EIC) upon electrolysis, as illustrated in Scheme 1. Thus, the amount of analyte converted, in combination with the conversion yield, can be used to calculate the total amount of analyte. In other words,

Total amount of analyte

=(amount of the oxidized/reduced analyte)/(the oxidation/ reduction yield)

=(Q/zF)/Δi

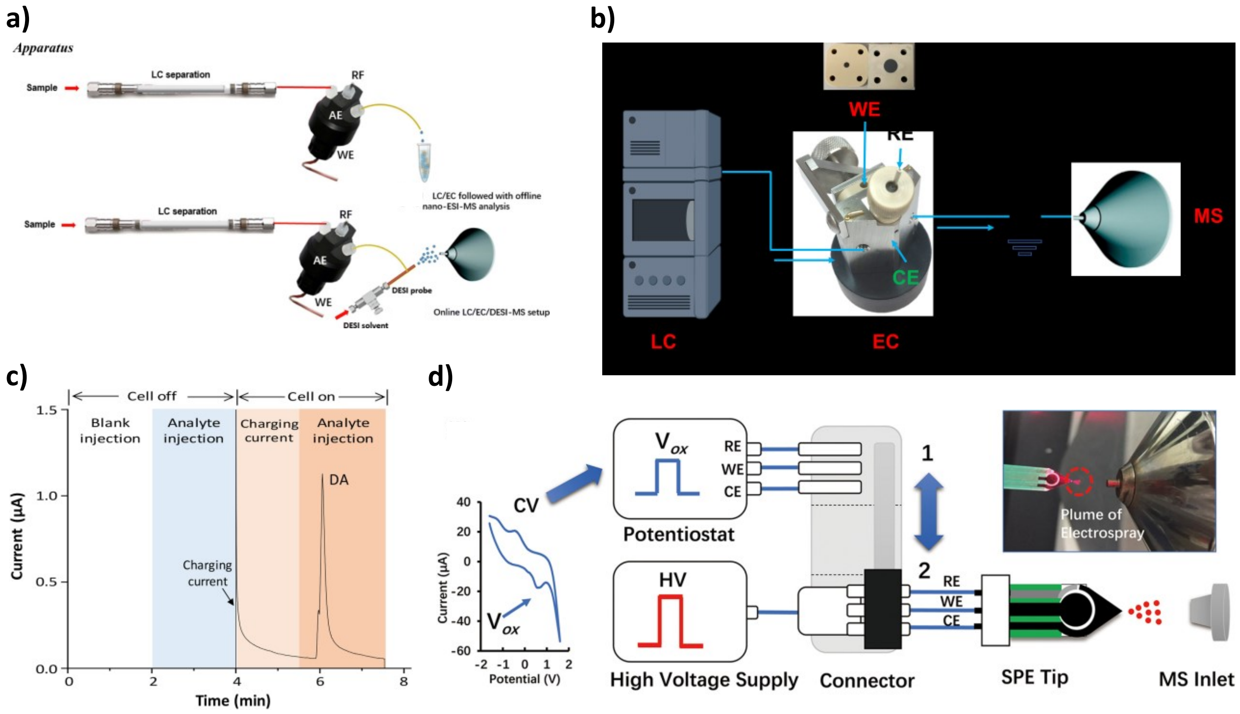
$=Q/(zF\Delta i)$



Scheme 1. Schematic showing the process of CMS including WE potential optimization by CV, coulometric measurement of converted analytes and MS measurement of redox reaction yields.

2.2. Instrumentation

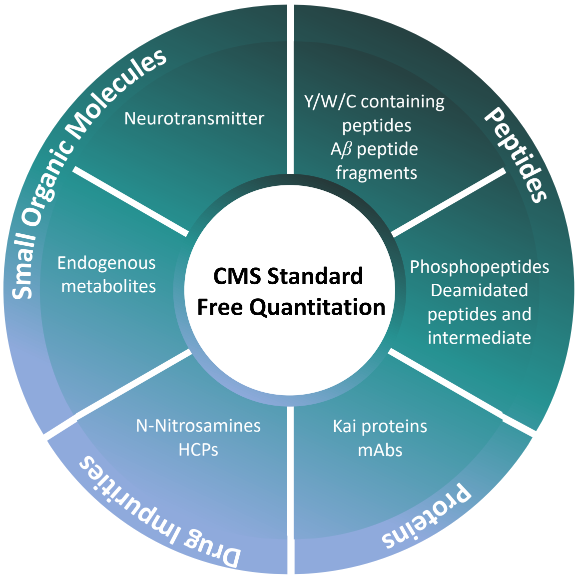
As shown in Scheme 2a, the original prototype of CMS is a coupled liquid chromatography/electrochemistry/mass spectrometry (LC/EC/MS) apparatus.[54] A liquid chromatography system equipped with a C18 column was used for separation, allowing the CMS quantitation of an individual analyte in a mixture sample. An electrochemical thin-layer flow cell equipped with a Magic Diamond (boron-doped diamond) disc electrode (i.d., 8 mm) or a glassy carbon disc (i.d., 3 mm and 6 mm) as the WE used for the oxidation process. A positive potential (ranging from + 1.0 to + 1.3 V) was applied to the WE electrode for oxidation of the LC-separated target compounds. The eluate flowing out of the cell was either collected and subsequently analyzed using nano-electrospray ionization mass spectrometry (nanoESI-MS, Scheme 2a) or directly monitored online using home-built liquid sample desorption spray ionization mass spectrometry[81] (LS-DESI-MS, Scheme 2b). The oxidation yield was calculated based on the EIC peak area or peak intensity change relative to an internal standard before and after electrolysis. In addition to DESI, a commercial heated electrospray ionization (HESI) source was used later for convenience [58]. For compounds that are already purified, a LC column is not necessary and can be removed (i.e., flow-through analysis), thus shortening the analysis time for each injected sample[55]. Using an LC auto-sampler, multiple samples can be injected sequentially, via flow injection (FI), to expand the application of CMS for high throughput quantitation and fast screening analysis. As shown in Scheme 2c, the whole injection sequence for one sample took 7.5 min where 0-2 min was a blank solvent injection for cleaning purpose followed by a 2 min analyte injection in the "cell-off" mode. The electrochemical cell was turned on for 1.5 min before the second injection in the "cell on" mode due to the charging current decay. Moreover, to further simplify the setup, a versatile three-electrode system screen printed on the polymer support acting both as the coulometry platform for electrochemical oxidation and the sample loading tip for spray ionization was reported[82]. The combination of CMS with direct ambient sampling methods facilitated the quantitation speed and simplicity with low cost, which showed the potential of CMS for rapid on-site testing.



Scheme 2. Schematic showing different CMS apparatus configurations: a) the prototype CMS setup involving online LS-DESI-MS or offline MS measurement; b) the LC/EC/HESI-MS apparatus used for CMS; c) the FI-CMS for high throughput quantitation of purified compounds; d) the conductive polymer spray ionization mass spectrometry (CPSI-MS) for performing CMS quantitation. Reprinted and slightly modified with permission from Ref[54], Ref[83], Ref[55] and Ref[82].

3. Applications

With rapid advances in instrumentation and technologies, mass spectrometers reporting the absolute quantity of target analytes have gained increasing popularity given its a wide range of applications such as drug discovery and development, biomarker verification/validation, and biological process exploration. However, MS-based quantitation strategies often rely on a reference material like isotope labeled standards, which is expensive, sometimes not available, or very time-consuming to synthesize. As an emerging and novel quantitative strategy, CMS has demonstrated its capabilities for absolute quantitation across different modalities such as small molecule metabolites, peptides, proteins with PTMs and large molecule biotherapeutics (Scheme 3). These applications showed promising results of CMS and its great potential to be used in different fields.



Scheme 3. Summary of current applications and advances of CMS absolute quantitation of small organic molecules, peptides, proteins and drug impurities

<u>3.1. Quantitation of small organic molecules</u>

Small molecule quantitation by MS has been widely carried out, mainly relying on structural similarities or isotope-labeled compounds as the internal standard due to MS signal fluctuations and the ion suppression effect from the matrices. In addition, MS-based absolute quantitation approaches require the construction of a calibration curve. Although LC coupled with electrochemical detection has been well established for absolute quantitation of electrochemically active analytes such as carbohydrates, antibiotics, and neurotransmitters based on the generated electric current response which is proportional to the quantity of analytes, authentic target compounds and the calibration curve are still needed. The traditional coulometric approach has been known for the direct quantitation of electroactive compounds based on Faraday's law. The absolute quantity of the analytes could be easily calculated if the targets undergo complete oxidation or reduction. However, full redox conversion is often difficult to achieve and control for multiple reasons. Therefore, the first prototype CMS experiment proposed that the absolute quantitation of analytes under partial electrolysis is still possible, if the conversion yield can be measured, for example, using mass spectrometry.[54] In preliminary trials, several electroactive small organic molecules such as dopamine (DA), norepinephrine (NE), rutin (RN), and urine acid (UA), were first tested and quantified using CMS. The obtained quantitation error was small and ranged from − 2.6 to + 4.6%.

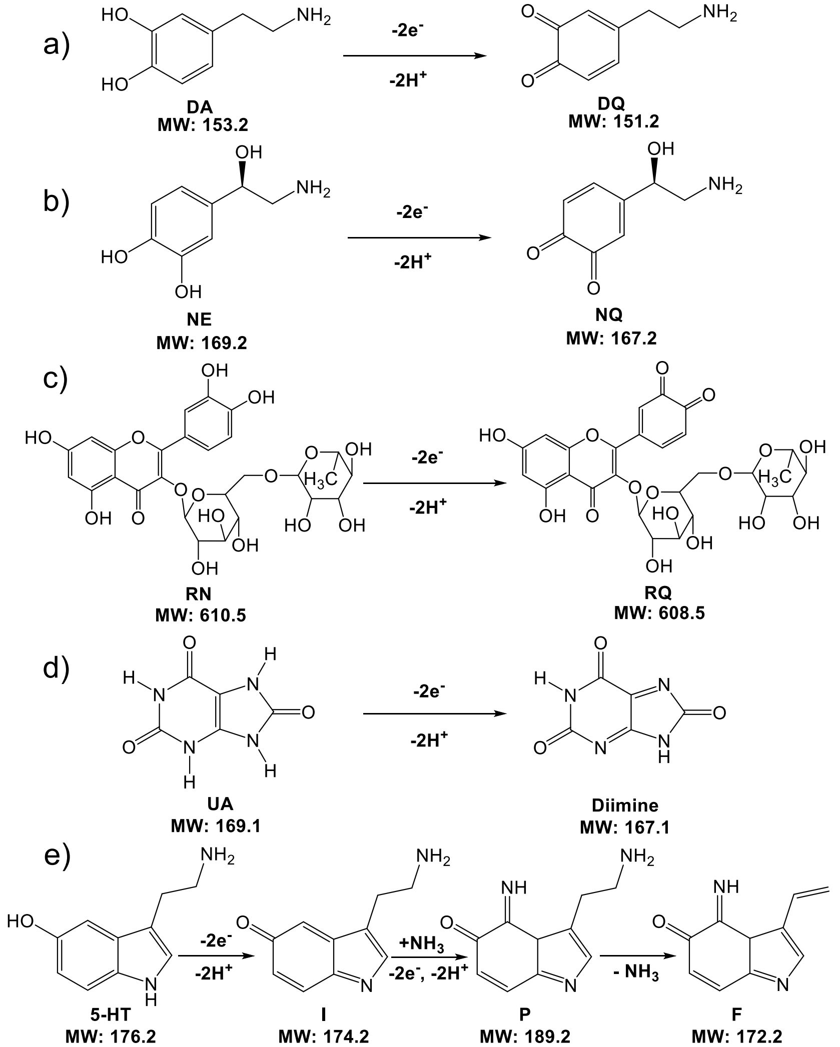


Figure 1. Equations showing the electrochemical oxidation of a) DA, b) NE, c) RN, d) UA and e) 5-HT. Reprinted and slightly modified with permission from Ref[54] and Ref[55].

Figure 1 shows the electrochemical oxidation reactions of these tested compounds. For example, neurotransmitter DA and NE as well as RN, a glycoside of the flavonoid quercetin containing two glucose units (structure shown in Figure 1a), are known to undergo a two-electron transfer electrochemical oxidation to produce quinone products. Figure 2 illustrates the CMS quantitation result of rutin, as an example. The protonated rutin was seen at *m/z* 611 (Figure 2a). After electrolysis (Figure 2b), a peak at *m/z* 609 was observed, corresponding to the oxidized product rutin quinone (RQ). Figure 2c, d shows the EIC (*m/z* 611, the protonated rutin observed at 1.4 min) of 50 μM rutin with an injection volume of 6 μL (injected amount: 300 pmol) at the mobile flow rate of 300 μL/min with the cell being turned off and on (applied potential: + 1.3 V), respectively. The EIC peak area shown in Figure 2d was smaller by 2.5%, in comparison with that of the peak shown in Figure 2c, indicating that the oxidation yield for rutin was 2.5%. On the other hand, a sharp electric oxidation current was detected after cell was turned on, as shown in Figure 2f–h (Figure 2e shows the background current diagram for blank solvent sample as a contrast). Based on the integration of the current peak area (Figure 2f–h), the amount of the oxidized rutin was calculated to be 7.3 pmol on average. Therefore, the measured amount of rutin was 7.3 pmol/2.5% = 292 pmol, which was close to the injection amount of 300 pmol with the measurement error to be − 2.6%.

It's noteworthy that the integration of MS with traditional coulometric approach not only allows the measurement of oxidation yield, but also enables monitoring of the electrochemical reaction products and confirms the number of electrons per molecule that are involved in the redox conversion. In terms of the oxidation yield measurement, it could be also performed with offline

MS analysis (Scheme 2a). For example, DA and NE were collected after flowing through LC and the electrochemical cell before and after electrolysis, spiked with arginine as a reference compound and injected for nano-ESI-MS analysis. The peak intensity ratio (relative to arginine) change provided the oxidation yield. Uric acid, another electroactive small molecule, was also quantified based on a two-electron electrochemical oxidation to generate a diimine product (Figure 1d). Elevated UA level in blood can trigger gout, aggregate in joints, and is related to kidney disease. Therefore, quantitative analysis of UA level in urine is meaningful and was also demonstrated by CMS. [54]

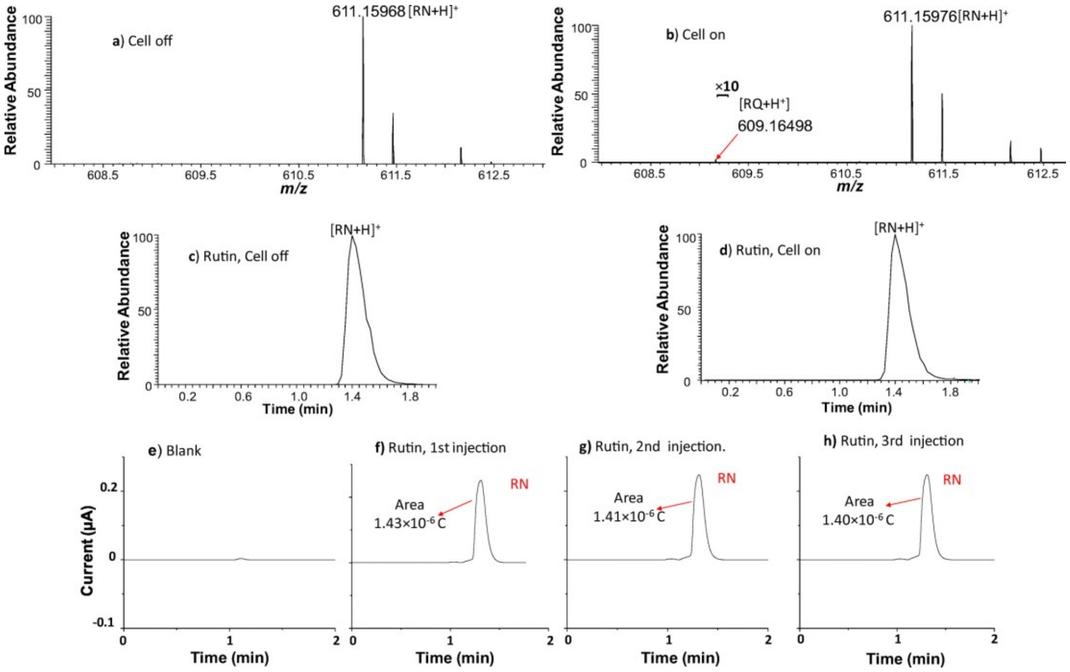


Figure 2. Online DESI-MS spectra of rutin when the applied potential was off (a) and turned on +1 .3 V (b). The peak of the rutin oxidation product, RQ, was seen at *m/z* 609 in (b). EIC of rutin recorded when the applied potential was off (c) and turned on + 1.3 V (d). electric current responses (e) due to the blank solvent and (f–h) the oxidation of rutin sample in triplicate measurements. Reprinted with permission from Ref[54].

With using LC, CMS absolute quantitation is applicable for mixture sample. As an example, 5-HT (structure shown in Figure 1e) and DA were quantified individually in a mixture sample following HILIC separation.[55] To further improve the application of CMS to accommodate the high throughput feature and fast screen analysis in drug discovery and development process, an improved version of CMS setup for fast quantitation of purified electroactive compounds was demonstrated with a 7.5 min cycle time for each analyte. In this experiment, a flow injection (FI) analysis sequence was developed using a LC auto-sampler and resulted in a significant decrease of analysis time for each injected, allowing sequential multiple sample quantitation. As illustrated in Scheme 2c, 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 carryover, 2) an analyte solution injection when the electrochemical cell was turned off (i.e., the "cell off" mode), and 3) an analyte solution injection when the electrochemical cell was turned on (i.e., the "cell on" mode). The running time for each injected sample was 2 min, but the electrochemical cell was turned on 1.5min before step 3) so that the charging current could fade away and not interfere with the analyte oxidation current.

Furthermore, ambient ionization methods, which can be used to ionize samples directly with no or little preparation under ambient conditions, are a new generation of ionization technologies for MS. With a novel ambient ionization method called conductive polymer spray ionization mass spectrometry (CPSI-MS), Song *et al.*[82] developed a three-electrode system screen printed on the conductive polymer support to serve as both the coulometry platform for electrochemical oxidation and the sample loading tip for spray ionization. As shown in Scheme 1d, 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 the WE, RE, and CE. This SPE polymer tip with a conducting layer can serve as both the WE and the high voltage triggered spray ionization tip. The CMS quantitation based on CPSI-MS platform includes two steps after loading a 10-μL pure solution or biological fluid onto the SPE polymer tip (Figure 3a). After loading the analyte solution onto the tip, the electrochemical oxidation potential was first optimized with CV 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 MS. The analyte's native concentration was quantified by dividing the oxidized product's concentration (based on Coulomb's law) and the oxidation yield (estimated from mass spectrometry assuming that the parent and oxidation product have nearly the same ionization efficiencies). Several model compounds including tyrosine, dopamine, and angiotensin II were tested (Figure 3b-e) and quantified with quantitation error within 11%.

Because of its simplification in sample processing, cost, and ease, this standard-free absolute quantitation platform is ideal for onsite analysis. In addition, the versatile polymer tip can be fabricated with slight modification to selectively extract the analyte and oxidize the target analyte in the presence of biological matrixes. For example, bilirubin, an important metabolite marker for indicating various liver and gallbladder diseases, was spiked in urine and successfully quantified with CPSI-MS platform due to the specific DODMAC/MWCNT modification on the WE. However, for real-world applications, the sample matrixes might be more complicated and thus sample pretreatment steps might be necessary before the measurement to remove electrochemically active interferences. Alternatively, introducing target-specific elements such as enzymes, antibodies, aptamers, lectins, or molecularly imprinted polymer cavities for the modification of WE would significantly increase the selectivity of EC reaction as well as the quantitation accuracy.

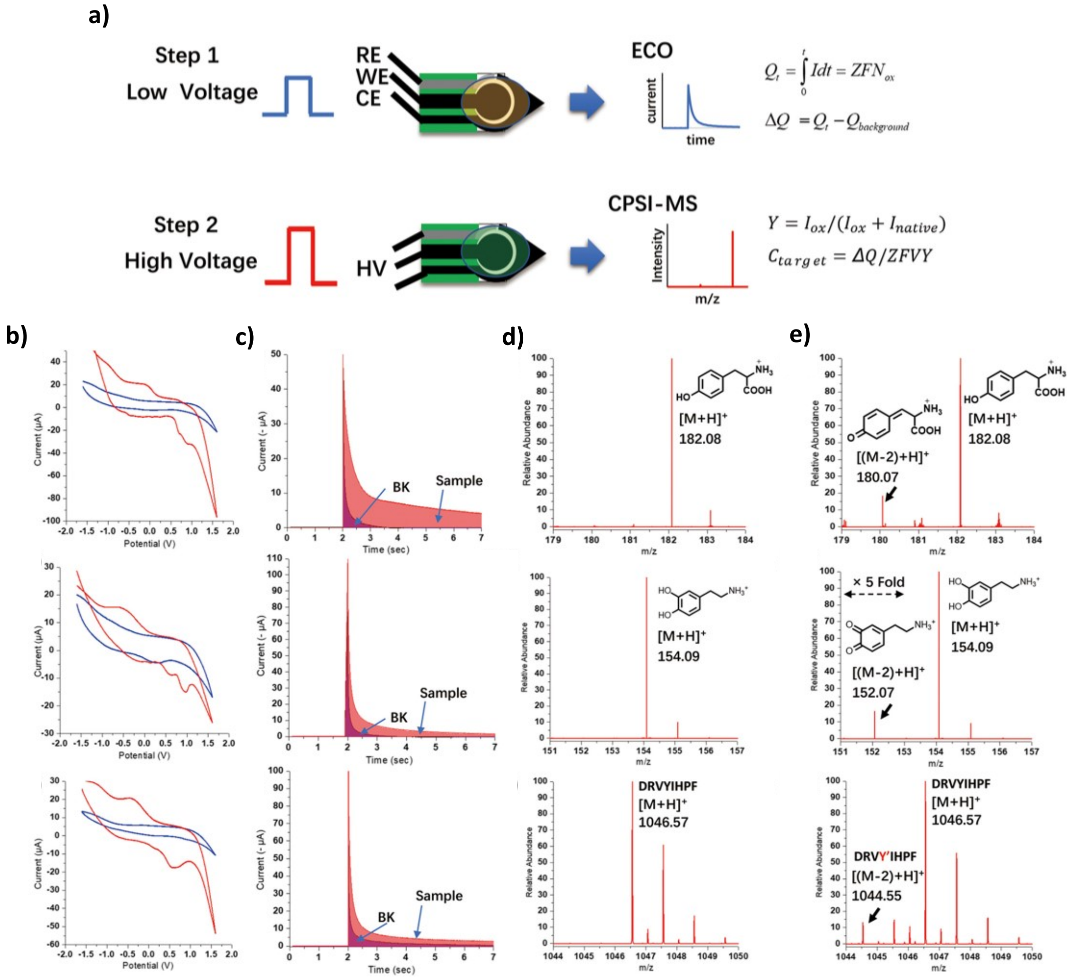


Figure 3. a) The general workflow of the CMS quantitation based on CPSI-MS platform. b) The cyclic voltammograms of tyrosine, dopamine, and angiotensin II on the nafion-modified screen-printed WE (red line) and the bare WE (blue line); c) the current responses versus with the electrochemical oxidation time for analyte solution (sample) and blank solvent (BK); d,e) mass spectra of dopamine, tyrosine, and angiotensin II acquired before and after electrochemical oxidation. Reprinted and slightly modified with permission from Ref[82].

3.2. Peptide quantitation

In addition to the quantitation of small molecules, CMS has been explored to quantify peptides without using peptide standards or isotope-labeled peptide standards. Among the 20 naturally occurring amino acids, four are oxidizable: cysteine (C), tyrosine (Y), tryptophan (W), and methionine (M), listed in the order of their increasing oxidation potential. Glutathione (GSH), a thiol-containing peptide, was first tested with CMS based on the electrochemical oxidation conversion of thiols into disulfides (Figure 4a). CMS quantitation of GSH is similar to small molecules, as shown in Figure 5. After electrolysis, a peak at *m/z* 307 was observed, corresponding to + 2 ion of the oxidized glutathione product GSSG (Figure 5d), as expected. The integrated current area and the measured oxidation yield were combined to successfully quantify the amount of native GSH with a measurement error of 4.6%.

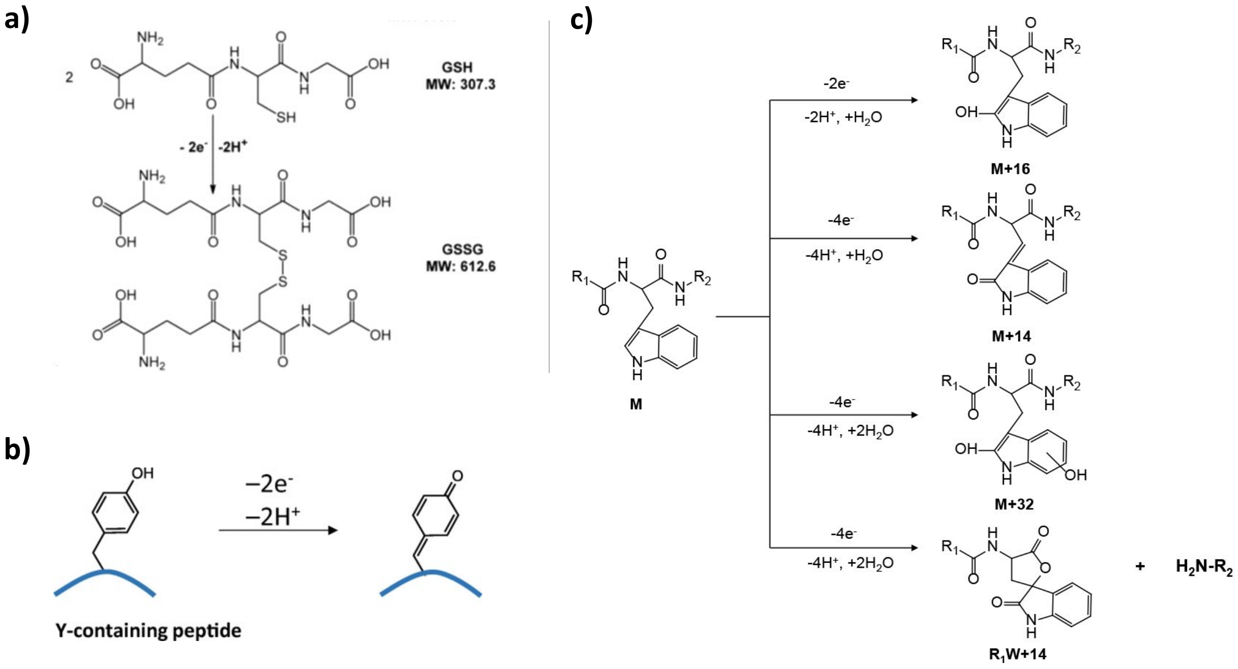


Figure 4. Equation showing electrochemical oxidation of a) GSH; b) a tyrosine-containing peptide; c) a tryptophan-containing peptide. Reprinted and slightly modified with permission from Ref[54], Ref[58] and Ref[59].

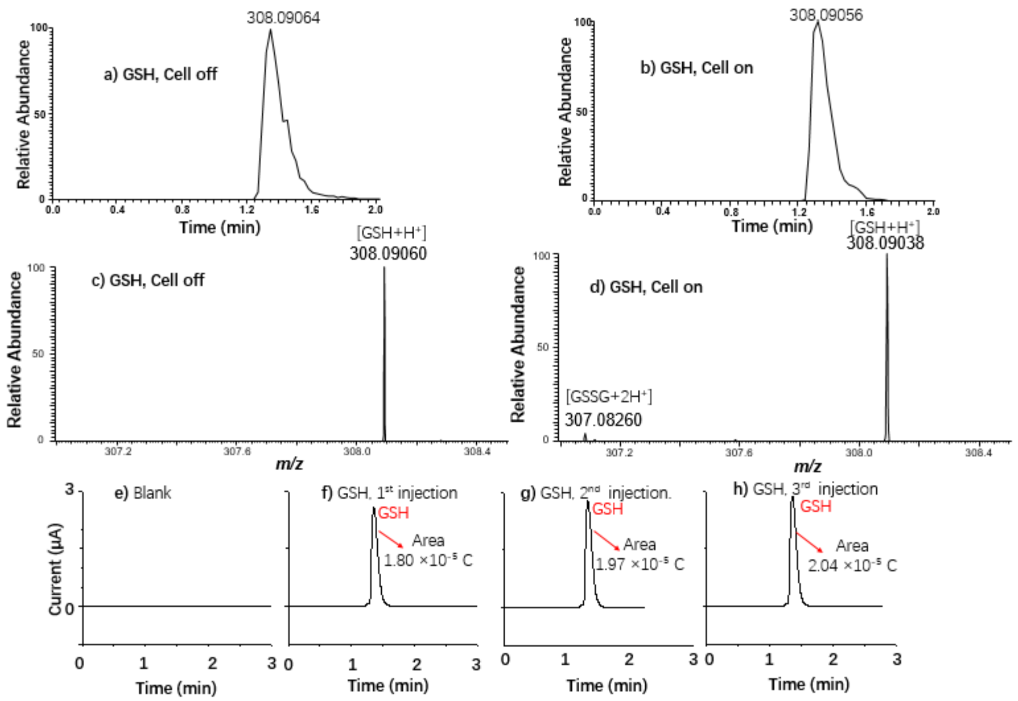


Figure 5. a) EIC of GSH (*m/z* 308, the protonated GSH) when the applied potential was off a) and turned on +1.3 V b); Online MS spectra of GSH without oxidation c) and with oxidation (applied potential: + 1.3 V); +2 ion of the oxidation product GSSG was seen at *m/z* 307 in d); Electric current response due to e) the blank sample and f-h) to the oxidation of GSH sample in triplicate measurements. Reprinted with permission from Ref[54].

Another oxidizable residue, tyrosine, with a relatively low oxidation potential and relatively high abundance in proteins was investigated and showed a reproducible and stable electric current for all the examined tyrosine-containing peptides. Under an appropriate oxidation potential (e.g., 1.00-1.05 V vs. Ag/AgCl), the phenol side chain of a peptide tyrosine residue can be oxidized into a semi-quinone by losses of two electrons and two protons (Figure 4b), in which a mass shift of 2 Da occurs, which can be readily monitored by MS analysis. For example, GGYR was used as a test sample. Tyrosine-containing peptide GGYR can undergo oxidation by giving up two electrons. In fact, the protonated GGYR was found at *m/z* 452 prior to electrolysis (Figure 6a). A new peak at *m/z* 450 was seen during electrolysis (Figure 6b), which corresponded to +1 ion of the oxidized GGYR product, GGY'R (Y' stands for the oxidized tyrosine residue). The EIC of 50 μM GGYR with an injection volume of 6 μL was shown in Figures 6c and d. The applied potentials were 0 V and +1.0 V. When compared to the peak in Figure 6c, the integrated area for the peak in Figure 6d was 5.2% smaller, showing that GGYR had a 5.2% oxidation yield (averaged in triplicate). The electric current seen in Figure 6f was likely the result of GGYR oxidation because there was no electric current peak seen for the solvent in contrast (Figure 6e). The average concentration of the oxidized GGYR was determined to be 14.9 pmol based on the integration of the present peak (Figure 6f). Therefore, the measured amount of GGYR was 285 pmol, which was near to the injection amount of 300 pmol with a measurement error of -5.0%.

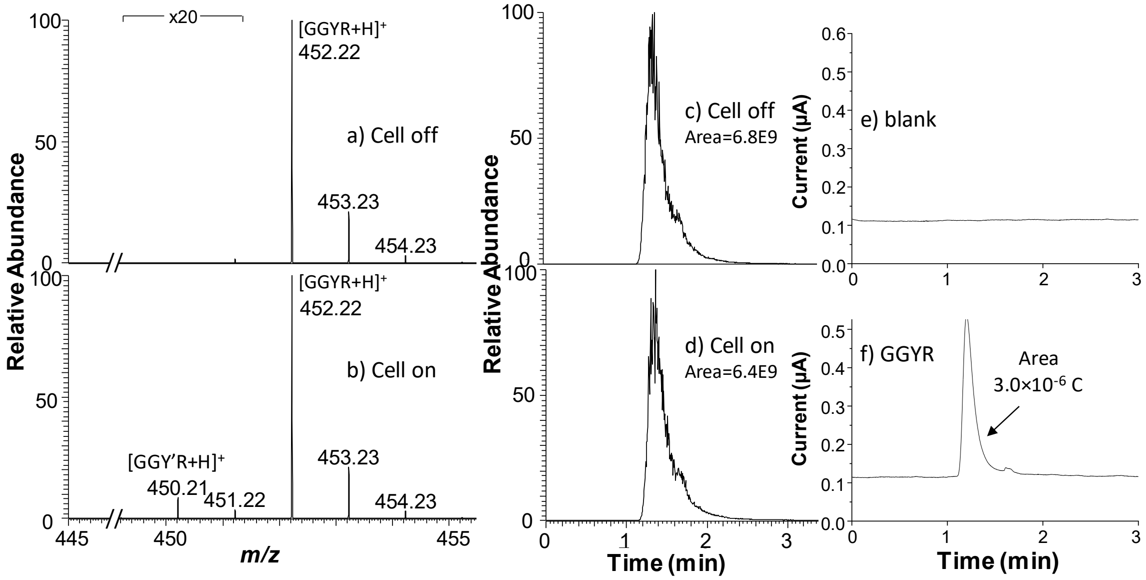


Figure 6. ESI-MS spectra of GGYR when the applied potential was a) 0 V and b) +1.0 V. The peak of the oxidized product GGY'R was clearly seen at *m/z* 450 in b). EIC of GGYR was recorded when the applied potential was c) 0 V and d) +1.0 V. Electric current responses were shown due to the oxidation of e) a blank solvent and f) GGYR peptide. Reprinted with permission from Ref[58].

In addition, the oxidized product (*m/z* 450) was subjected to MS/MS analysis to ensure that it was indeed generated via the electrochemical oxidation of tyrosine in the GGYR (Figure 7). In this experiment, a syringe pump was used to introduce the GGYR sample into an electrochemical flow cell for oxidation at +1.0 V potential. The final product was collected and re-ionized using nESI, and the resulting oxidation product, GGY'R was detected at *m/z* 450. To compare with the intact peptide GGYR ion at *m/z* 452, collision-induced dissociation (CID) data of *m/z* 450 was recorded. As seen in Figure 7, the oxidized peptide ion (*m/z* 450) produced the fragment ion y_1 (*m/z* 175) and y_2' (*m/z* 336) while the intact peptide (*m/z* 452) produced y_1 (*m/z* 175) and y_2 (*m/z* 338), demonstrating the occurrence of the oxidation to the tyrosine residue where 2 Da mass shift took place. Further proof that oxidation does in fact occur to the side chain of tyrosine in this peptide can be seen in the fragment ion of *m/z* 344 that results from dissociation of *m/z* 450 by loss of the oxidized tyrosine side chain $O{=}C_6H_4{=}CH_2$, as illustrated in Figure 7a.

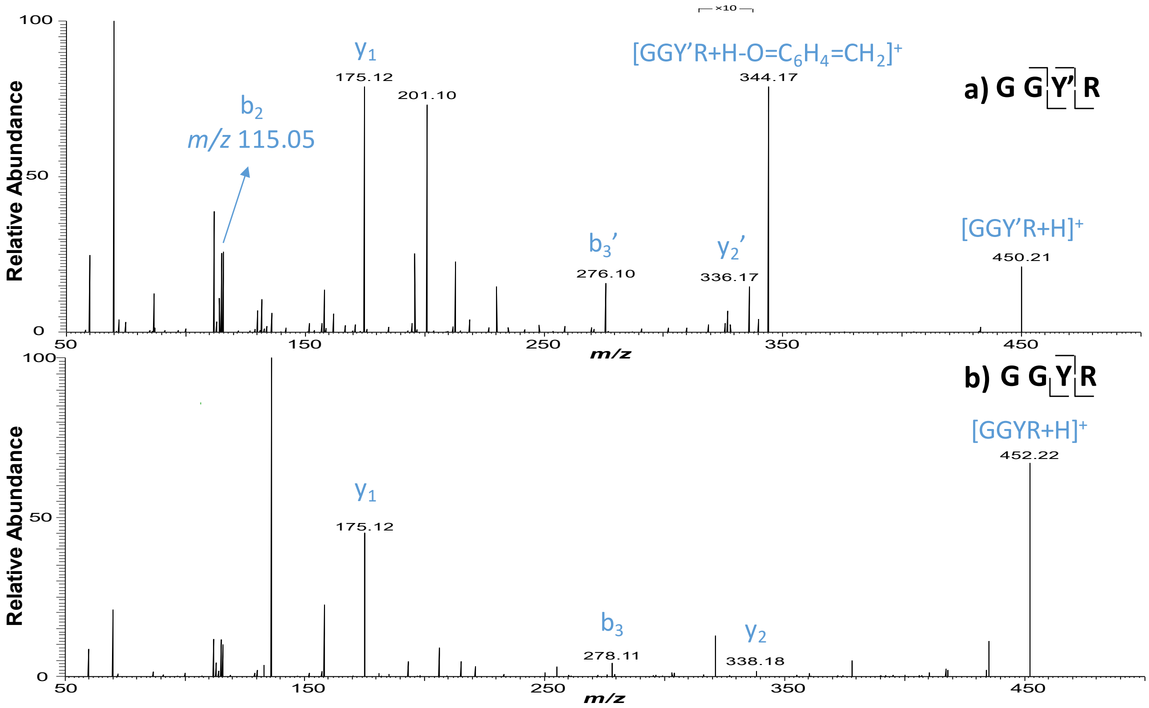


Figure 7. CID MS/MS spectra of a) the oxidized peptide ion $[GGY'R+H]^+$ (*m/z* 450) and b) the intact peptide ion $[GGYR+H]^+$ (*m/z* 452). Fragment ion of *m/z* 344 in a) resulted from the loss of the oxidized tyrosine side chain from *m/z* 450. Reprinted with permission from Ref[58].

To demonstrate how CMS is also applicable to peptides containing more than one tyrosine residue, angiotensinogen (1–14), a known tetra decapeptide (TDP) with the sequence

DRVYIHPFHLLVYS, was studied. It was found that oxidation occurred dominantly to one tyrosine of the peptide, due to a short residence time in the electrochemical flow cell with using a high sample flow rate. Oxidation of two tyrosine residues could also take place. In such cases, CMS quantitation of peptide containing more than one tyrosine residue is still possible based on the assumption that the intensities of the different oxidized products represent a good approximation for the concentrations of the different oxidized product forms. For instance, prior to electrolysis, the +4 charged TDP ion was identified at *m/z* 440.49 (Figure 8a). A peak at *m/z* 439.98 was found after electrolysis (Figure 8b), matching to the +4 ion of oxidized TDP product originating from the oxidation of one of the tyrosines. A second, smaller peak at *m/z* 439.98 resulted from the oxidation of both tyrosines (Figure 8b), with an intensity ratio of 0.12:1 between *m/z* 439.48 and *m/z* 439.98. Figures 8c and d displayed the EIC of *m/z* 440.49, the +4 charged TDP ion, as a result of the injection of 6 μL of 50 μM TDP (300 pmol in total) at 0 V and +1.05 V (vs. Ag/AgCl), respectively. The integrated area of the peak depicted in Figure 8d was 2.6% less than that of the peak shown in Figure 8c, indicating that the oxidation yield for TDP was 2.6%. Figure 8f showed the TDP oxidation current peak (Figure 8e showed the background current pattern for blank solvent sample at +1.05 V potential). The total electric charge Q involved in the TDP oxidation, leading to the generation of *m/z* 439.98 via one tyrosine oxidation (2e$^-$ per mole peptide) and *m/z* 439.48 via two tyrosine oxidation (4e$^-$ per mole peptide), could be computed by integrating the current peak area. Taking into account the intensity ratio of 0.12:1 between *m/z* 439.48 and *m/z* 439.98 and the similarity of the structures of the two oxidation products, the molar ratio of the two oxidation products was roughly 0.12:1. Therefore, the total amount of oxidized peptide was calculated to be 7.2 pmol. Considering the oxidation yield of 2.6%, the amount of TDP we measured was 283 pmol. Compared to the injection quantity of 300 pmol, the quantitation error was -5.5%. This result demonstrated that CMS can successfully quantify peptides with multiple tyrosine residues. In addition to GGYR and TDP, several model peptides containing tyrosine residues were quantified with good accuracy and results are summarized in Table 1.

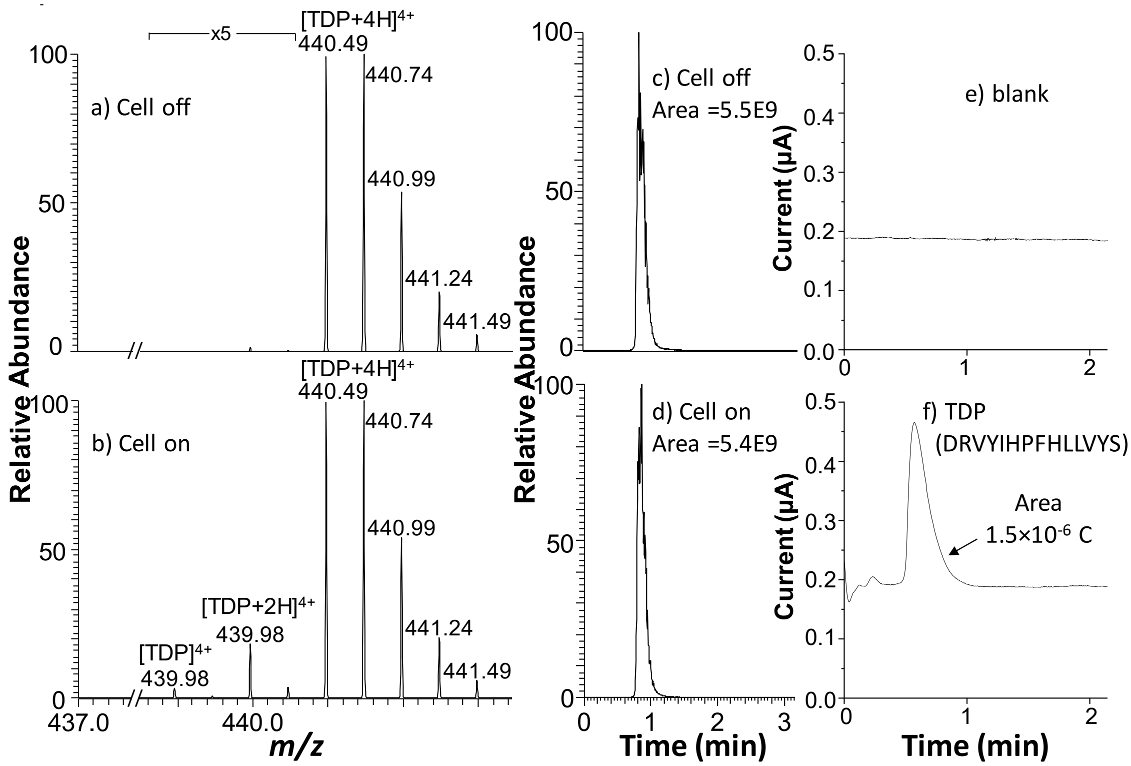


Figure 8. ESI-MS spectra of TDP when the applied potential was a) 0 V and b) +1.05 V. The major peak of the oxidized product of TDP was seen at *m/z* 439.98 (+4 charged) in b). EIC of the +4 charged TDP ion was recorded when the applied potential was c) 0 V and d) +1.05 V. Electric current responses were shown e) due to the blank solvent and f) the oxidation of TDP peptide. Reprinted with permission from Ref[58].

Table 1. List of Tyr and Trp-containing peptides quantified by CMS

#	Name	Peptide sequence	Molecular weight (Da)	Quantitation error (%)
1	Gly-Gly-Tyr-Arg	GG**Y**R	451.5	-5.0
2	Asp-Arg-Val-Tyr	DRV**Y**	551.6	-7.5
3	Oxytocin	C**Y**IQNCPLG, disulfide bond 1–6	1007.2	2.4
4	[Arg8]-vasotocin	C**Y**IQNCPRG, disulfide bond 1–6	1050.2	0.8
5	Angiotensinogen (1–14)	DRV**Y**IHPFHLLV**Y**S	1760.1	-5.5
6	Phosphorylated UOM9	KRPpSQRHGSK**Y**	1422.5	-5.8
7	L-tryptophylglycylglycine	**W**GG	318.3	-2.1

8	Fibronectin adhesion-promoting peptide	**W**QPPRARI	1023.2	4.3
9	gp 100 (25−33) human	KVPRNQD**W**L	1155.3	1.5
10	BDC2.5 mimotope	RTRPL**W**VRME	1343.6	-2.3
11	delta sleep inducing peptide	**W**AGGDASGE	848.8	-4.5

To further extend the CMS application, we also investigated the possibility of using CMS for absolute quantitation of tryptophan-containing peptides. Tryptophan is known to be electroactive and has a relatively low oxidation potential (+1.01 V, vs NHE).[84] The major oxidation products and pathways of tryptophan-containing peptides were proposed and validated by MS analysis.[85] As depicted in Figure 4c, the major oxidation products are the singly hydroxylated M+16 (2e$^-$ oxidation product) and the ketone product M+14 (4e$^-$ oxidation product). Minor oxidation products include the doubly hydroxylated M+32 (4e$^-$ oxidation product) and the cleavage product R1W+14 (4e$^-$ oxidation product). Online CMS allows for simple monitoring of the different oxidation products. On the basis of the similar structures of these oxidation products, we hypothesized that the intensity ratios of the various oxidized products are approximately equivalent to their molar ratios, as shown in eq. 1-3. Assuming that the moles of the products M+16, M+14, M+32, and R_1W+14 are n_1, n_2, n_3, and n_4, the total mole of the oxidized peptide is $n = n_1+n_2+n_3+n_4$. According to Faraday's Law, Q is derived from each of the four oxidation pathways (eq 4). From equations 1 through 4, the total amount of oxidized peptide n can be calculated using equation 5, where Q is obtained experimentally by integrating the Faradaic current over time and F is the Faraday constant. Upon obtaining n and the oxidation yield, the total amount of the peptide can be calculated as the ratio of the amount of oxidized peptide n and the oxidation yield Δi (i.e., n/Δi).

$$\frac{n_2}{n_1} = \frac{[M + 14]}{[M + 16]} \qquad (1)$$

$$\frac{n_3}{n_1} = \frac{[M + 32]}{[M + 16]} \tag{2}$$

$$\frac{n_4}{n_1} = \frac{[R_1W + 14]}{[M + 16]} \tag{3}$$

$$Q = F(2n_1 + 4n_2 + 4n_3 + 4n_4) \tag{4}$$

$$n = n_1 + n_2 + n_3 + n_4 = \frac{Q}{2F} \times \frac{1 + ([M + 14] + [M + 32] + [R_1W + 14])/([M + 16])}{1 + 2 \times ([M + 14] + [M + 32] + [R_1W + 14])/([M + 16])} \tag{5}$$

To testify this hypothesis, a tripeptide WGG was chosen to determine the viability of CMS peptide quantification based on tryptophan oxidation. No electric current peak was seen when the oxidation potential of +1.00 V was applied to a blank solvent sample (Figure 9e). In contrast, a sharp electric current peak was found after the injection of WGG sample at the same oxidation potential, showing that the current peak resulted from the electrochemical oxidation of WGG (Figure 9f). The protonated WGG was discovered at *m/z* 319.14 prior to electrolysis (Figure 9a). The oxidation products WGG+14, WGG+16, and WGG+32 were detected at *m/z* 333.12, 335.13, and 351.11, respectively, after electrolysis (Figure 9b). Specifically, the ion intensities of WGG+14 and WGG+16 were significantly greater than those of WGG+32. The integrated EIC peak area of *m/z* 319.14 in Figure 12d was 4.4% less than in Figure 9c, indicating that WGG's oxidation yield was 4.4%. On the basis of eq (5) and the integration of the current peak area as well as the ion intensity ratios of the oxidized products, the quantity of WGG that was oxidized was determined to be 3.8 pmol. In conjunction with the oxidation yield of 4.4%, the amount of WGG was measured to be 87.1 pmol, which was fairly close to the injection amount of 89.0 pmol (measurement error of 2.1%).

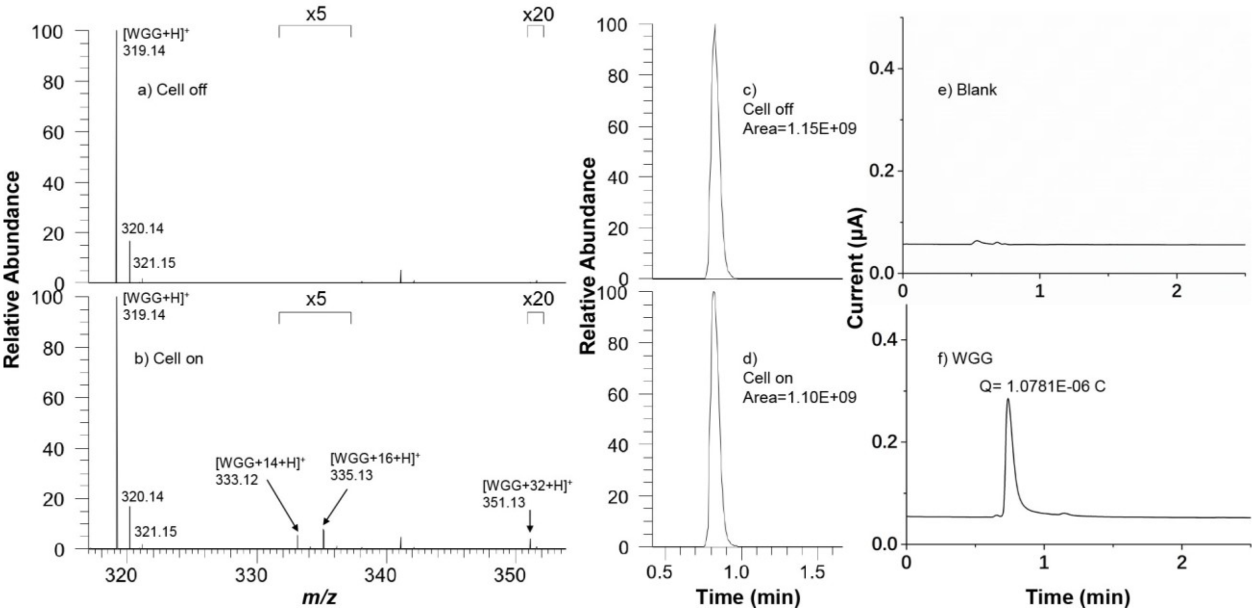
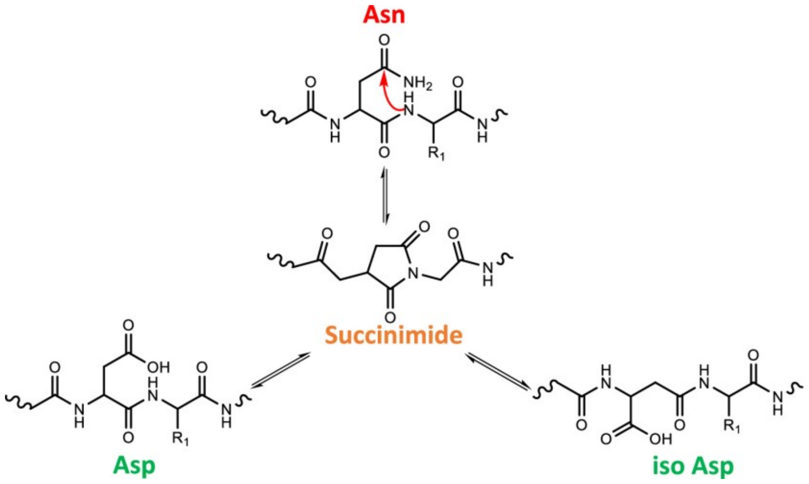


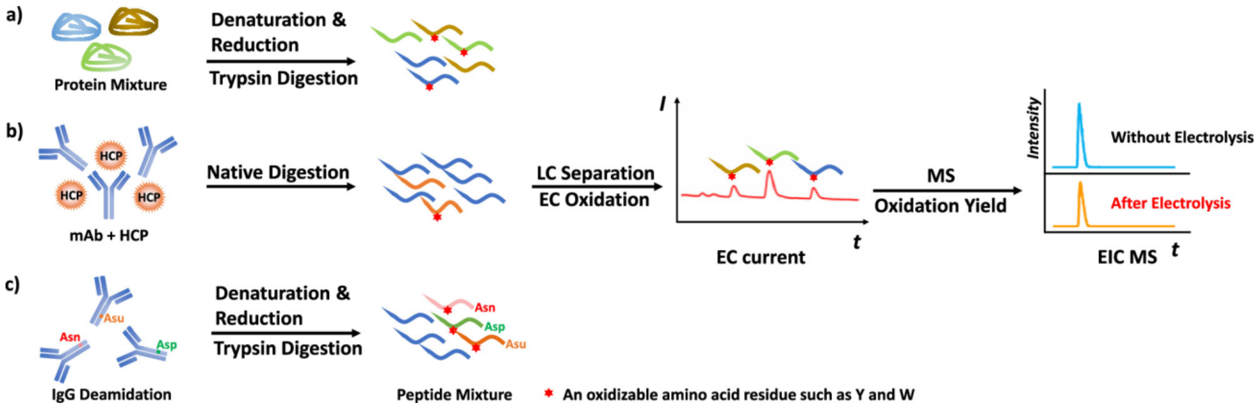
Figure 9. MS spectra of WGG a) when the cell was off and b) when the cell was turned on (applied potential: +1.00 V). The oxidation products of WGG were detected at *m/z* 333.12, 335.13, and 351.13. EICs of WGG were acquired c) when the cell was off and d) when the cell was turned on (applied potential: +1.00 V). Electric current diagrams were collected from (e) blank solvent and (f) the oxidation of WGG. Reprinted with permission from Ref[59].

Several other peptides such as WGG, WQPPRARI, KVPRNQDWL, RTRPLWVRME and WAGGDASGE were also successfully quantified, with the quantitation errors ranging from -4.5 to 4.3% in triplicate measurements (as summarized in Table 1).

After a demonstration of the feasibility of CMS for peptide quantitation, several important applications were addressed, including the absolute quantitation of peptides containing PTMs like phosphorylation[58] and deamidation[83] as well as the quantitation of amyloid beta (A*β*) peptide fragments,[59] an important risk factor having a central role in the onset and progression of Alzheimer's disease (AD). Phosphorylation is an important post-translation modification and has been widely researched in proteomics studies. Absolute quantitation of phosphopeptides often involves isotope-labeled peptides, which typically requires multiple-step synthesis with high cost. To solve this problem, we first tested one phosphopeptide RRLIEDAEpYAARG, whose tyrosine reside was phosphorylated. Interestingly, such a peptide did not display an oxidation current upon oxidation probably due to the covered electroactive phenol group. Another phosphopeptide which was phosphorylated in its serine residue and contained one free tyrosine residue was also tested and successfully quantified with CMS. Therefore, we assume that absolute quantitation of phosphopeptides without using any standards is still possible if one free oxidizable residue exists.



Scheme 4. Mechanism of asparagine deamidation to aspartic acids via a succinimide intermediate. Reprinted with permission from Ref[83].



Scheme 5. Workflows showing absolute quantitation of a) multiple proteins in a protein mixture, b) HCPs in mAb, and c) mAb deamidation by CMS. Reprinted with permission from Ref[83]

Another CMS application is the quantitation of antibody deamidation. Monoclonal antibodies (mAbs) and their related formats representing a large category of biopharmaceutical products are meeting an undeniable success as therapeutic agents in different fields such as oncology, immune disorders, and, more recently, the treatment of the SARS-CoV-2 virus. However, these recombinant therapeutic proteins contain a variety of chemical and physical modifications. Common examples include glycan structural differences, deamidation, oxidation, and glycation. Thus, to ensure patient safety, it is crucial to control and minimize this heterogeneity for product reliability and consistency across the entire product life cycle. Deamidation of asparagine (Asn) residues to Asp or isoAsp (Scheme 4) is a common chemical modification and degradation pathway that occurs during the manufacturing and storage of monoclonal antibodies (mAbs).[86, 87] Previous studies have shown that the degradation products as well as the succinimide intermediate can cause serious quality and safety issues such as aggregation, intermolecular crosslinking, activity loss, and immunogenicity. Therefore, mAb deamidation needs to be closely monitored. So far, absolute quantitation of deamidation is rare and absolute quantitation of the succinimide intermediate were not done due to the lack of standards. The simultaneous identification and absolute quantification of mAb deamidation degradation can be performed by CMS, as illustrated in the workflow of Scheme 5c. As a result, the simultaneous quantitation of native and deamidated peptides as well as the succinimide intermediate from deamidation of Asn 318 residue of NIST 8671 mAb can be completed. As shown in Figure 10, the residue N318 site from the heavy chain (HC, Figure 10a) was chosen for examination, and Figure 10b–d shows the EICs of four different peptide forms, including the unmodified peptide

VVSVLTVLHQDWLN$_{318}$GK, two deamidation peptides VVSVLTVLHQDWLisoD$_{318}$GK and VVSVLTVLHQDWLD$_{318}$GK, and the succinimide intermediate VVSVLTVLHQDWLSuc$_{318}$GK from a 30-min LC gradient elution. No oxidation current was observed for a blank solvent (Figure 10e) when the cell was turned on at +1.05 V, while after injection of the NIST mAb digest sample, four oxidation current peaks corresponding to four peptides were recorded shown in Figure 10f. At the same time, the oxidation products were monitored with online MS detection, which further confirmed the occurrence of electrochemical oxidation of those tryptophan-containing peptides. For example, as shown in Figure 10g, before electrolysis, a +3 ion of VVSVLTVLHQDWLSuc$_{318}$GK was observed at *m/z* 597.67; after electrolysis, the oxidation product ions at *m/z* 602.33 (M + 14 ion), *m/z* 603.00 (M + 16 ion), and *m/z* 608.33 (M + 32 ion) were observed in Figure 10h. The amount of oxidized VVSVLTVLHQDWLSuc$_{318}$GK was calculated as 0.050 pmol based on the charge (Q = 1.51 × 10–8C, Figure 10f) and the intensity ratios of the oxidation products observed. Based on the EIC peak change for *m/z* 597.67 upon oxidation (Figures 10g and h), the oxidation yield for VVSVLTVLHQDWLSuc$_{318}$GK was measured as 20.1% . Therefore, the total amount of VVSVLTVLHQDWLSuc$_{318}$GK was measured to be 0.25 pmol. Simultaneously, the deamidated peptides as well as the unmodified peptide were also quantified by CMS. These results indicate that CMS can be used for monitoring mAb deamidation degradation and also for the quantitation of peptides with modifications when standards are hard to access. Furthermore, since deamidation is a nonenzymatic and spontaneous PTM which is also strongly associated with aging proteome instability and degenerative diseases, novel quantitation strategies such as CMS may facilitate the investigation of the disease mechanisms and the monitoring of the aging process, which would be also useful for forensic analysis.

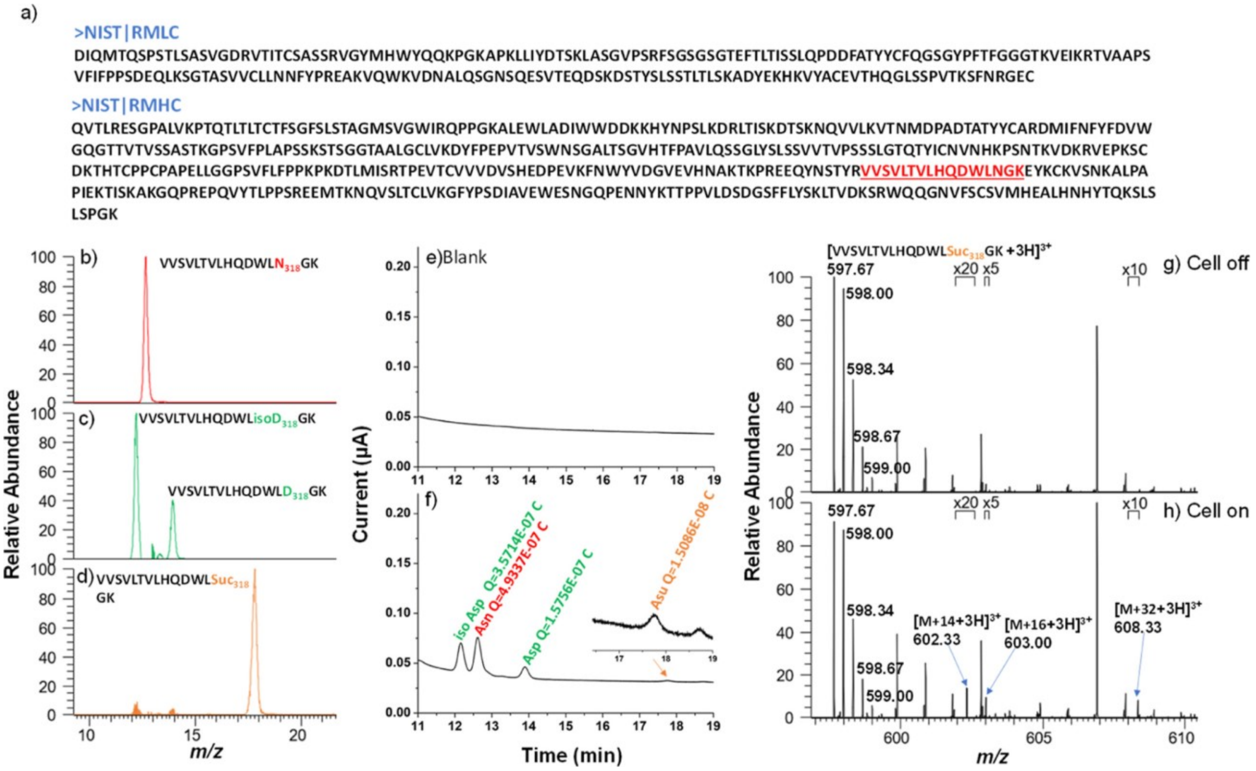


Figure 10. a) Sequence of an NIST 8671 light chain and heavy chain (the chosen N318 surrogate peptide VVSVLTVLHQDWLN$_{318}$GK from HC is highlighted in red). EICs of b) unmodified peptide VVSVLTVLHQDWLN$_{318}$GK and c) deamidated peptides VVSVLTVLHQDWLisoD$_{318}$GK and VVSVLTVLHQDWLD$_{318}$GK and d) succinimide intermediate VVSVLTVLHQDWLSuc$_{318}$GK. Electric oxidation current diagrams are shown due to the oxidation of e) solvent blank and f) mAb digest. The MS spectra of the succinimide intermediate VVSVLTVLHQDWLSuc$_{318}$GK was recorded g) without oxidation and h) with oxidation (applied potential: +1.05 V). Reprinted with permission from Ref[83].

In addition, CMS was also used to quantify amyloid beta (Aβ)-related peptides. Alzheimer's disease (AD) is becoming a major public health problem worldwide. Aβ peptide and its peptide fragments, derived from the proteolytic processing of amyloid precursor protein (APP), have been considered as the diagnostic biomarker and therapeutic target of AD. [88-90] Therefore, quantification of those different Aβ isoforms and their fragments is significant for early diagnosis of AD. Quantification also facilitates the investigation of disease mechanism and thus benefits drug discovery. MS is widely used and represents an important tool in the field of AD due to its capability of providing both qualitative and quantitative information on the Aβ involved in AD.

However, for traditional MS-based absolute quantitation of Aβ peptides, isotopically-labeled Aβ peptides are often needed as internal standards, which are time-consuming and expensive. In one experiment,[59] we reported successful quantifications of two long Aβ peptide fragments, Aβ_{1-16} and Aβ_{1-28}, using CMS based on the electrochemical oxidation of tyrosine residue without adding isotope labeling standards, for the first time. This result also demonstrated that CMS could also be used for very long peptides.

3.3. Protein quantitation

MS-based absolute quantitation of protein concentration in biological samples plays a significant role in clinical and pharmaceutical applications such as the evaluation of clinical biomarker candidates[91] and biotherapeutic bioanalysis/DMPK.[92] Popular methods for absolute protein quantitation by MS involve the digestion of target proteins and employ isotope-labeled peptide internal standards to quantify chosen surrogate peptides, which are mature and widely used in regulated bioanalysis.[93] Although these methods have gained success, the synthesis of isotope-labeled peptides are time-consuming and costly. Therefore, it is only feasible to quantify a limited number of proteins at a time due to the high cost and complexity of sample preparation. Thus, the development of methods for accurate protein quantitation without the use of isotope-labeled standards is largely in need. To circumvent this limitation, CMS was also applied for protein quantitation by selecting a surrogate peptide containing electrochemically active residue (e.g., tyrosine, tryptophan and cysteine) to present the corresponding proteins. To evaluate the feasibility of CMS for protein quantitation, several model proteins like β-casein, apomyoglobin, and cytochrome c were first quantified based on tyrosine or tryptophan- containing surrogate peptides and validated with traditional IDMS method with good accuracy. The basic workflow of CMS quantitation is very similar to the commonly used target proteomics quantitation

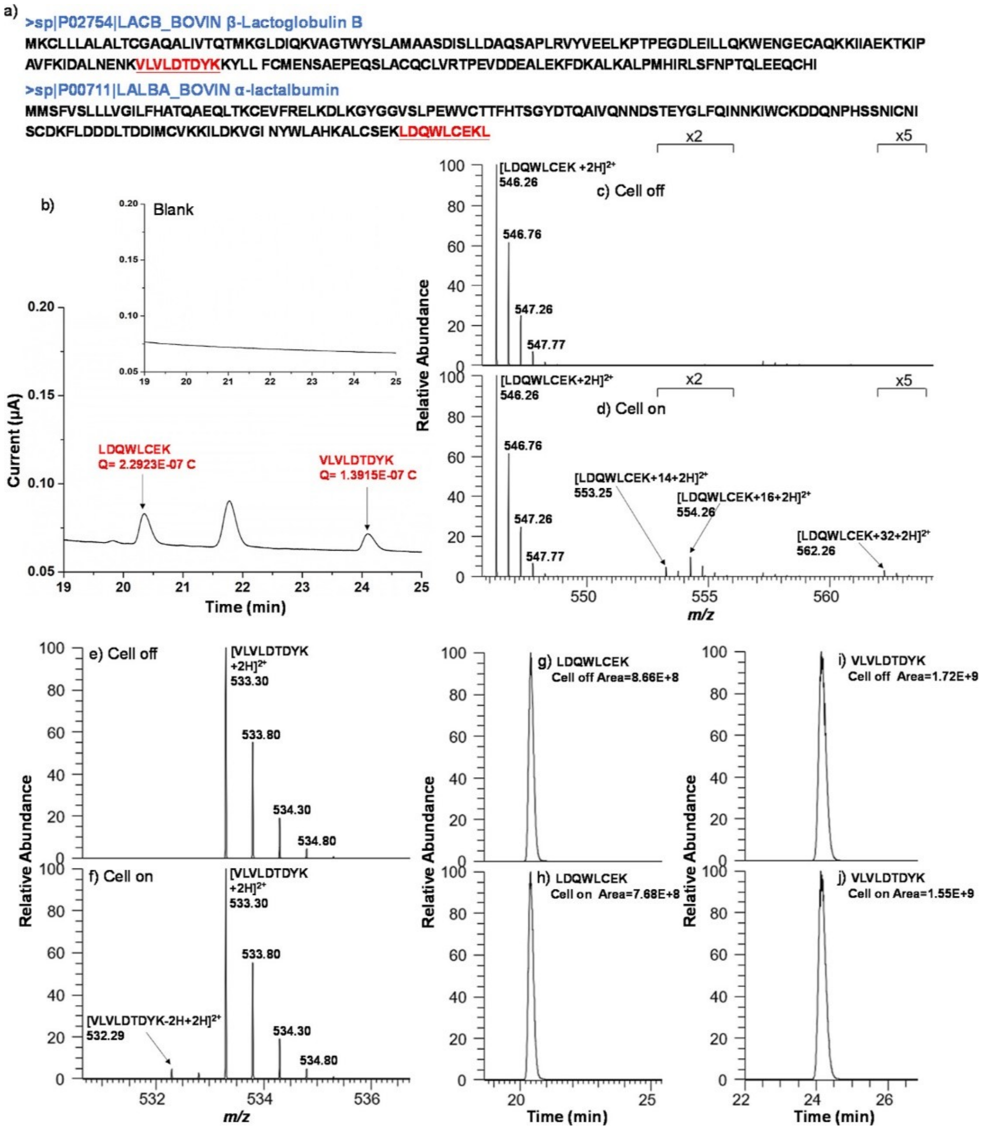


Figure 11. a) Sequences of β-lactoglobulin B and α-lactalbumin (the chosen surrogate peptides VLVLDTDYK and LDQWLEK for CMS quantitation are highlighted in red); b) electric current diagrams were collected from oxidation of blank solvent (inset) and the digested protein sample after LC separation; MS spectra of LDQWLEK c) without oxidation and d) with oxidation (applied potential: +1.05 V). MS spectra of VLVLDTDYK e) without oxidation and f) with oxidation (applied potential: + 1.05 V). EICs of LDQWLEK are shown in g) without oxidation and h) with oxidation (applied potential: + 1.05 V); EICs of VLVLDTDYK were acquired i) without oxidation and j) with oxidation (applied potential: + 1.05 V). Reprinted with permission from Ref[83].

(e.g., SRM/PRM) including protein extraction, selective enrichment, tryptic digestion, chromatographic separation, and MS detection. To further expand the application of CMS for absolute quantitation of multiple proteins from a mixture sample in one run, three proteins, β-lactoglobulin B (178 amino acids, MW 18,276 Da), α-lactalbumin (142 amino acids, MW 14,175 Da), and carbonic anhydrase (260 amino acids, MW 29,100 Da), in a mixture sample were first digested, and three resulting oxidizable surrogate peptides containing either tyrosine or tryptophan, VLVLDTDYK, LDQWLCEK, and DGPLTGTYR, were selected for CMS analysis (Figure 11).[83] The CMS quantitation result was validated using a traditional isotope dilution method with good accuracy, indicating the capability of CMS for multiplex protein quantitation as long as multiple surrogate peptides representing different proteins can be LC-separated.

To further explore the CMS utility for real biological sample quantitation, two of the essential circadian clock proteins isolated from *Escherichia coli*, KaiB and KaiC, were also tested with CMS. [57, 83] The circadian clock is an endogenous timekeeping mechanism that provides many advantages for life in a rhythmically changing environment. Disruption of the circadian clock in humans is correlated with many health issues, such as cancer, heart attacks, obesity, diabetes, fatigue, mood disorders, and, most notably, jet lag. Till now, there is no commercially available protein standard for absolute quantitation. Because proteins are the major determinant of cell physiology, the timely measurement of the protein amount is extremely helpful in understanding the circadian regulation of gene expression and the physiology of cyanobacteria. Therefore, the successful quantitation of KaiB[57] and KaiC[83] after purification suggests the potential of CMS for real sample quantitation.

Additionally, to further test the capability of CMS for large molecule quantitation, Vectibix (panitumumab, approximate molecular weight 147 kDa), a recombinant human IgG2 kappa monoclonal antibody that binds specifically to the human epidermal growth factor receptor (EGFR), was also tested and quantified with CMS. LLIYDASNLETGVPSR, a tyrosine-containing peptide from antibody light chain was identified and separated by LC/MS analysis and used for quantitation by CMS. Without oxidation (Figure 12a), peptide LLIYDASNLETGVPSR, chosen as the surrogate peptide, was observed at *m/z* 874.5 (+2 ion). As shown in Figure 12b, after electrolysis, the oxidized product of LLIYDASNLETGVPSR (+2 ion) was detected at *m/z* 873.5, due to two hydrogen losses from tyrosine oxidation. Figure 12c and d show the EIC the +2 ion of LLIYDASNLETGVPSR (*m/z* 874.5) from 3 µL of 3.4 µM IgG2 antibody digest (10.2 pmol) without and with oxidation, respectively. Note that one IgG2 molecule contains two identical light chains in which the surrogate peptide fragment is located. Therefore, the theoretical amount of surrogate peptide was 20.4 pmol per injection by calculation. The oxidation yield for LLIYDASNLETGVPSR was suggested to be 8.2%, by comparing the peak area of *m/z* 874.5 before and after oxidation (Figure 12c and d). Meanwhile, the amount of the oxidized LLIYDASNLETGVPSR was calculated to be 1.28 pmol, based on the area integration of electric current peak observed from peptide oxidation (Figure 12f). Therefore, the measured amount of LLIYDASNLETGVPSR was 15.7 pmol (triplicate average: 15.0 pmol). Compared to the theoretical amount (20.4 pmol) of this surrogate peptide, the measurement error was −26.4%. The successful quantification of mAbs without standards displays the immense potential of CMS for the quantification of large molecule biotherapeutics, which would be extremely useful in the pharmaceutical field like quantitative analysis in bioanalytical and clinical applications. Notably, a major disadvantage of surrogate peptide-based quantitation is the peptide recovery during preparation steps prior to MS, particularly for tryptic digestion. In the case of antibody quantitation by CMS after digestion, the relatively large error of -26.4% could be due to relatively low digestion efficiency for a large antibody protein peptide.[94] Combining CMS with novel digestion strategies like using surfactants to improve digestion efficiency would improve quantitation accuracy.

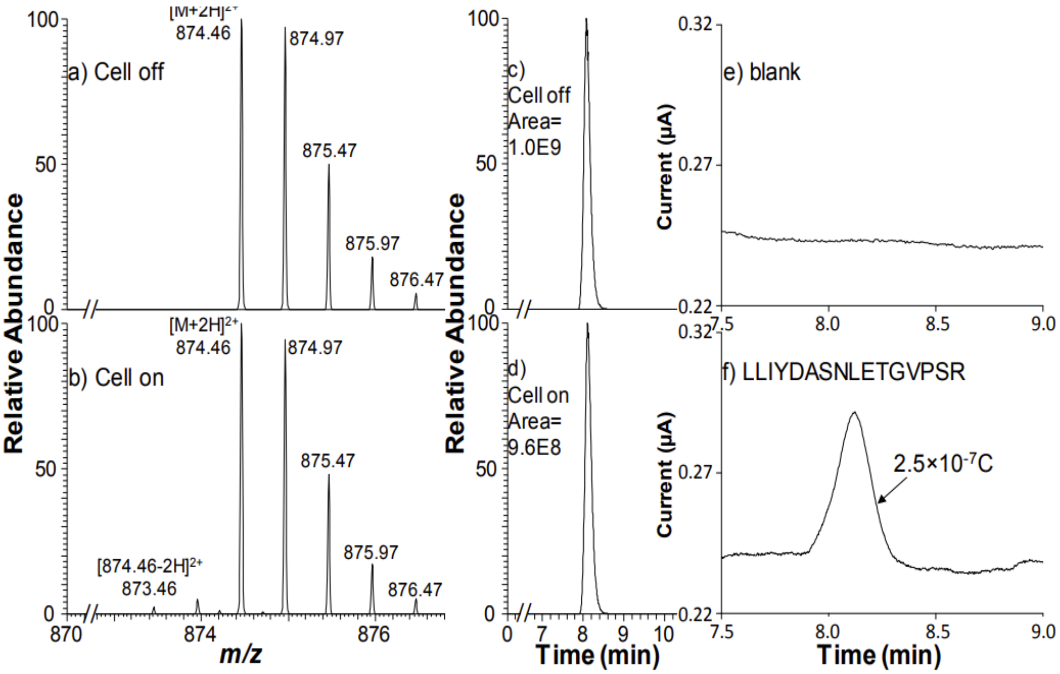


Figure 12. ESI-MS spectra of LLIYDASNLETGVPSR from the IgG2 tryptic digest when the applied potential was a) 0 V and b) +1.05 V. The peak of the oxidation product was seen at *m/z* 873.46 in b). EIC of LLIYDASNLETGVPSR was recorded when the applied potential was c) 0 V and d) +1.05 V. Electric current responses were due to the oxidation of e) the blank solvent and f) LLIYDASNLETGVPSR. Reprinted with permission from Ref[57].

3.4. Quantifying drug impurities of antibody HCP proteins and nitrosamines

The fact that CMS eliminates the need for standards or a calibration curve makes it ideal as a rapid quantitation method for drug development and the process of monitoring and clearing high drug impurities in a timely manner. Host cell proteins (HCPs) are a major class of impurities derived from biotherapeutics production processes. HCPs that remain in the final drug product, especially for degrading or immunogenic proteins such as cathepsin D or phospholipase B like 2 (PLBL2), have been shown to affect product quality and patient safety due to the immune response.[95, 96] The current standard approach to quantify HCPs is based on enzyme-linked immunosorbent assay (ELISA)[97, 98] which has drawback in its detection selectivity. MS is emerging as an alternative way to characterize HCP contents, but the existing MS methods might be hard to implement since either peptide or protein internal standards are needed for HCP quantitation. These standards take a significant amount time to synthesize. It is extremely important to find a rapid quantitation method—without the use of standards—for timely process optimization and risk assessment so that high-risk HCPs can be tracked and cleared within days of discovery, especially during disease outbreaks such as the COVID-19 pandemic. CMS is an attractive method for this task because it does not require standards. As a demonstration, we applied CMS for the absolute quantitation of PLBL2 spiked in NIST mAb 8671 at two spiking levels (i.e., mAb/PLBL2 = 200:1 and 2000:1).[83] The workflow was shown in Scheme 5b. NPALWK, a surrogate peptide identified from PLBL2 (sequence shown in Figure 13a), was separated and quantified with CMS. When the cell was off (Figure 13b), the +2 ion of NPALWK was observed at *m/z* 364.71. After electrolysis (Figure 13c), the oxidation product peaks corresponding to M + 14, M + 16, and M + 32 were observed at *m/z* 371.70, 372.71, and 380.70, respectively. The oxidation yield for NPALWK was measured to be 16.3% by comparing the EIC peak area of *m/z* 364.71 before (Figure 13d) and after oxidation (Figure 13e). Figure 13g shows the electric current peak from oxidation of NPALWK, from which the amount of oxidized NPALWK was calculated to be 0.64 pmol. Therefore, the measured amount of NPALWK was 3.9 pmol. For confirmation, an isotope-labeled peptide NPALW$\hat{K}$(labeled at lysine, ^{13}C6, and ^{15}N4) was purchased and used as an internal standard to quantify NPALWK in the same digest sample. By this traditional isotope dilution method, NPALWK was measured to be 4.1 pmol. Again, the discrepancy of the quantitation results between CMS and IDMS was small (−4.9%). These results indicate that CMS could be used for absolute quantitation of very low abundant proteins (i.e., PLBL2) in the presence of a highly abundant predominant protein (NIST mAb 8671). CMS thus might be used to track the clearance of a specific known HCP under various process conditions after shotgun proteomics identification. No need for standards would make this CMS strategy more suitable to meet the need for rapid quantitation of HCPs after their identification.

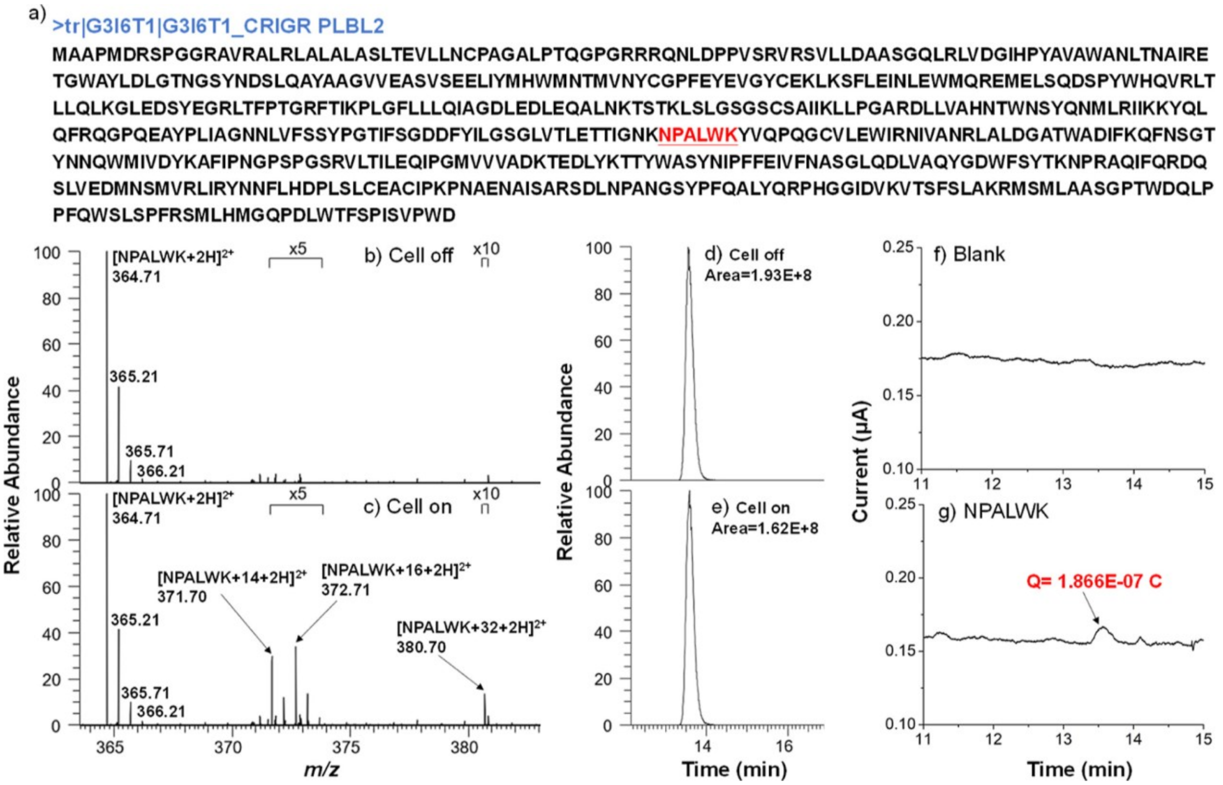
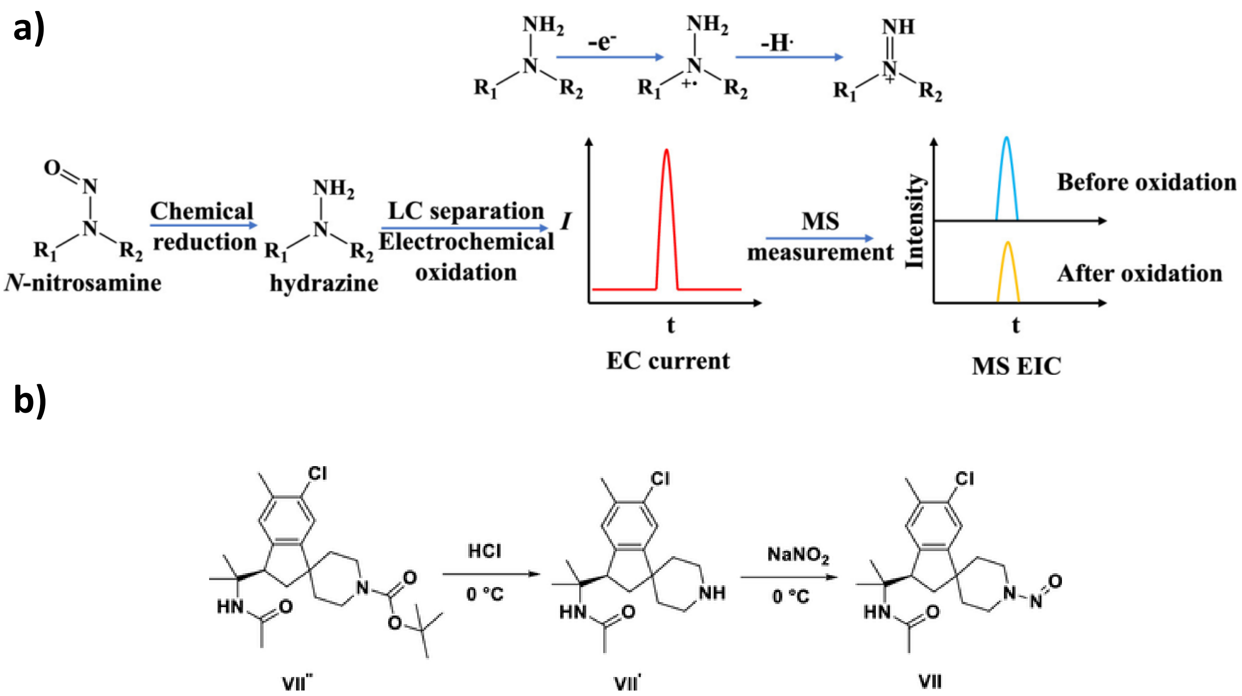


Figure 13. a) Sequence of PLBL2 (the chosen surrogate peptide NPALWK is highlighted in red). MS spectra of NPALWK from the digested sample (mAb/PLBL2 = 200:1) b) when the cell was off and c) when the cell was turned on (applied potential: +1.05 V). The oxidation products of NPALWK were detected at *m/z* 371.70, 372.71, and 380.70. EICs of NPALWK were acquired d) when the cell was off and e) when the cell was turned on (applied potential: +1.05 V). Electric current diagrams were collected from oxidation of f) blank solvent and g) NPALWK. Reprinted with permission from Ref[83].

CMS can also be used for quantifying small molecular drug impurities such as *N*-nitrosamines. Carcinogenic *N*-nitrosamines were recently found in the sartan family of drugs and caused many drug recalls.[99] The detection and quantification of these chemicals is therefore especially important. Methods reported for *N*-nitrosamine quantitation rely on the use of standards and are just applicable to simple *N*-nitrosamines;[100-103] there is an urgent need to quantify *N*-nitrosamines derived from drugs with complicated structures that lack standards. In our study,[60] CMS was applied for the absolute quantitation of six simple *N*-nitrosamines: *N*-nitrosodiethylamine (NDEA), *N*-nitroso-4-phenylpiperidine (NPhPIP), *N*-nitrosodiphenylamine (NDPhA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosodipropylamine (NDPA), and *N*-nitrosopiperidine (NPIP). This quantification resulted in an excellent measurement accuracy (quantitation error ≤1.1%). Briefly, *N*-nitrosamine is first converted into electrochemically active hydrazine via zinc reduction under acidic condition and the resulting hydrazine can then be easily quantified using CMS (Scheme 6a).



Scheme 6. Workflow for absolute quantitation of *N*-nitrosamines by CMS a); Synthesis route of *N*-nitrosamine **VII** b). Reprinted and slightly modified with permission from Ref[60].

In addition, as a demonstration of the method utility, a drug-like *N*-nitrosamine, (R)-N-(2-(6-chloro-5-methyl-1′-nitroso-2,3-dihydrospiro[indene-1,4′-piperidin]-3-yl)propan-2-yl)acetamide (*N*-nitrosamine **VII**), was also synthesized (Scheme 6b) and successfully quantified using CMS at 15 ppb level in a complex formulation matrix, following solvent extraction, *N*-nitrosamine isolation, and reductive conversion to hydrazine **7**. As shown in Figures 14 a and b, the oxidation product ion at *m/z* 348 had increased intensity when +0.3 V potential (vs Ag/AgCl) was applied to WE. The protonated hydrazine **7** ion of *m/z* 350 shown in Figure 14d was smaller by 16.9% than that of the peak shown in Figure 14c, suggesting the oxidation yield to be 16.9%. On the other hand, the oxidation current peak was detected, as shown in Figure 14f (Figure 14e shows the background current diagram for the blank solvent sample under the same +0.3 V potential as a contrast). On the basis of the integration of the current peak area, the amount of the oxidized **7** was calculated to be 10.0 pmol (about 3.5 ng). Considering the extraction and isolation yield (43.9%), the side product ratio (4.4%), the reduction yield (58.1%), and the dilution factor 10, the CMS measured amount of *N*-nitrosamine **VII** was 410 pmol on the average from a triplicate measurement. The measurement error was −1.1% compared with the theoretical amount of 412 pmol. The standard-free feature of CMS makes it a simple and powerful approach for *N*-nitrosamine absolute quantitation. It has great potential for analysis of other drug impurities or metabolites, which could significantly impact pharmaceutical quality control, food safety analysis, and water treatment applications in the future. Although zinc-based chemical reduction shows some limitations, especially for controlling the *N*-nitrosamine and zinc reaction ratio, other reductants with better selectivity could overcome this issue. Besides, modified electrodes could be used for directly reducing or oxidizing N-nitrosamines, potentially simplifying the quantitation process.

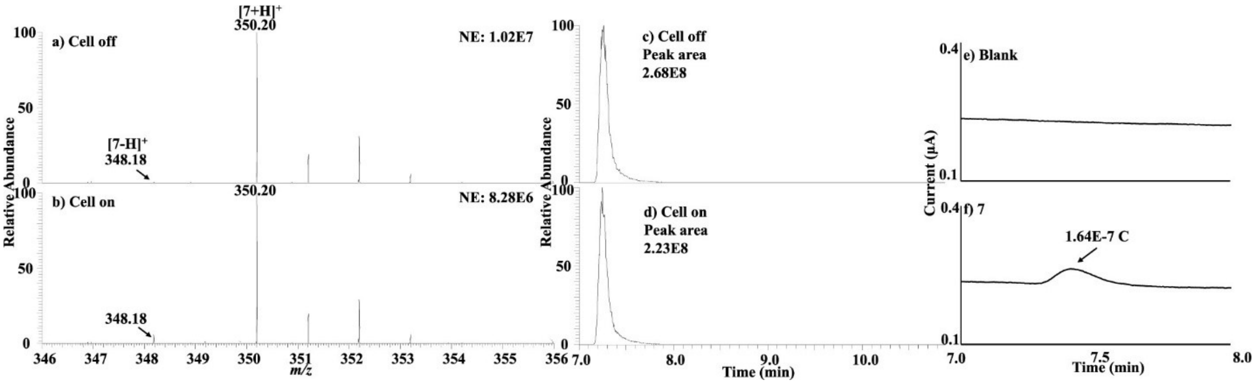


Figure 14. ESI-MS spectra of hydrazine **7** (from reduction of *N*-nitrosamine **VII** in the test sample with drug matrix) when the applied potential was a) 0 V and b) +0.3 V. EIC of **7** at *m*/*z* 350 was recorded when the applied potential was c) 0 V and d) + 0.3 V (vs Ag/AgCl). Electric current responses were shown due to the oxidation of e) a blank solvent and f) **7**. Reprinted with permission from Ref[60].

4. Challenges and future perspectives

To summarize, the method of CMS is surveyed in this review by addressing applications ranging from small molecule metabolites to large molecule biotherapeutics in a detailed manner. Standard-free CMS is well suitable for the absolute quantitation of target molecules for which the generation of standards is often difficult or not available, such as small drug molecules with complicated structures and peptides with PTMs like phosphopeptides and deamidated peptides. No need for standards and calibration curves would significantly shorten the process of method development compared to traditional quantitation strategies. It enables the fast-screening analysis during drug discovery as well as the rapid quantification and monitoring of high-risk drug impurities for timely process optimization and quality control. Moreover, coupling CMS with versatile direct ambient sampling methods like CPSI-MS makes it feasible for on-site quantitation and forensic analysis. The selective redox reaction could be achieved by specific modification (e.g., enzymes, antibodies, aptamers, lectins, or molecularly imprinted polymer cavities) on WE or simply by varying the electrochemical redox potential.

However, although the above CMS publications showed promising results, we have to admit that there are some bottlenecks in terms of the current CMS format which need more development to be widely used. One major challenge is that real biological samples can sometimes be very complex. Although combining CMS with LC separation has been used in our previous work, resolving all electroactive target analytes as well as the interferences would still be challenging due to the fact that unlike MS, the overlapped current response cannot be differentiated. Thereby, for extremely complicated clinical samples such as biomarker quantitation in human body fluids and large molecule bioanalysis/DMPK, pre-treatment steps prior to CMS including immune-affinity enrichment, protein/peptide fractionation, or multidimensional online separation would be helpful and make CMS powerful tool to reduce time and cost, especially for large-scale quantitation. Another drawback of CMS is that only the analytes with electroactive functional groups could be quantified, thus hindering the expansion of CMS. To address this, we are developing electrochemically active tags which can covalently link to non-active target analytes such as peptides that lack Y, W, or C amino acid residues. For example, this tag can be designed with an amine-reactive group that specifically labels lysine residue, allowing the absolute quantitation of any tryptic peptides. We believe that this can make CMS an excellent quantitation tool for a very broad range of analytes regardless of the electrochemically reactive groups.

In terms of quantitation sensitivity, peptides such as WGG can be well quantified at an amount as low as 75 fmol. Further possible improvement in CMS quantitation sensitivity could be achieved using microelectrode instead of currently used millimeter sized electrodes. We believe this standard-free quantitation approach has the potential for more interesting applications in the near future.

Acknowledgements

This work was supported by NSF (CHE-2203284) and NIH (1R21GM148874-01).

Abbreviations and Acronyms

AQUA	Absolute quantification strategy
CAD	Charged aerosol detector
CE	Counter Electrode
CHO	Chinese Hamster Ovary
CMS	Coulometric Mass Spectrometry
CPSI	Conductive Polymer Spray Ionization
DA	Dopamine
DESI	Desorption Electrospray Ionization
DQ	Dopamine o-Quinone
EC	Electrochemistry
EIC	Extracted Ion Chromatogram
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporative light scattering detector
ESI	Electrospray Ionization
FI	Flow injection
GSH	Glutathione
GSSG	Glutathione disulfide
HCP	Host Cell Protein
HESI	Heat Electrospray Ionization
HPLC	High Performance Liquid Chromatography
5-HT	5-Hydroxytryptamine
ICAT	Isotope-Coded Affinity Tags
ICPL	Isotope-Coded Protein Labeling
iTRAQ	isobaric Tags for Relative and Absolute Quantitation
LC	Liquid Chromatography
mAb	Monoclonal Antibody
MS	Mass Spectrometry
MWCNT	Multi-walled Carbon Nanotubes
NE	Norepinephrine
nESI-MS	nano Electrospray Ionization Mass Spectrometry
PET	Polyethylene Terephthalate
PLBL2	Phospholipase B-like 2 Protein
PSAQ	Protein standard absolute quantification
PTFE	Polytetrafluoroethylene
PTM	Post-translational modification

QconCAT	Quantification conCATamer
RE	Reference electrode
RI	Refractory Index
RN	Rutin
RQ	Rutin Quinone
SILAC	Stable Isotope Labeling by Amino acids in Cell culture
SRM	Selected Reaction Monitoring
TDP	Tetradecapeptide
UA	Uric Acid
UPLC	Ultra Performance Liquid Chromatography
UV-Vis	Ultraviolet Visible
W	Tryptophan
WE	Working Electrode
Y	Tyrosine

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