ORIGINAL PAPER

Investigation of the electrochemical behavior of cysteine by hyphenation of electrochemistry and mass spectrometry
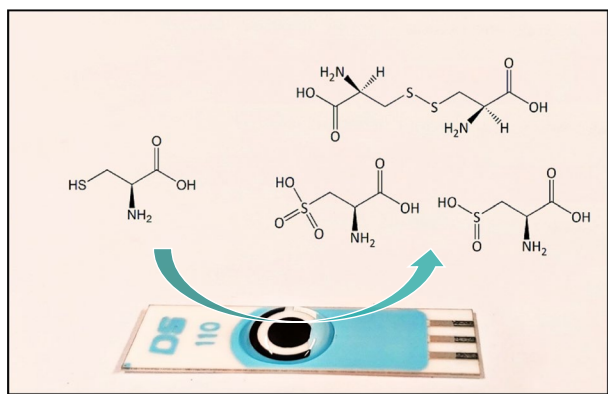
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Abstract

The identification of the reaction product species stands out as one of the main limitations of the classical electrochemical techniques. This can be overcome by the combination of electrochemistry (EC) to mass spectrometry (MS). Moreover, the method can be further enhanced by implementing a separation technique between EC and MS. In the present work, the oxidation behavior of cysteine with coupling EC directly to MS (real-time EC–MS) or by implementing capillary electrophoresis (CE), to separate the analytes before the injection into the mass spectrometer, is investigated. Electrochemical measurements and pre-treatment were applied on screen-printed electrodes (SPEs) based on carbon. Direct EC–MS measurements were carried out with a modified flow cell, while online EC–CE–MS studies of cysteine oxidation were conducted with a custom-made setup. An electrochemical conversion yield for cysteine of up to 69% was found. Cystine, cysteic acid, and cysteine sulfinic acid were found as oxidation products. The identification of these product species was carried out according to their migration behavior in CE, and mass-to-charge ratios in addition to their isotopic patterns shown in the MS spectra.

Graphical abstract

Introduction

From the beginning of the introduction of EC–MS by Bruckenstein and Gadde in 1971 [1], it has become a strong method for both qualitative and quantitative studies of redox processes [2, 3]. In the mentioned hyphenation, MS is being used because of its high sensitivity and specificity, providing structural information unavailable from EC methods. Here electrochemical reactions are used

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to oxidize or reduce analytes to improve the ionization of analytes for MS analysis [4].

Coupling the electrochemical flow cell directly to a mass spectrometer is the general mode of EC–MS. Such an arrangement is characterized by the rapid transfer of electro-generated species to the mass spectrometer. Therefore, it can detect even short-lived intermediates and enables real-time EC–MS studies [5].

By applying a potential sweep and simultaneously recording the MS spectra, information regarding the electrochemical product formation depending on the potential can be generated. In such a system, all the electro-generated species in the solution will reach the detector simultaneously, as no separation technique is implemented between the redox reaction at the surface of the electrode and the detection site in the mass spectrometer. This can cause the discrimination of analytes because of the ion suppression effects in electrospray ionization (ESI), and subsequently, the efficiency of quantitative information will be limited [6]. In 2003, a novel approach to overcome this limitation in terms of hyphenating EC to CE was reported by Matysik [7]. This concept was termed electrochemically assisted injection (EAI) [7]. In 2011, Scholz et al. introduced the combination of EAI–CE to MS with the help of a manually controlled injection cell for the first time [8]. This method was further improved by Palatzky et al. [9, 10] and led to the development of an automated injection cell, which was used in several further studies, e.g., in the simulation of oxidative stress on guanosine and 8-oxo-7,8-dihydroguanosine [11]. Another biologically interesting analyte is cysteine, which is one of the electrochemically active amino acids, and the sulfhydryl group on its side chain provides it with redox properties suitable for electrochemical conversions [12]. Moreover, cysteine is involved in different biological processes, for example as a part of the reactive center of enzymes and in the antioxidant glutathione. Along with cystine (a dimer form of cysteine), it shows a decisive impact on proteins' structure by forming disulfide bonds [13]. The oxidation products of cysteine, such as cysteic acid and cysteine sulfinic acid, play a role in current research as potential biomarkers [14] in addition to metabolic studies [15, 16]. There is an interest in the study of the conversion yield of the target analytes which can be investigated by applying different oxidation potentials and using the peak areas of the used method with and without electrochemical pretreatment [17].

Within the scope of this contribution, we focused on the electrochemical simulation of oxidative stress on cysteine and the analysis of the formation of oxidation products by means of MS. An electrochemical flow cell coupled to a time-of-flight MS was employed to investigate the potential-dependent formation of product species. A separation step, in the form of CE between the electrochemical generation and the detection in the MS, was implemented to identify the reaction products.

Results and discussion

Identification of the oxidation products of cysteine by direct EC–MS measurements

Direct EC–MS measurements were performed to provide information about the relation between potential and the formation of oxidation products of cysteine. Mass voltammograms (containing current–potential characteristics as well as MS information (m/z, MS signal intensity)) [5] obtained from experiments with cysteine solutions adjusted to acidic (A), neutral (B), or basic (C) pH are illustrated in Fig. 1.

In the measurement of the cysteine solution adjusted to pH 5 (Fig. 1A), two additional oxidation products apart from cystine were found. The results show that the generation of species with m/z of 154.02 began at an applied potential of 0.4 V. The next product with m/z of 170.01 started to form at a potential of 0.8 V. Similarly, both species were found in the experiments on cysteine solutions at neutral and basic pH (Fig. 1B, C). Cystine is present in all three samples due to the partial oxidation of cysteine by dissolved oxygen. It has to be noted that the potential of the quasi-reference electrode of the SPE depends on pH as well as on the presence/ absence of redox couples like cysteine/ cystine. In the experimental section, we specify the shift of the quasi-reference potential for the experimental conditions applied in this study. Under basic conditions, the formation of cystine was indicated by rather high intensities of the mass trace $m/z = 241.03$. In contrast, MS signals with $m/z = 154.02$ and $m/z = 170.01$ appeared just at more positive potentials. Thus, we conclude that cystine generation is more favorable at higher pH values. On the contrary, in acidic and neutral media, the generation of the additional two product species started at comparable potentials as the cystine formation and resulted in higher signal intensities than under basic conditions. The signals of these two product species were assigned to cysteine sulfinic acid ($m/z = 154.02$) and cysteic acid ($m/z = 170.01$). This assignment was confirmed by CE-MS experiments. The measured mass-to-charge ratios with the proposed oxidation product species and the potential ranges of their formation are summarized in Table 1.

Identification of the oxidation products of cysteine by online EC–CE–MS

To confirm the identification of the two additional oxidation products as cysteine sulfinic acid and cysteic acid, online EC–CE–MS measurements were performed. Figure 2A
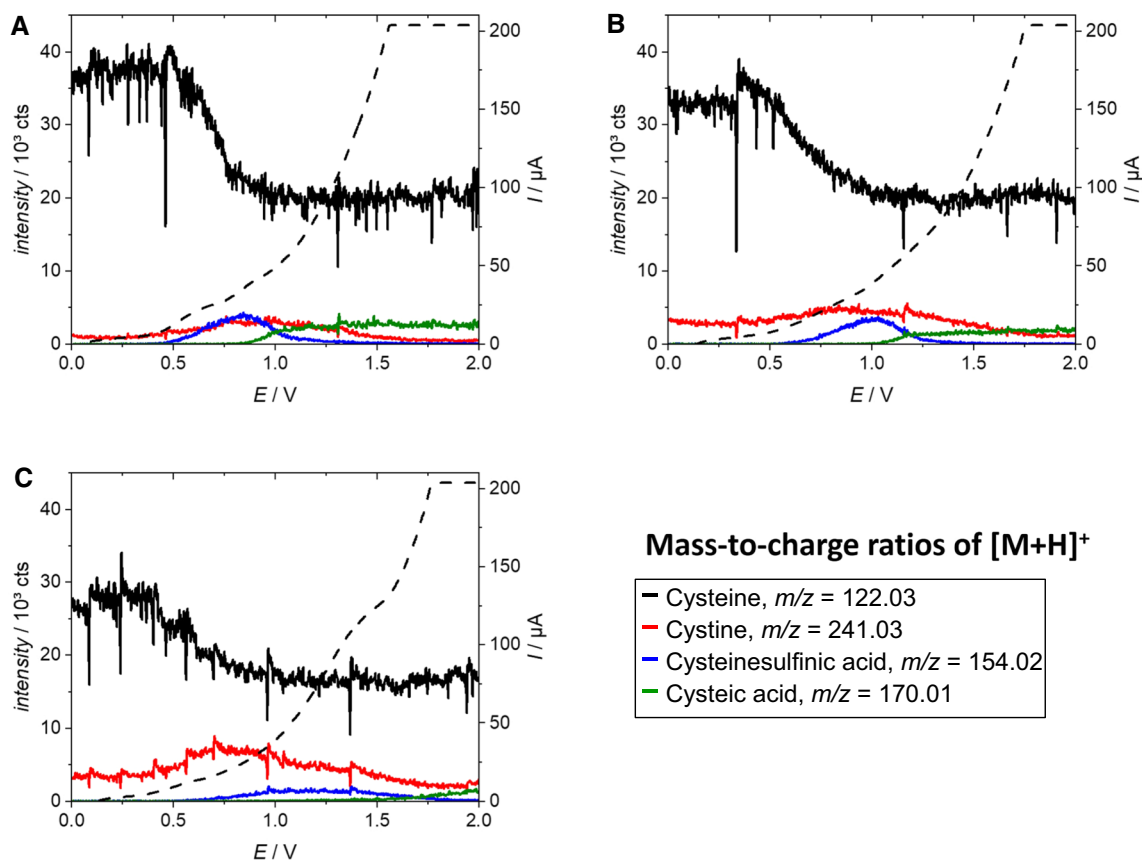
Fig. 1 Mass voltammograms of a 1 mM cysteine solution in 50 mM ammonium acetate adjusted to pH 5 (**A**), pH 7 (**B**), and pH 9 (**C**). The measurements were conducted on a carbon-based SPE implemented in a flow cell. Linear sweep voltammograms were performed from 0 to 2.0 V at a scan rate of 10 mV s^{-1}. Currents recorded during measurements are shown as a dashed line. The current recording is leveled off at 200 μA but the potentiostatic control was still ensured. The flow rate of sample solutions was 16 mm^3 min.$^{-1}$. The transfer capillary had an ID of 50 μm and a length of 21 cm
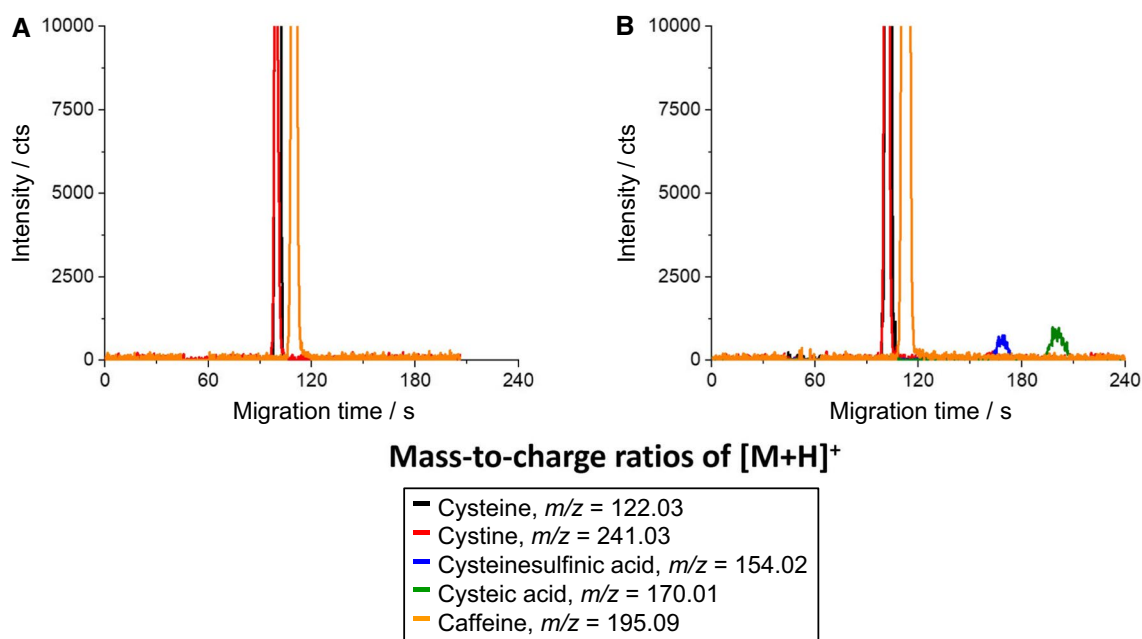
Table 1 Proposed oxidation products of cysteine and potential ranges of their formation at the respective pH

m/z of $[M+H]^+$	Proposed oxidation species	Potential range for product formation		
		pH 5	pH 7	pH 9
241.03	Cystine	0.5–1.4 V	0.4–1.4 V	0.3–1.5 V
154.02	Cysteine sulfinic acid	0.4–1.6 V	0.5–1.6 V	0.6–2.0 V
170.01	Cysteic acid	0.8–2.0 V	1.0–2.0 V	1.1–2.0 V

shows an enlarged electropherogram resulting from a measurement without electrochemical pre-treatment. In Fig. 2B, a potential of 1.1 V was applied for 15 s prior to the CE separation. The electropherograms were enlarged to highlight the two oxidation products, as they had comparably low signal intensities.

Without an applied potential (Fig. 2A), no signals for cysteine sulfinic and cysteic acid were observed. After the electrochemical sample pre-treatment (Fig. 2B) both analytes could be detected with migration times of 171 s (cysteine sulfinic acid) and 203 s (cysteic acid). These products migrated as anionic species under the applied separation conditions, as both migrated after caffeine, the added electroosmotic flow marker. However, the application of positive electrospray ionization (+ESI) resulted in the formation of $[M+H]^+$ species with sufficient intensity to be recorded. Comparative measurements with added reference substances were conducted under the same conditions. The measured mass-to-charge ratios, the migration times, and the isotopic patterns of electro-generated products matched with the reference substances.

Mass-to-charge ratios of [M+H]⁺

- Cysteine, *m/z* = 122.03
- Cystine, *m/z* = 241.03
- Cysteinesulfinic acid, *m/z* = 154.02
- Cysteic acid, *m/z* = 170.01
- Caffeine, *m/z* = 195.09

Fig. 2 Enlarged electropherograms for EC–CE–MS measurements of a 1 mM cysteine solution (pH 7, 50 mM ammonium acetate, 250 μM caffeine) without electrochemical pre-treatment (**A**) and with an applied potential of 1.1 V (**B**). The potential was applied for 15 s. Hydrodynamic injection lasted 10 s. The separation capillary had an ID of 50 μm and a length of 35 cm. The separation voltage was 20 kV and the BGE was a 500 mM acetic acid solution

The effect of the electrochemical pre-treatment potential on the conversion yield of cysteine by online EC–CE–MS

The change in the conversion yield of cysteine was studied by applying different potentials during the electrochemical pre-treatment. The conversion yield is defined as the percentage of cysteine that is converted to its oxidation products. It can be determined using the CE peak areas corresponding to cysteine with and without electrochemical pre-treatment. According to the potential ranges of the products' formation (Table 1), 3 different potentials were chosen to be applied. The results are shown in Table 2.

The results from Table 2 show that by increasing the electrochemical pre-treatment potential from 1.1 to 1.5 V, the conversion of cysteine to its oxidation products increases about 20%. The results indicate that for the application of the highest potential (1.5 V), 69.1% of cysteine is converted to its oxidation products but the remaining 30.9% of cysteine is not being oxidized. However, using higher electrochemical pre-treatment potentials is not practically favorable as the anodic breakdown potential of the electrolyte solution is approached. All in all, the measurements show that high percentages of cysteine can be converted to the corresponding oxidation products by including electrochemical pre-treatment.

Table 2 Total conversion rate of 1 mM cysteine solution (pH 7, 50 mM ammonium acetate) at different potentials (*n* = 3)

Applied potential / V	Total conversion rate % (*n* = 3)
1.1	48.6 ± 2.9
1.3	53.1 ± 1.0
1.5	69.1 ± 2.1

The potentials were applied for 15 s and the hydrodynamic injection lasted for 10 s. A separation capillary with an ID of 50 μm and a length of 30 cm was used. A separation voltage of 25 kV was applied and a BGE of 500 mM acetic acid was used

Conclusion

In summary, this study presents novel results concerning the electrochemical behavior of cysteine. To identify and classify the electrochemical oxidation products of cysteine, we present the real-time EC-MS and online EC-CE-MS measurements. The proposed method shows apart from cystine as the main oxidized species of cysteine, cysteine sulfinic acid, and cysteic acid as additionally formed products. First, the potential-dependent formation of the product species was examined under different

pH conditions. The results indicate, in contrast to cystine which was shown to be the dominant oxidation product at basic pH, cysteic acid and cysteine sulfinic acid were more likely to be formed at acidic pH. Second, the oxidation products were electro-generated, separated, and detected by online EC–CE–MS. Electrochemically formed species were identified according to their migration behavior in CE, the determination of highly accurate mass-to-charge ratios, and isotopic patterns compared to their reference substances. Finally, we focused on studying the effect of the electrochemical pre-treatment potential on the conversion yield of cysteine by online EC–CE–MS. It is shown that high percentages of cysteine can be converted to the proposed oxidation products by including an electrochemical pre-treatment protocol.

Experimental

All chemicals used were of analytical grade. The following solutions were prepared: 0.1 M sodium hydroxide solution, formic acid, ammonia ammonium acetate, (Merck, Germany), acetic acid, L-cystine (Mann Research, USA), L-cysteine, L-cysteic acid monohydrate, L-cysteine sulfinic acid monohydrate (Sigma Aldrich, USA), isopropanol (Roth, Germany), caffeine (ABCR, Germany). All solutions were prepared using ultra-pure water provided by a Milli-Q Advantage A10 system (Merck, Germany).

In this study, fused silica capillaries (Polymicro Technologies, USA) with an inner diameter (ID) of 50 µm and an outer diameter of 360 µm were used. The polyimide coating of both ends was removed about 0.5 cm. Then the injection side of the capillary tip was polished at a 15-degree angle and the detection side of it was prepared by polishing to a plain edge.

Following protocol was applied for conditioning the capillaries before beginning each session of the measurements: First, 0.1 M sodium hydroxide solution was used to flush the capillary for 10 min, followed by 5 min of flushing with ultra-pure water, and finally for more than 30 min with separation buffer (500 mM acetic acid). Disposable screen-printed electrodes (SPEs), of the type DRP-110 (Metrohm DropSens, Spain) with counter and working electrodes carbon-based and a silver quasi-reference electrode, were used for the electrochemical measurements. All utilized potentials in this work refer to the mentioned electrode system. However, the potential of the quasi-reference electrode depends on pH and on the presence / absence of redox couples. For the relevant solutions used in this study, the following potential shifts of the quasi-reference electrode of the SPE sensor versus an Ag/AgCl/sat. KCl reference electrode (Forschungsinstitut Meinsberg, Germany) were measured: Acetate buffer of pH 5 containing 1 mM cysteine, $\Delta E = -0.0428$ V

(RSD = 3.7%, $n = 5$); acetate buffer of pH 7 containing 1 mM cysteine, $\Delta E = -0.1566$ V (RSD = 1.5%, $n = 5$); acetate buffer of pH 9 containing 1 mM cysteine, $\Delta E = -0.2676$ V (RSD = 0.3%, $n = 5$).

Cysteine's electrochemical conversion in EC-MS measurements was conducted using a modified flow cell (Metrohm DropSens, Spain) [18] for SPEs. A µStat 200 potentiostat (Metrohm DropSens, Spain) was used for controlling the potential. The sample solution flowed through the electrochemical flow cell by implementing a syringe pump of type UMP3 with a Micro2T pump controller (World Precision Instruments, USA) which was equipped with a 1 cm^3 glass syringe. A micrOTOF time-of-flight mass spectrometer (Bruker Daltonics, Germany) equipped with a grounded CE-ESI–MS sprayer interface (Agilent Technologies, Germany) was used for the detection. A fused silica capillary (with a length of 21 cm) was used for the connection between the flow cell and the MS. Sample solutions containing both 1 mM cysteine and 50 mM ammonium acetate were prepared with pH values of 5, 7, and 9. For the operation of the ESI interface, a sheath liquid consisting of water, isopropanol, and formic acid (49.9/49.9/0.2 v/v/v) in measurements with applying positive ion mode of electrospray ionization, while a sheath liquid containing water, isopropanol, and ammonia (49.9/49.9/0.2 v/v/v) were used for the measurements in negative ion mode. A constant flow rate of 8 mm^3 min^{-1} was provided by a syringe pump (KD Scientific, USA). On the other hand, the flow rate of the sample solution was 16 mm^3 min^{-1}. A potential range of 0 to 2.0 V and a scan rate of 0.01 V s^{-1} were used to record the linear sweep voltammograms and simultaneously the mass spectra were registered. The MS detection parameters were set to be for the mass range of $m/z = 50$–500, spectra rate 5 Hz, dry gas 4.0 cm^3 min^{-1}, nebulizer gas 1.0 bar.

Figure 3 shows an automated, custom-made system for CE introduced by Palatzky et al. [10] in 2013. This setup was used for online EC–CE–MS measurements. The main part of the system is a horizontally movable Teflon desk with specific positions for a buffer vial and an SPE. A laboratory-constructed high voltage source is then connected to the buffer vial with a Pt electrode.

The separation capillary can be installed via a polyether ether ketone sleeve and can be moved in the vertical direction. Because of the high voltage and due to safety reasons, the setup was installed in a safety box made from plexiglass. For the online EC–CE–MS experiments, the same detector and Potentiostat as the EC–MS setup were used. The separation capillaries had the length of 30 and 35 cm. Two different sample solutions were used in this part of the work, first sample consisted of 1 mM cysteine, 50 mM ammonium acetate, and 250 µM caffeine as the electroosmotic flow (EOF) marker. The same solution additionally containing 100 µM cysteic acid and 100 µM cysteine sulfinic acid was used as
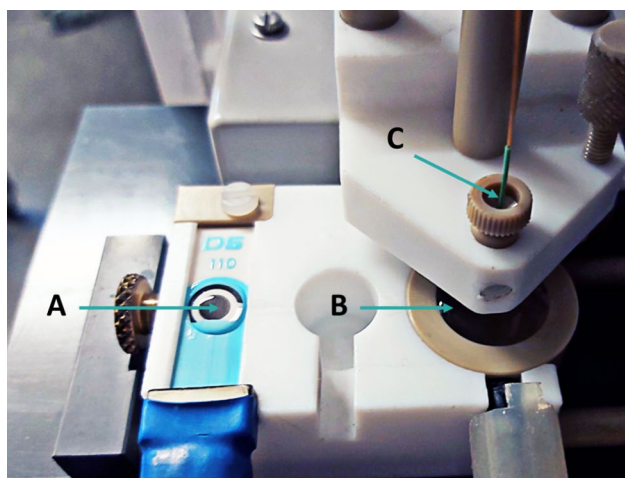
Fig. 3 Detailed view of the EC–CE–MS configuration used in this work. **A** SPE with 60 mm^3 of sample drop, **B** buffer reservoir with integrated Pt electrode, and **C** capillary holder with fixed capillary

the second sample solution. For online EC–CE–MS measurements, 60 mm^3 of the prepared solutions were placed on top of the electrode, and by applying 15 s of a constant potential the oxidation was started. 5 s after the start of the oxidation, the hydrodynamic sample injection was started by an automatic movement of the capillary tip into the sample droplet for 10 s. Afterward, the capillary was automatically moved back into the buffer vial where the separation happens by applying the separation voltage. A solution of 500 mM acetic acid was used as the separation buffer. The sheath liquid composition and MS detection parameters were set to be the same as the EC–MS measurements.

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