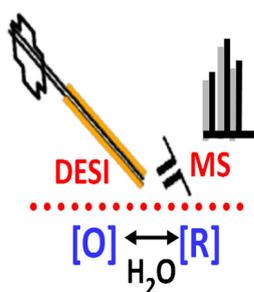


Online Investigation of Aqueous-Phase Electrochemical Reactions by Desorption Electrospray Ionization Mass Spectrometry

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Abstract. Electrochemistry (EC) combined with mass spectrometry (MS) is a powerful tool for elucidation of electrochemical reaction mechanisms. However, direct online analysis of electrochemical reaction in aqueous phase was rarely explored. This paper presents the online investigation of several electrochemical reactions with biological relevance in the aqueous phase, such as nitrosothiol reduction, carbohydrate oxidation, and carbamazepine oxidation using desorption electrospray ionization mass spectrometry (DESI-MS). It was found that electroreduction of nitrosothiols [e.g., nitrosylated insulin B (13-23)] leads to free thiols by loss of NO, as confirmed by online MS analysis for the first time. The characteristic mass shift of 29 Da and the reduced intensity provide a quick way to identify nitrosylated species. Equally importantly, upon

collision-induced dissociation (CID), the reduced peptide ion produces more fragment ions than its nitrosylated precursor ion (presumably the backbone fragmentation cannot compete with the facile NO loss for the precursor ion), thus facilitating peptide sequencing. In the case of saccharide oxidation, it was found that glucose undergoes electro-oxidation to produce gluconic acid at alkaline pH, but not at neutral and acidic pHs. Such a pH-dependent electrochemical behavior was also observed for disaccharides such as maltose and cellobiose. Upon electrochemical oxidation, carbamazepine was found to undergo ring contraction and amide bond cleavage, which parallels the oxidative metabolism observed for this drug in leucocytes. The mechanistic information of these redox reactions revealed by EC/DESI-MS would be of value in nitroso-proteome research and carbohydrate/drug metabolic studies.

Key words: Electrochemistry, Mass spectrometry, Desorption electrospray ionization, Nitrosothiol reduction, Carbohydrate oxidation

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Introduction

Online coupling of electrochemistry (EC) with mass spectrometry (MS) is powerful for probing redox reaction mechanisms. This is because MS can serve as a highly sensitive detector providing molecular weight and structural information for intermediates and stable products of electrochemical reactions. Various EC/MS techniques have been designed, using different ionization methods such as electron impact (EI) [1],

thermospray (TS) [2, 3], fast atom bombardment (FAB) [4], and electrospray ionization (ESI) [5–8]. However, the online monitoring of electrolysis in aqueous solution has been very limited [1, 2, 9, 10] and organic solvent was often doped into sample solution for electrolysis in order to enhance ionization efficiency in the previous EC/MS studies. As electrochemical reactions are traditionally carried out in aqueous solution (for the reason that water is an ideal medium for dissolving electrolyte salts and maintaining solution conductivity) [11], and biomolecules occur in aqueous media, it is necessary to extend the EC/MS investigation to aqueous electrochemical reactions.

Ambient ionization methods [12–14] such as desorption electrospray ionization (DESI) [15] introduced by Cooks et al. are among a family of the latest ionization methods that can be used to directly ionize samples with no or little

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preparation under ambient conditions. With the advent of ambient ionization methods, EC/MS coupling has been diversified, using liquid sample DESI [10, 16–22], nanospray DESI [23], or flow atmospheric pressure afterglow (FAPA) ionization [24]. In particular, liquid sample DESI was developed for directly analyzing liquid samples in our and other laboratories [16, 25–28]. The ionization occurs via the interaction of liquid sample with charged droplets generated by the DESI spray and the resulting ions are collected and analyzed by a mass spectrometer. It is useful in directly analyzing samples including large proteins/protein complexes from their native environments [16, 29–34]. Furthermore, it is possible to use liquid sample DESI-MS for studying fast reaction kinetics in submillisecond time resolution [35] or coupling with liquid chromatography (LC) [18, 36–38], microfluidics [26], and microextraction [39]. In terms of combination with EC [10], it was shown that liquid sample DESI-MS can be used to capture transient intermediates [20]. The combined EC/DESI-MS (i.e., the combination of EC with liquid sample DESI-MS) is also useful for the structural analysis of disulfide bond-containing proteins in either top-down [19] or bottom-up approaches [17, 21] and for probing protein 3D-structures and protein–protein interactions in combination with cross-linking chemistry [22].

In this study, we investigated several electrochemical reactions in the aqueous phase using EC/DESI-MS, including nitrosothiol reduction as well as carbohydrate and carbamazepine oxidation. These redox reactions were selected because of their biological importance (see detailed discussion below) and the fact that their reaction mechanisms have not been examined using online MS techniques yet. For the first time, it is shown that nitrosothiols such as *S*-nitroso-*N*-acetyl-penicillamine (SNAP), *S*-nitrosoglutathione (GSNO), and nitrosylated insulin B (13-23) can be electrochemically reduced into free thiols via NO loss and detected online by MS. Also, pH-dependent electrochemical reactivities are observed for saccharide oxidation. In addition, carbamazepine exhibits an electrochemical oxidation pathway that is similar to the metabolic process reported in leukocytes [40]. In addition to providing these mechanistic information, the study also uncovers the related analytical applications (e.g., for quickly identifying and sequencing nitrosylated peptides) and shows the applicability of DESI-MS for online analysis of electrochemical reactions in aqueous solution, at different pHs.

Experimental

Chemicals

S-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetyl-penicillamine (SNAP), ubiquitin from bovine erythrocytes, trypsin from porcine pancreas, formic acid, 9-acridine carboxaldehyde, acridine, acridone, glucose, maltose, and cellobiose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrosylated insulin B (13-23) was purchased from Protea (Morgantown, WV, USA). Acetic acid and HPLC-grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA) and GFS Chemicals (Columbus, OH, USA), respectively. Deionized

water used for sample preparation was obtained using a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA, USA).

Digestion of Ubiquitin

Digestion of ubiquitin were carried out using TPCK-treated trypsin with a ratio of 1:50 (enzyme/protein) in 50 mM ammonium bicarbonate aqueous solution overnight at 38°C incubation to finish digestion.

Online EC/DESI-MS Apparatus

A home-built apparatus for coupling a thin-layer electrochemical flow cell with either a Waters Xevo QToF mass spectrometer (Milford, MA, USA) or a Thermo Scientific LCQ DECA mass spectrometer (San Jose, CA, USA) by liquid DESI was used (apparatus shown in Scheme 1S, Supporting Information) and described previously in detail [20]. The Reactor Cell (Antec BV, Zoeterwoude, The Netherlands) equipped with a gold disc working electrode (i.d. 8 mm) was used for nitrosothiol reduction and saccharide oxidation. A HyREF electrode was used as the reference electrode, and carbon-loaded PTFE was used as a counter electrode. The μ -PrepCell (Antec BV, Zoeterwoude, The Netherlands) equipped with a glassy carbon working electrode (30 × 12 mm²) was employed for carbamazepine oxidation. A HyREF electrode was used as the reference electrode and titanium was used as the auxiliary electrode. The electrochemically oxidized or reduced products flowed out of the cell via a short piece of fused silica connection capillary (i.d. 0.1 mm, length 4.0 cm) and underwent interactions with the charged microdroplets from the DESI spray for ionization. The capillary outlet was placed about 1 mm downstream from the DESI spray probe tip and kept in line with the DESI sprayer tip and the mass spectrometer's inlet. For the positive ion detection mode, the spray solvent for DESI was methanol/water (1:1 by volume) containing 1% acetic acid, and a high voltage of 5 kV was applied to the spray probe. For the negative ion mode used for saccharide oxidation monitoring, the spray solvent for DESI was methanol/water (1:1 by volume) containing 1% ammonia, and a high voltage of –5 kV was applied to the spray probe. Injection flow rates for both the DESI spray solvent and the sample solution for electrolysis were 10 μ L/min.

In this study, all of the sample solutions except carbamazepine were prepared using water as solvent with the pH adjusted by HCOOH, NH₄OH, or NaOH. In the case of carbamazepine, because of its low solubilities in water, stock solutions had to be prepared in methanol and diluted using water. However, the final content of methanol in the sample solutions used for electrolysis was negligible (less than 2% by volume).

Results and Discussion

Nitrosothiol Reduction

Nitrosothiol compounds (RSNOs) are attracting increasing attention because of their important biological activities [41].

Most of their biological functions are triggered via the cleavage of the S–N bond to release nitric oxide, which plays a significant regulatory role in biological systems [42]. RSNOs are also potentially implicated in the post-translational modification of protein cysteine residues [41, 43]. Recently, the homolytic cleavage of S–N bonds of nitrosothiol ions upon gas-phase dissociation was used to generate sulfur-based radical ions, the fragmentation behaviors and reactivities of which were extensively investigated [44–46]. Among various methods developed for analyses of RSNOs, electrochemical detection based on reduction of RSNOs is sensitive and selective [47–49]. However, it is difficult to identify the reaction products using only an electrochemical detector. In this regard, MS could provide more direct evidence for the reaction mechanism elucidation. In this work, the electrochemical reduction of three nitrosothiols, including SNAP, GSNO, and nitrosylated insulin B (13-23), followed by online mass spectrometric detection were first investigated, using our EC/DESI-MS method.

Figure 1a shows the DESI-MS spectrum acquired when a solution of 100 μM SNAP in H_2O containing 0.5% HCOOH flowed through the thin-layer electrochemical cell with no potential being applied. The protonated SNAP was detected at m/z 221, along with its in-source CID fragment ion, the sulfur radical ion of m/z 191, as a result of NO loss. This NO loss is characteristic for the protonated nitrosothiol, which was utilized to produce sulfur based radical ions [44–46]. As shown in Figure 1b, when a negative potential (-1.4 V) was applied to the gold working electrode, the intensity of m/z 221 decreased significantly, while the abundance of the ion at m/z 192,

corresponding to the electrochemical reduction product of SNAP, increased. The mass shift of 29 Da is due to NO loss and subsequent hydrogen addition, which confirms the previously proposed electrochemical reduction mechanism for nitrosothiol compounds as shown in Scheme 1 [50]. Upon CID, m/z 221 mainly dissociates to the sulfur radical cation at m/z 191 by loss of NO (structure shown in Figure 1S-a, Supporting Information), which is typical for nitrosothiol compounds because of the labile nature of S–N bond. CID of m/z 191 generated from in-source CID of the protonated SNAP shows the radical induced loss of $(\text{CH}_3)_2\text{C}=\text{S}$ to give rise to m/z 117 (Figure 1S-b, Supporting Information). In contrast, CID of the reduced product ion at m/z 192 mainly leads to loss of small molecules such as H_2O , $\text{CH}_2=\text{C}=\text{O}$, and HCOOH to form fragment ions at m/z 174, 150, and 146, respectively. The results suggest that the reduced product ion has a different dissociation behavior from both the intact precursor ion and the sulfur radical cation resulting from the precursor ion by loss of NO.

Nitrosothiol peptide GSNO was also examined using EC/DESI-MS. This compound is important for NO metabolism and mediates many signaling pathways via protein post-translational modification [49]. Figure 1c shows the DESI-MS spectrum acquired when a solution of 100 μM GSNO in H_2O containing 0.5% HCOOH flowed through the thin-layer electrochemical cell with no potential applied. Similar to SNAP, the protonated GSNO is detected at m/z 337, along with its in-source CID fragment ion m/z 307 by loss of NO (structure shown as inset of Figure 1c). As shown in Figure 1d, when a -1.4 V was applied to the gold working electrode, m/z

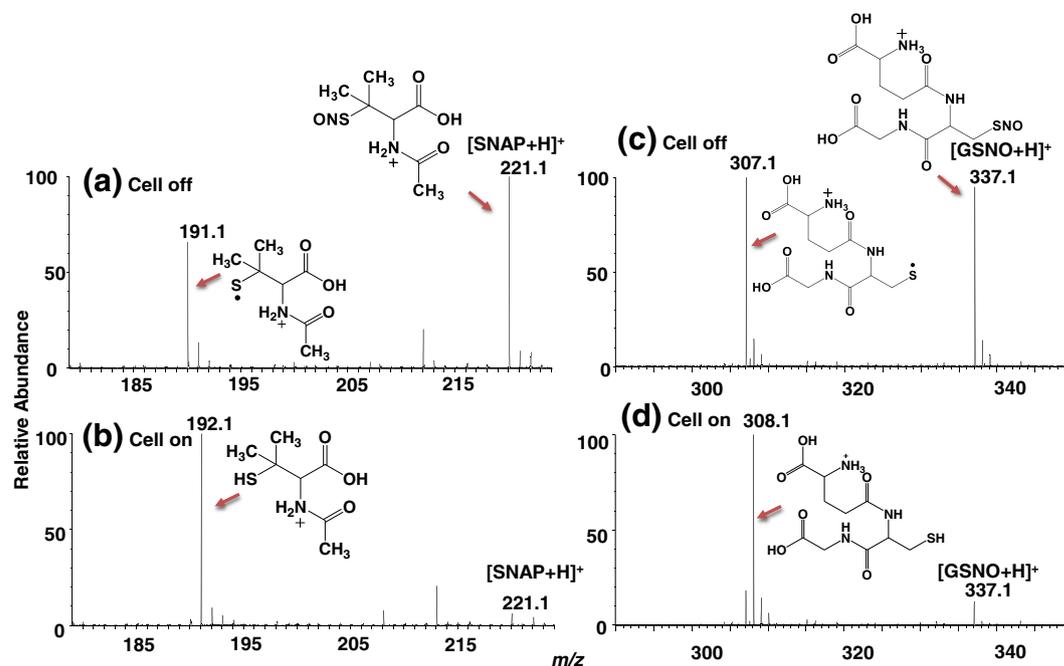
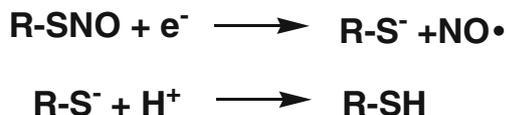


Figure 1. DESI-MS spectra acquired when a solution of (a) 100 μM SNAP and (c) 100 μM GSNO in water containing 0.5% HCOOH flowed through the thin-layer electrochemical cell with no potential applied. DESI-MS spectra acquired when a solution of (b) 100 μM SNAP and (d) 100 μM GSNO in water containing 0.5% HCOOH flowed through the thin-layer electrochemical cell with an applied potential of -1.4 V



Scheme 1. Electrochemical reduction mechanism for S-nitrosothiol compounds

337 decreased considerably while the protonated glutathione (GSH, m/z 308) was produced, as a result of electroreduction of GSNO. The sulfur radical cation m/z 307 is detected as the predominant fragment ion of the protonated GSNO (m/z 337) by characteristic NO loss (Figure 2S-a, Supporting Information). CID of m/z 307 shows abundant fragment ion $[\text{b}_2\text{-H}]^+$ (m/z 232) by loss of glycine and m/z 289 and 271 attributable to water losses (Figure 2S-b, Supporting Information). Another fragment ion m/z 202 arises from m/z 289 because of further loss of $\text{CH}_2=\text{C}(\text{NH}_2)\text{COOH}$. The same fragmentation behavior was reported [51]. Electroreduction of GSNO generates free thiol species GSH and the protonated reduction product GSH produces b_2 and y_2 ions upon CID (Figure 2S-c, Supporting Information), which agrees with the MS/MS behavior of the protonated GSH generated by ESI of authentic GSH compound (data not shown), and confirms the formation of free thiol species after EC reduction. In this case of the nitrosylated tripeptide GSH, CID of the protonated GSNO does not give much structure information as NO loss is the predominant dissociation pathway, whereas the protonated reduction product GSH gives rise to more structurally informative fragment ions upon CID. This is also true for other peptides [e.g., nitrosylated insulin B (13-23) shown below], and shows the potential application of the online EC/DESI-MS method in structure analysis of nitrosylated peptides. Again, the characteristic mass shift of 29 Da (attributable to NO loss and H addition) between the protonated intact GSNO and the protonated reduction product GSH upon electrolysis indicates a simple and fast way to identify nitrosylated species.

Another nitrosylated peptide, nitrosylated insulin B (13-23), was examined using EC/DESI-MS. Insulin B (13-23) peptide is a fragment of the insulin B chain, which contains 11 amino acids, and its seventh amino acid cysteine is nitrosylated (sequence shown in Figure 2a). Figure 2b shows the DESI-MS spectrum acquired when a solution of 10 μM nitrosylated insulin B (13-23) in water containing 0.5% HCOOH flowed through the electrochemical cell with no potential applied. Only doubly charged peptide ion $[\text{M} + 2\text{H}]^{2+}$ was detected at m/z 619.8 with high intensity. When a negative potential of -2 V was applied to the working electrode to trigger electroreduction, m/z 619.8 decreased and a new doubly charged product was detected at m/z 605.3 (Figure 2c). Again, the mass difference of the peptide before and after reduction is 29 Da, indicating NO loss and H addition.

Figure 2d shows the acquired CID MS/MS spectrum of the doubly charged peptide ion $[\text{M} + 2\text{H}]^{2+}$ at m/z 619.8, which again shows the facile NO loss to produce sulfur radical ion at m/z 604.8. MS³ of this sulfur radical cation (Figure 2e) shows

HS loss (m/z 588.3), which is induced by the sulfur radical and consistent with the previous literature report [52]. Besides, additional fragment ions such as b_4 , $b_{10} + \text{H}_2\text{O}^{2+}$, y_6 , b_6 , y_7 , and y_9 are also detected (Figure 2e). The electrochemical reduction of nitrosylated insulin B (13-23) generates the native peptide, and the CID MS/MS spectrum of the reduced production (m/z 605.3) is shown in Figure 2f. Obviously, there are more observed fragment ions resulting from peptide backbone dissociation in comparison to CID of either the nitrosylated peptide ion or the sulfur radical peptide ion. The observed fragment ions, including b_3 , b_4 , b_5 , b_6 , b_7 , b_8 , b_9 , b_{10}^{2+} , y_2 , y_3 , y_4 , y_5 , y_6 , y_7 , y_8 , y_9 , and y_{10}^{2+} cover all of the backbone cleavages from which the peptide full sequence can be determined. The enhanced peptide sequence coverage upon CID for the electroreduced product ion is due to the absence of NO compared with the nitrosylated peptide ion. Although CID of the sulfur radical peptide ion also produces some fragment ions from backbone cleavages (Figure 2e), the radical induced fragmentation via HS loss dominates the dissociation pathway so that less fragment ions are observed in comparison to the reduced product ion.

Our results show that EC/DESI-MS method is advantageous in quickly identifying the nitrosylated species based on their response to electroreduction (including the characteristic mass decrease of 29 Da and intensity drop upon electroreduction) and is helpful to peptide sequencing, which suggests its potential application in nitroso-proteome research. Indeed, the response to electrolysis can serve as a simple and fast way to identify nitrosylated peptides from other non-nitrosylated peptides in a mixture. As a demonstration for this application, a mixture of peptides from trypsin-digested ubiquitin and the nitrosylated insulin B (13-23) was subjected to EC/DESI-MS analysis (Figure 3S, Supporting Information). Before electrolysis, the DESI-MS spectrum (Figure 3S-a, Supporting Information) shows singly and doubly charged peptides from ubiquitin digestion as well as the doubly charged nitrosylated insulin B (13-23) at m/z 619.8. With a reduction potential applied to the cell (Figure 3S-b, Supporting Information), only the nitrosylated peptide ion at m/z 619.8 displays a large relative intensity change (decreased by 61%; using non-nitrosylated peptide ion $[\text{LIFAGK} + \text{H}]^+$ m/z 648.4 as the reference), whereas other nitroso-free peptide ions have little relative intensity change from -2% to $+9\%$ (Table 1S, Supporting Information) probably because of the MS response fluctuation. Meanwhile, the reduced peptide at m/z 605.3 with a mass shift of 29 Da from the precursor ion was observed as a result of NO loss and H addition from the nitrosylated insulin B (13-23).

Carbohydrate Oxidation

Electrochemical oxidation of carbohydrates such as glucose has been of great interest over the years and shows potential applications in areas including blood sugar sensor and fuel cell research [53], but it has not been examined using online MS techniques [54–57]. Previous electrochemical studies show

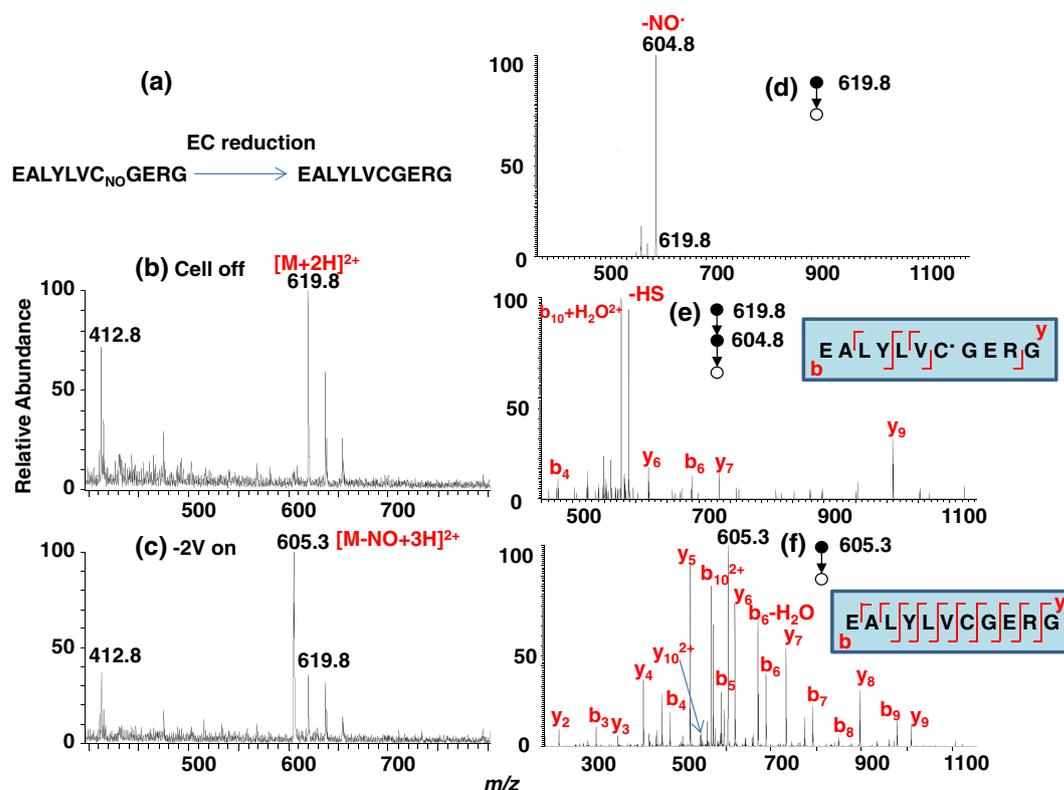


Figure 2. (a) Scheme showing peptide before and after EC reduction; DESI-MS spectra acquired when a solution of 10 μM nitrosylated insulin B (13-23) in water containing 0.5% HCOOH flowed through the electrochemical cell (b) with no potential applied and (c) with applied potential of -2 V. CID MS/MS spectra of (d) doubly charged nitrosylated insulin B (13-23) $[\text{M} + 2\text{H}]^{2+}$ (m/z 619.8) before reduction, (e) the peptide radical ion at m/z 604.8, and (f) doubly charged reduction product at m/z 605.3

that glucose is poorly oxidized on gold surface in acidic solution because of the low concentration of OH adsorbed on a gold electrode at low pH [54], but a gold electrode is attractive for glucose oxidation in neutral and alkaline solutions as it shows more negative oxidative potential for glucose and higher oxidation current compared with other metal electrodes [57]. In this study, the direct electrochemical oxidation of glucose in alkaline solution with a gold electrode was performed and monitored using online DESI-MS. Figure 3a shows the DESI-MS spectrum acquired when a solution of 500 μM glucose in water containing 2 mM NaOH (pH 11) flowed through the thin-layer electrochemical cell with no potential being applied. Without the cell energized, only the deprotonated glucose at m/z 179 was detected. When an oxidation potential of 1.5 V was applied to the working electrode of the cell, the deprotonated oxidation product, gluconic acid, was observed at m/z 195. The CID of this oxidation product ion was compared with the authentic gluconic acid sample, and the result is shown in Figure 3c and d. An identical fragmentation pattern showing consecutive losses of H_2O and HCHO was observed. This result confirms the structure of the electrogenerated glucose oxidation product as gluconic acid. Acidic and neutral pH solvents were also tested, and the results are shown in Figure 3e–h. In stark contrast with alkaline pH, no oxidation product was detected at either acidic or neutral pH, which confirms that the lower pH does not favor glucose electro-oxidation.

Two other disaccharides, maltose and cellobiose, in alkaline solution were also examined using our online EC/DESI-MS method, and the results are shown in Figure 4S (Supporting Information). As shown in Figure 4S-a, without the cell turned on, only the deprotonated maltose $[\text{maltose-H}]^-$ at m/z 341 was observed. With 1.5 V being applied to the cell, the deprotonated maltobionic acid (m/z 357), the major oxidation product of maltose, was detected successfully. CID of m/z 357 is shown in Figure 4S-c, in which the losses of $\text{C}_5\text{H}_{12}\text{O}_4$, $\text{C}_5\text{H}_{12}\text{O}_6$, $\text{C}_6\text{H}_{12}\text{O}_6$, water, and further losses of HCHO and CO were observed. Another disaccharide cellobiose was also tested and gave results similar to those observed with maltose (Figure 4S-d, S-e, and S-f, Supporting Information).

Note that these experiments represent some of the very few reported examples of using online MS techniques to investigate electrochemical reactions in alkaline pH conditions [58–60] (one reason for this is that alkaline pH does not favor the formation of positive ions by MS). In the case of using DESI-MS, the electrolysis pH does not affect the ionization as the DESI spray pH could be adjusted depending on the ionization mode in need [38].

Carbamazepine Oxidation

Carbamazepine electro-oxidation in water was also examined using EC/DESI-MS in our study. Carbamazepine is a tricyclic

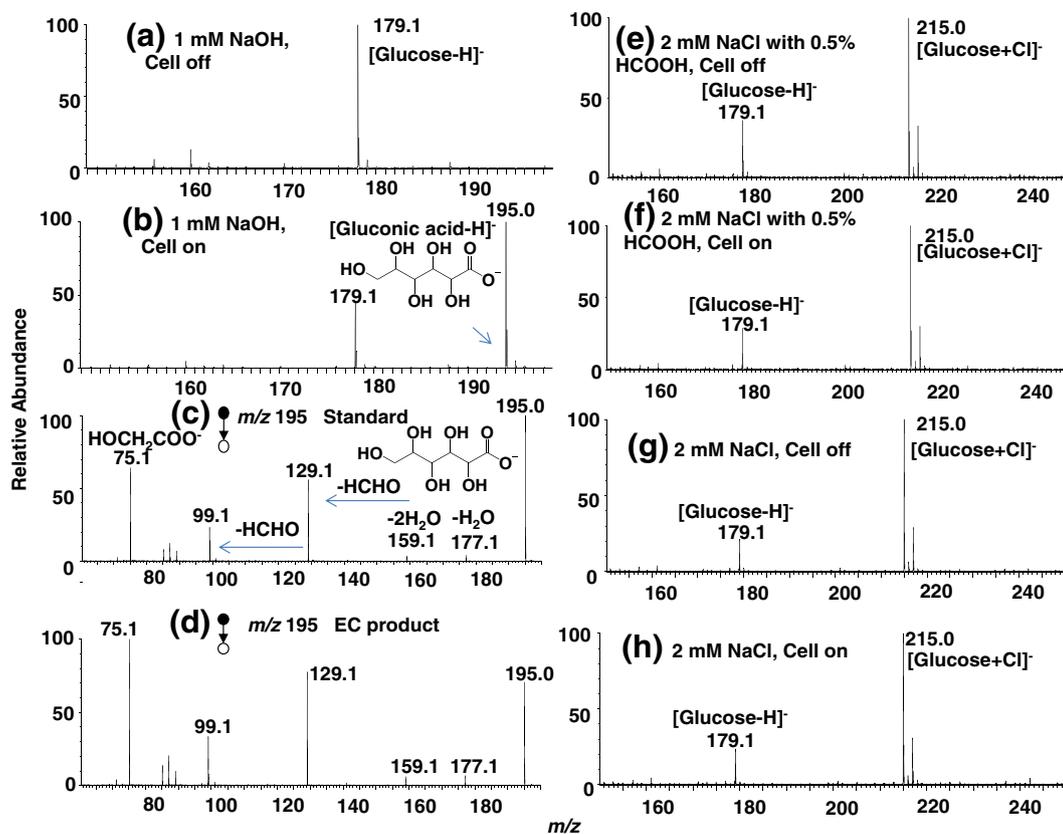


Figure 3. DESI-MS spectra acquired when a solution of 500 μM glucose in water containing 1 mM NaOH flowed through the thin-layer electrochemical cell at a rate of 5 $\mu\text{L}/\text{min}$ with an applied potential of (a) 0.0 V, and (b) 1.5 V. CID MS/MS spectra of m/z 195 generated (c) by ESI of authentic gluconic acid sample, and (d) by electro-oxidation of glucose. DESI-MS spectra acquired when a solution of 500 μM glucose in water containing 2 mM NaCl in 0.5% HCOOH with applied potential of (e) 0.0 V, and (f) 1.5 V; DESI-MS spectra acquired when a solution of 500 μM glucose in water containing 2 mM NaCl with applied potential of (g) 0.0 V, and (h) 1.5 V

compound used as an antiepileptic drug in the treatment of epilepsy and bipolar disorders [61]. More than 30 carbamazepine metabolites have been detected in the human body, some of which are pharmacologically active or genotoxic [62]. As shown in Scheme 2S-a (Supporting Information) [63], carbamazepine is

mainly metabolized via three pathways in the liver. The primary route is the production of the carbamazepine-10,11-epoxide; a second route is the formation of hydroxylated compounds. The third route is the formation of iminostilbene. While in the leucocytes with strong oxidizing properties, carbamazepine

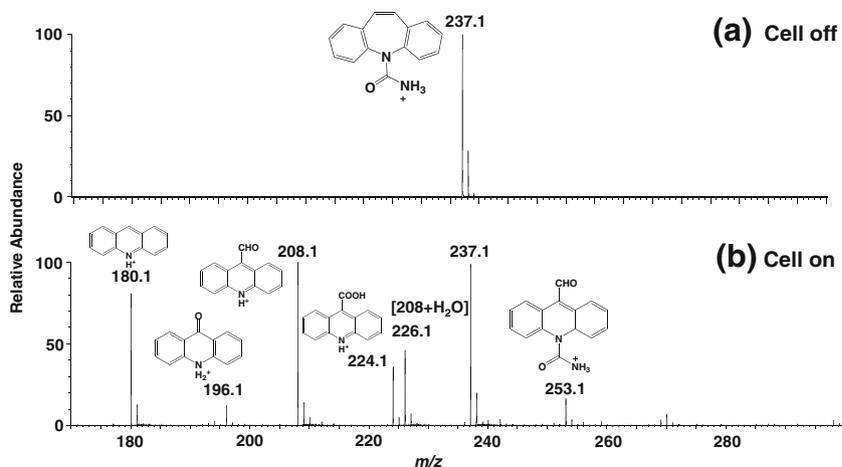
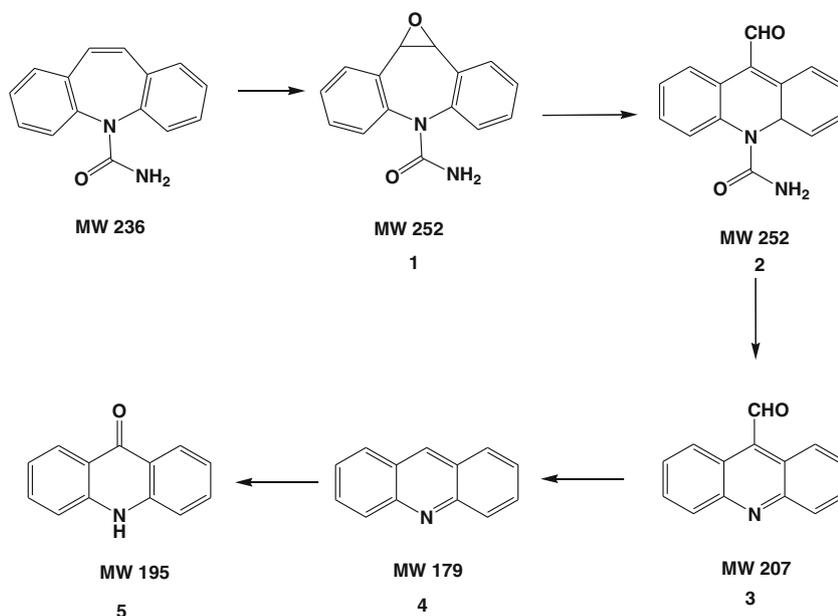


Figure 4. DESI-MS spectra acquired when a solution of 200 μM carbamazepine in 20 mM HCOONH_4 in H_2O flowed through the thin-layer electrochemical cell at a rate of 40 $\mu\text{L}/\text{min}$ with an applied potential of (a) 0.0 V, and (b) 1.3 V



Scheme 2. Proposed electrochemical oxidation pathway for carbamazepine

could be converted to various metabolites including an intermediate aldehyde, 9-acridine carboxaldehyde, acridine, and

acridone (Scheme 2S-b, Supporting Information) [40, 63]. The determination of carbamazepine and its metabolites in biological

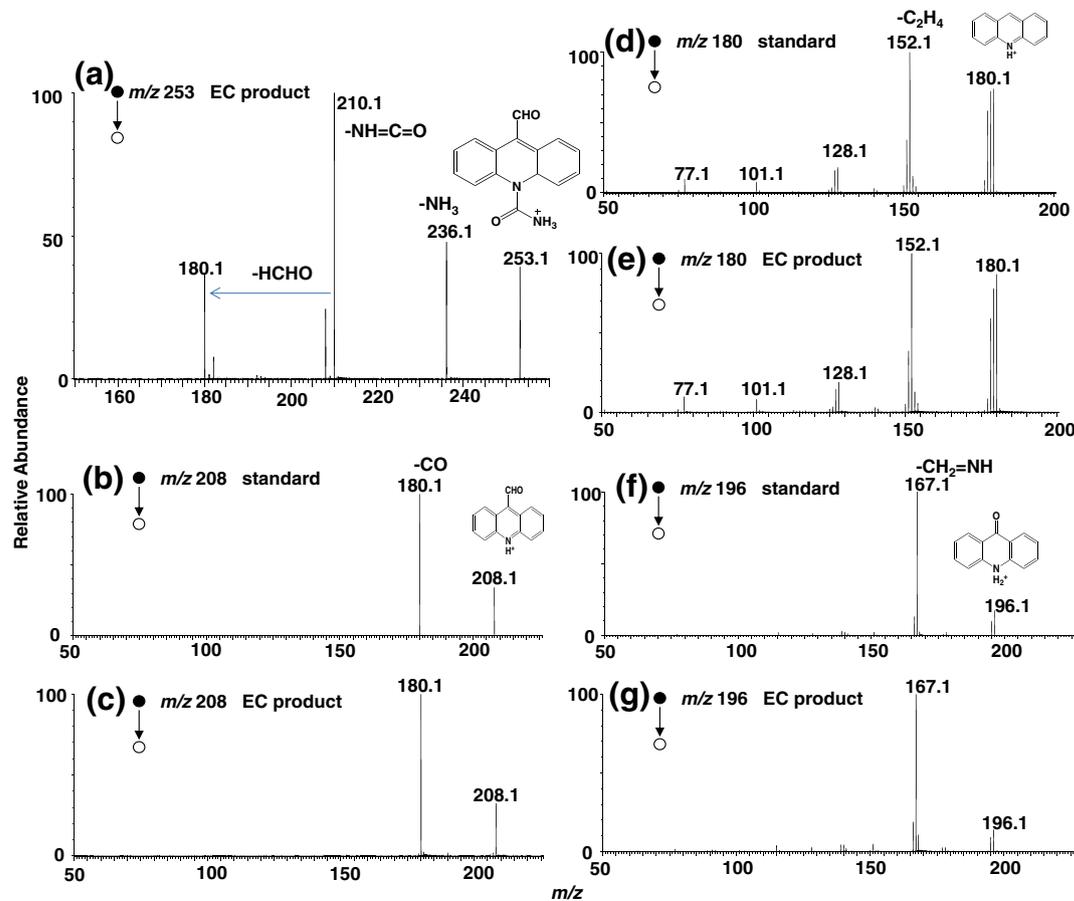


Figure 5. CID MS/MS spectra of the protonated electrochemical oxidation products of (a) m/z 253, (c) m/z 208, (e) m/z 180, (g) m/z 196, and the protonated standard compounds of (b) 9-acridine carboxaldehyde (m/z 208), (d) acridine (m/z 180), and (f) acridone (m/z 196)

fluids using chromatography [64, 65] and electrochemical detection [61, 66, 67] was reported. However, these methods lack chemical specificity. EC/MS is a valuable instrumental method to mimic metabolic pathways of drug compounds since most metabolism pathways involve redox reactions. Electrochemical simulation is a fast method and can potentially provide metabolic information comparable to an in vitro study [68–71]. Although most EC/MS work use organic solvent to facilitate MS detection, it would make more sense to use water as the electrolysis solvent for mimicking drug metabolism in aqueous media. In a previously reported study of the electrochemical behavior of carbamazepine, initially a one-electron oxidation of carbamazepine was observed to form a radical that dimerizes rapidly; the dimer itself can then undergo further oxidation [72].

Figure 4 shows the experimental results for online EC/DESI-MS monitoring of carbamazepine electro-oxidation. First, EC/DESI-MS was tested for a 200 μM carbamazepine in 20 mM HCOONH_4 in H_2O (pH 6.8) using a flow rate of 40 $\mu\text{L}/\text{min}$. With the electrochemical cell not energized, only the protonated carbamazepine (m/z 237) was detected with high intensity (Figure 4a). When a positive potential of 1.3 V was applied to the working electrode of the cell, the intensity of m/z 237 ion decreased and other ions of m/z 180, 196, 208, 224, 226, and 253 were detected (Figure 4b).

The electro-oxidation mechanism of carbamazepine is proposed in Scheme 2. First, carbamazepine undergoes epoxidation to generate carbamazepine-10,11-epoxide **1**, which isomerizes to an intermediate aldehyde **2** via ring contraction. The detected ion of m/z 253 appears to be unstable as it could not be detected with lower sample injection flow rate through electrochemical cell during the experiment. This excludes the possibility that the observed m/z 253 is from the stable species carbamazepine-10,11-epoxide, which is commercially available. Instead, we propose that it is the protonated intermediate aldehyde **2**. CID of m/z 253 yields the fragment ions of m/z 236, 210, and 180 by consecutive losses of NH_3 , NHCO , and HCHO , consistent with the assigned structure (Figure 5a). As proposed in Scheme 2, loss of the CONH_2 substituent from **2** affords 9-acridine carboxaldehyde **3** (detected at m/z 208). Subsequent loss of CO gives rise to acridine **4** (detected at m/z 180). Acridine can then be further oxidized to form the corresponding acridone **5** (detected at m/z 196). The comparison of MS/MS spectra between authentic compounds and the assigned products, 9-acridine carboxaldehyde, acridine and acridone, is also shown in Figure 5. The protonated 9-acridine carboxaldehyde (m/z 208) dissociates into m/z 180 by loss of CO (Figure 5b and c). The protonated acridine (m/z 180) dissociates into m/z 152 by loss of C_2H_4 . CID of the acridone ion (m/z 196) yields fragment ion of m/z 167 by loss of $\text{CH}_2=\text{NH}$ (Figure 5f and g). The fragmentation pathways observed for the electrogenerated products are the same as those of authentic compounds, which supports the proposed structures and thereby the proposed oxidative reaction mechanism shown in Scheme 2. As shown in Figure 4b, there are another two new ions observed with m/z 224 and 226. It is likely that m/z 224 results from oxidation of 9-acridine carboxaldehyde **3** to carboxylic acid.

The m/z 226 might be a water adduct of m/z 208. The observed electrochemical oxidation of carbamazepine in this study appears to be similar to the reported oxidative metabolic pathway for carbamazepine in leucocytes (Scheme 2S-b, Supporting Information), providing an additional example showing that electrochemical mass spectrometry could be used for in vivo drug metabolism simulation.

Conclusions

Electrochemical reactions of several organic compounds with biological relevance in aqueous solution, including the reduction of nitrosothiols and the oxidation of carbohydrates and carbamazepine, were examined using EC/DESI-MS. Several interesting findings were uncovered in this study. First, it was found that electroreduction of nitrosothiols leads to free thiols involving characteristic mass decrease of 29 Da and a large intensity drop, providing a quick way to identify nitrosylated species. Also, the reduced nitrosylated peptide gives more sequence information upon CID. For oxidation of glucose, maltose, and cellobiose in alkaline solution, the corresponding carboxylic acid products were detected successfully. In contrast, no oxidation products were observed at neutral or acidic pH. For carbamazepine electrochemical oxidation, a similar oxidative metabolism pathway as in leucocytes was revealed using EC/DESI-MS. The mechanistic information of these redox reactions revealed by EC/DESI-MS would be of value in the nitroso-proteome research and carbohydrate/drug metabolic studies. In addition, the study also suggests that EC/DESI-MS is suitable for investigating different electrochemical reactions in aqueous solution, at both acidic and alkaline pHs.

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References

1. Bruckenstein, S., Gadde, R.R.: Use of a porous electrode for in situ mass spectrometric determination of volatile electrode reaction products. *J. Am. Chem. Soc.* **93**, 793–794 (1971)
2. Hambitzer, G., Heitbaum, J.: Electrochemical thermospray mass spectrometry. *Anal. Chem.* **58**, 1067–1070 (1986)
3. Dewald, H.D., Worst, S.A., Butcher, J.A., Saulinskas, E.F.: Separation and identification of isoflavones with on-line liquid chromatography-electrochemistry-thermospray mass spectrometry. *Electroanalysis* **3**, 777–782 (1991)
4. Bartmess, J.E., Phillips, L.R.: Electrochemically assisted fast atom bombardment mass spectrometry. *Anal. Chem.* **59**, 2012–2014 (1987)
5. Zhou, F.M., Vanberkel, G.J.: Electrochemistry combined online with electrospray mass spectrometry. *Anal. Chem.* **67**, 3643–3649 (1995)
6. Lu, W., Xu, X., Cole, R.B.: On-line linear sweep voltammetry-electrospray mass spectrometry. *Anal. Chem.* **69**, 2478–2484 (1997)
7. Deng, H., Van Berkel, G.J., Takano, H., Gazda, D., Porter, M.D.: Electrochemically modulated liquid chromatography coupled with on-line with electrospray mass spectrometry. *Anal. Chem.* **72**, 2641–2647 (2000)

8. Bökman, C.F., Zettersten, C., Sjöberg, P.J.R., Nyholm, L.: A setup for the coupling of a thin-layer electrochemical flow cell to electrospray mass spectrometry. *Anal. Chem.* **76**, 2017–2024 (2004)
9. Stassen, I., Hambitzer, G.: Anodic oxidation of aniline and *N*-alkylanilines in aqueous sulphuric acid studied by electrochemical thermospray mass spectrometry. *J. Electroanal. Chem.* **440**, 219–228 (1997)
10. Li, J., Dewald, H.D., Chen, H.: Online coupling of electrochemical reactions with liquid sample desorption electrospray ionization-mass spectrometry. *Anal. Chem.* **81**, 9716–9722 (2009)
11. Creager, S.: Solvents and Supporting Electrolytes. In: Zoski, C.G. (ed.) *Handbook of Electrochemistry*, p. 57. Elsevier, Amsterdam (2007)
12. Cooks, R.G., Ouyang, Z., Takats, Z., Wiseman, J.M.: Ambient mass spectrometry. *Science* **311**, 1566–1570 (2006)
13. Huang, M.-Z., Yuan, C.-H., Cheng, S.-C., Cho, Y.-T., Shiea, J.: Ambient ionization mass spectrometry. *Annu. Rev. Anal. Chem.* **3**, 43–65 (2010)
14. Ifa, D.R., Wu, C., Ouyang, Z., Cooks, R.G.: Desorption electrospray ionization and other ambient ionization methods: current progress and preview. *Analyst* **135**, 669–681 (2010)
15. Takáts, Z., Wiseman, J.M., Gologan, B., Cooks, R.G.: Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science* **306**, 471–473 (2004)
16. Miao, Z., Chen, H.: Direct analysis of liquid samples by desorption electrospray ionization-mass spectrometry (DESI-MS). *J. Am. Soc. Mass Spectrom.* **20**, 10–19 (2009)
17. Zhang, Y., Dewald, H.D., Chen, H.: Online mass spectrometric analysis of proteins/peptides following electrolytic cleavage of disulfide bonds. *J. Proteome Res.* **10**, 1293–1304 (2011)
18. Zhang, Y., Yuan, Z.Q., Dewald, H.D., Chen, H.: Coupling of liquid chromatography with mass spectrometry by desorption electrospray ionization (DESI). *Chem. Commun.* **47**, 4171–4173 (2011)
19. Zhang, Y., Cui, W., Zhang, H., Dewald, H.D., Chen, H.: Electrochemistry-assisted top-down characterization of disulfide-containing proteins. *Anal. Chem.* **84**, 3838–3842 (2012)
20. Lu, M., Wolff, C., Cui, W., Chen, H.: Investigation of some biologically relevant redox reactions using electrochemical mass spectrometry interfaced by desorption electrospray ionization. *Anal. Bioanal. Chem.* **403**, 355–365 (2012)
21. Zheng, Q., Zhang, H., Chen, H.: Integration of online digestion and electrolytic reduction with mass spectrometry for rapid disulfide-containing protein structural analysis. *Int. J. Mass Spectrom.* **353**, 84–92 (2013)
22. Zheng, Q., Zhang, H., Tong, L., Wu, S., Chen, H.: Cross-linking electrochemical mass spectrometry for probing protein three-dimensional structures. *Anal. Chem.* **86**, 8983–8991 (2014)
23. Liu, P., Lanekoff, I.T., Laskin, J., Dewald, H.D., Chen, H.: Study of electrochemical reactions using nanospray desorption electrospray ionization mass spectrometry. *Anal. Chem.* **84**, 5737–5743 (2012)
24. Smoluch, M., Mielczarek, P., Reszke, E., Hieftje, G.M., Silberring, J.: Determination of psychostimulants and their metabolites by electrochemistry linked on-line to flowing atmospheric pressure afterglow mass spectrometry. *Analyst* **139**, 4350–4355 (2014)
25. Miao, Z., Chen, H.: Proceedings of the 56th ASMS Conference on Mass Spectrometry and Allied Topics. Denver, CO, June 1–5 (2008)
26. Ma, X., Zhao, M., Lin, Z., Zhang, S., Yang, C., Zhang, X.: Versatile platform employing desorption electrospray ionization mass spectrometry for high-throughput analysis. *Anal. Chem.* **80**, 6131–6136 (2008)
27. Chipuk, J.E., Brodbelt, J.S.: Transmission mode desorption electrospray ionization. *J. Am. Soc. Mass Spectrom.* **19**, 1612–1620 (2008)
28. Moore, B.N., Hamdy, O., Julian, R.R.: Protein structure evolution in liquid DESI as revealed by selective noncovalent adduct protein probing. *Int. J. Mass Spectrom.* **330/332**, 220–225 (2012)
29. Miao, Z., Wu, S., Chen, H.: The study of protein conformation in solution via direct sampling by desorption electrospray ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **21**, 1730–1736 (2010)
30. Zhang, Y., Chen, H.: Detection of saccharides by reactive desorption electrospray ionization (DESI) using modified phenylboronic acids. *Int. J. Mass Spectrom.* **289**, 98–107 (2010)
31. Pan, N., Liu, P., Cui, W., Tang, B., Shi, J., Chen, H.: Highly efficient ionization of phosphopeptides at low pH by desorption electrospray ionization mass spectrometry. *Analyst* **138**, 1321–1324 (2013)
32. Ferguson, C.N., Benchaar, S.A., Miao, Z., Loo, J.A., Chen, H.: Direct ionization of large proteins and protein complexes by desorption electrospray ionization-mass spectrometry. *Anal. Chem.* **83**, 6468–6473 (2011)
33. Yao, Y., Shams-Ud-Doha, K., Daneshfar, R., Kitova, E., Klassen, J.: Quantifying protein–carbohydrate interactions using liquid sample desorption electrospray ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **26**, 98–106 (2015)
34. Liu, P., Zhang, J., Ferguson, C.N., Chen, H., Loo, J.A.: Measuring protein–ligand interactions using liquid sample desorption electrospray ionization mass spectrometry. *Anal. Chem.* **85**, 11966–11972 (2013)
35. Miao, Z., Chen, H., Liu, P., Liu, Y.: Development of submillisecond time-resolved mass spectrometry using desorption electrospray ionization. *Anal. Chem.* **83**, 3994–3997 (2011)
36. Liu, Y., Miao, Z., Lakshmanan, R., Loo, R.R.O., Loo, J.A., Chen, H.: Signal and charge enhancement for protein analysis by liquid chromatography-mass spectrometry with desorption electrospray ionization. *Int. J. Mass Spectrom.* **161**, 161–166 (2012)
37. Cai, Y., Adams, D., Chen, H.: A new splitting method for both analytical and preparative LC/MS. *J. Am. Soc. Mass Spectrom.* **25**, 286–292 (2014)
38. Cai, Y., Liu, Y., Helmy, R., Chen, H.: Coupling of ultrafast LC with mass spectrometry by DESI. *J. Am. Soc. Mass Spectrom.* **25**, 1820–1823 (2014)
39. Sun, X., Miao, Z., Yuan, Z., Harrington, P.B., Colla, J., Chen, H.: Coupling of single droplet micro-extraction with desorption electrospray ionization-mass spectrometry. *Int. J. Mass Spectrom.* **301**, 102–108 (2011)
40. Furst, S.M., Uetrecht, J.P.: Carbamazepine metabolism to a reactive intermediate by the myeloperoxidase system of activated neutrophils. *Biochem. Pharmacol.* **45**, 1267–1275 (1993)
41. Arulsamy, N., Bohle, D.S., Butt, J.A., Irvine, G.J., Jordan, P.A., Sagan, E.: Interrelationships between conformational dynamics and the redox chemistry of *S*-nitrosothiols. *J. Am. Chem. Soc.* **121**, 7115–7123 (1999)
42. Stampler, J.S., Jaraki, O., Osborne, J., Simon, D.I., Keaney, J., Vita, J., Singel, D., Valeri, C.R., Loscalzo, J.: Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso-adduct of serum albumin. *PNAS* **89**, 7674–7677 (1992)
43. Hogg, N.: The biochemistry and physiology of *S*-nitrosothiols. *Annu. Rev. Pharmacol. Toxicol.* **42**, 585–600 (2002)
44. Ryzhov, V., Lam, A.K.Y., O’Hair, R.A.J.: Gas-phase fragmentation of long-lived cysteine radical cations formed via NO loss from protonated *S*-nitrosocysteine. *J. Am. Soc. Mass Spectrom.* **20**, 985–995 (2009)
45. Lam, A.K.Y., Ryzhov, V., O’Hair, R.A.J.: Mobile protons versus mobile radicals: gas-phase unimolecular chemistry of radical cations of cysteine-containing peptides. *J. Am. Soc. Mass Spectrom.* **21**, 1296–1312 (2010)
46. Osburn, S., O’Hair, R.A.J., Ryzhov, V.: Gas-phase reactivity of sulfur-based radical ions of cysteine derivatives and small peptides. *Int. J. Mass Spectrom.* **316/318**, 133–139 (2012)
47. Vukomanovic, D.V., Hussain, A., Zoutman, D.E., Marks, G.S., Brien, J.F., Nakatsu, K.: Analysis of nanomolar *S*-nitrosothiol concentrations in physiological media. *J. Pharmacol. Toxicol. Methods* **39**, 235–240 (1998)
48. Cha, W., Anderson, M.R., Zhang, F., Meyerhoff, M.E.: Amperometric *S*-nitrosothiol sensor with enhanced sensitivity based on organoselenium catalysts. *Biosens. Bioelectron.* **24**, 2441–2446 (2009)
49. Yap, L.-P., Sancheti, H., Ybanez, M.D., Garcia, J., Cadenas, E., Han, D.: Chap. 6 - Determination of GSH, GSSG, and GSNO using HPLC with Electrochemical Detection. In *Methods Enzymol.* Enrique, C., Lester, P., Eds. Academic Press: Vol. 473, pp. 137–147 (2010)
50. Hou, Y., Wang, J., Arias, F., Echegoyen, L., Wang, P.G.: Electrochemical studies of *S*-nitrosothiols. *Bioorg. Med. Chem. Lett.* **8**, 3065–3070 (1998)
51. Zhao, J., Siu, K.W.M., Hopkinson, A.C.: Glutathione radical cation in the gas phase; generation, structure and fragmentation. *Org. Biomol. Chem.* **9**, 7384–7392 (2011)
52. Hao, G., Gross, S.S.: Electrospray tandem mass spectrometry analysis of *S*- and *N*-nitrosopeptides: facile loss of NO and radical-induced fragmentation. *J. Am. Soc. Mass Spectrom.* **17**, 1725–1730 (2006)
53. Pasta, M., Ruffo, R., Falletta, E., Mari, C.M., Pina, C.D.: Alkaline glucose oxidation on nanostructured gold electrodes. *Gold Bull.* **43**, 57–64 (2010)
54. Vassilyev, Y.B., Khazova, O.A., Nikolaeva, N.N.: Kinetics and mechanism of glucose electrooxidation on different electrode-catalysts: Part II: Effect of the nature of the electrode and the electrooxidation mechanism. *J. Electroanal. Chem.* **196**, 127–144 (1985)
55. Parpot, P., Kokoh, K.B., Belgsir, E.M., Le’Ger, J.M., Beden, B., Lamy, C.: Electroanalytical oxidation of sucrose: analysis of the reaction products. *J. Appl. Electrochem.* **27**, 25–33 (1997)
56. Aoun, S.B., Bang, G.S., Koga, T., Nonaka, Y., Sotomura, T., Taniguchi, I.: Electroanalytical oxidation of sugars on silver-UPD single crystal gold electrodes in alkaline solutions. *Electrochem. Commun.* **5**, 317–320 (2003)
57. Pasta, M., La Mantia, F., Cui, Y.: Mechanism of glucose electrochemical oxidation on gold surface. *Electrochim. Acta* **55**, 5561–5568 (2010)

58. Mautjana, N.A., Estes, J., Eyler, J.R., Brajter-Toth, A.: One-electron oxidation and sensitivity of uric acid in on-line electrochemistry and in electrospray ionization mass spectrometry. *Electroanalysis* **20**, 2501–2508 (2008)
59. Baumann, A., Lohmann, W., Schubert, B., Oberacher, H., Karst, U.: Metabolic studies of tetrazepam based on electrochemical simulation in comparison to in vivo and in vitro methods. *J. Chromatogr. A* **1216**, 3192–3198 (2009)
60. Arakawa, R., Yamaguchi, M., Hotta, H., Osakai, T., Kimoto, T.: Product analysis of caffeic acid oxidation by on-line electrochemistry/electrospray ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **15**, 1228–1236 (2004)
61. Pruneanu, S., Pogacean, F., Biris, A.R., Ardelean, S., Canpean, V., Blanita, G., Dervishi, E., Biris, A.S.: Novel graphene-gold nanoparticle modified electrodes for the high sensitivity electrochemical spectroscopy detection and analysis of carbamazepine. *J. Phys. Chem. C* **115**, 23387–23394 (2011)
62. Lertratanangkoon, K., Horning, M.G.: Metabolism of carbamazepine. *Drug Metab. Dispos.* **10**, 1–10 (1982)
63. Breton, H., Cociglio, M., Bressolle, F., Peyriere, H., Blayac, J.P., Hillaire-Buys, D.: Liquid chromatography-electrospray mass spectrometry determination of carbamazepine, oxcarbazepine and eight of their metabolites in human plasma. *J. Chromatogr. B* **828**, 80–90 (2005)
64. Takayasu, T., Ishida, Y., Kimura, A., Nosaka, M., Kuninaka, Y., Kawaguchi, M., Kondo, T.: Distribution of carbamazepine and its metabolites carbamazepine-10,11-epoxide and iminostilbene in body fluids and organ tissues in five autopsy cases. *Forensic Toxicol.* **28**, 124–128 (2010)
65. Mandrioli, R., Albani, F., Casamenti, G., Sabbioni, C., Raggia, M.A.: Simultaneous high-performance liquid chromatography determination of carbamazepine and five of its metabolites in plasma of epileptic patients. *J. Chromatogr. B Biomed. Sci. Appl.* **762**, 109–116 (2001)
66. Veiga, A., Dordio, A., Carvalho, A.J., Teixeira, D.M., Teixeira, J.G.: Ultra-sensitive voltammetric sensor for trace analysis of carbamazepine. *Anal. Chim. Acta* **674**, 182–189 (2010)
67. Kalanur, S.S., Jaldappagari, S., Balakrishnan, S.: Enhanced electrochemical response of carbamazepine at a nano-structured sensing film of fullerene-C60 and its analytical applications. *Electrochim. Acta* **56**, 5295–5301 (2011)
68. Jurva, U., Wikstrom, H.V., Bruins, A.P.: In vitro mimicry of metabolic oxidation reactions by electrochemistry/mass spectrometry. *Rapid Commun. Mass Spectrom.* **14**, 529–533 (2000)
69. Lohmann, W., Karst, U.: Biomimetic modeling of oxidative drug metabolism: strategies, advantages, and limitations. *Anal. Bioanal. Chem.* **391**, 79–96 (2008)
70. Jahn, S., Baumann, A., Roscher, J., Hense, K., Zazzeroni, R., Karst, U.: Investigation of the biotransformation pathway of verapamil using electrochemistry/liquid chromatography/mass spectrometry—a comparative study with liver cell microsomes. *J. Chromatogr. A* **23**, 9210–9220 (2011)
71. Faber, H., Melles, D., Brauckmann, C., Wehe, C., Wentker, K., Karst, U.: Simulation of the oxidative metabolism of diclofenac by electrochemistry/liquid chromatography/mass spectrometry. *Anal. Bioanal. Chem.* **403**, 345–354 (2012)
72. Kalanur, S.S., Seetharamappa, J.: Electrochemical oxidation of bioactive carbamazepine and its interaction with DNA. *Anal. Lett.* **43**, 618–630 (2010)