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Cephalosporin Antibiotics: Electrochemical Fingerprints and Core Structure Reactions Investigated by LC-MSMS

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**ABSTRACT:** Electrochemistry and exploiting electrochemical fingerprints is a potent approach to address newly emerging surveillance needs, for instance for antibiotics. However, a comprehensive insight in the electrochemical oxidation behaviour and mechanism is required for this sensing strategy. To address the lack in knowledge of the voltammetric behaviour of the cephalosporins antibiotics, a selection of cephalosporin antibiotics and two main intermediates were subjected to an electrochemical study of their redox behaviour by means of pulsed voltammetric techniques and small-scale electrolysis combined with HPLC-MS/MS analyses. Surprisingly, the detected oxidation products did not fit the earlier suggested oxidation of the sulfur group to the corresponding sulfoxide. The influence of different side chains, both at the three and the seven position of the β-lactam core structure on the electrochemical fingerprint were investigated. Additional oxidation signals at lower potentials were elucidated and linked to different side chains. These signals were further exploited to allow simultaneous detection of different cephalosporins in one voltammetric sweep. These fundamental insights can become the building blocks for a new on-site screening method.

β-lactam antibiotics (penicillin), the wonder drug discovered by Alexander Fleming in the 1920s brought about the greatest revolution in medicine of the 20\textsuperscript{th} century. After further development and the discovery of other antibiotics, common, yet deadly, diseases could be cured and the risk of infection after surgery could be significantly minimised\textsuperscript{1}. As a result, industrialization of production and the concomitant overuse of (semi)synthetic derivatives of the naturally occurring antibiotics began. Consequently, the natural process ‘survival of the fittest’ exerted itself, as all bacteria have the potential to mutate and render our drugs ineffective\textsuperscript{2,3}. This unfortunate effect of evolution is known as antimicrobial resistance (AMR) and one of the major health problems concerning modern society.

However due to the lack of new antibiotics reaching the market, it is imperative to conserve the effectiveness of existing antibiotics\textsuperscript{4}. Antimicrobial-resistant infections already claim at least 700,000 lives each year across the world with dire prospects of 10 million people dying every year by 2050\textsuperscript{5}. The world health organisation WHO has been leading multiple initiatives to address antimicrobial resistance, such as changing prescription policies, reduction of preventive use in animal feed and improve disposal control. One aspect in all of these is the pressing need for better surveillance and monitoring of antibiotics in our environment; aimed at rapid, sensitive and selective detection of antibiotics\textsuperscript{6}.

In light of misuse and overuse of antibiotics which led to the severe threat of AMR today, many attempts have been made to develop efficient methods to detect antibiotic residues. A common analytical approach for the determination of antibiotics is liquid chromatographic separation coupled with (tandem) mass spectrometry or UV/Vis-detection (LC-MS/MS or LC-UV/Vis), preceded by an extensive pre-concentration, such as solid phase extraction\textsuperscript{7-9}. These lab-based methodologies are functional, but time-consuming and costly, including, need for specialized analysts and sophisticated instruments. Other approaches in literature include optical\textsuperscript{10,11} and electrochemical biosensors\textsuperscript{12-14} and capillary electrophoresis (CE)\textsuperscript{15}, suffering from other shortcomings such as complicated modification procedures and high pH-dependance. Microbial screening assays are employed as a practical method for on-site detection of antibiotic residues. The microbial tests rely on a simple colour change, but no further information can be given like concentration or nature of the antibiotic(s). A well-known example of microbial tests is the Delvotest\textsuperscript{®}\textsuperscript{16}.

The use of electrochemistry could be an promising approach to address the on-site surveillance need of antibiotics, more specifically with square wave voltammetry (SWV). Selectivity and sensitivity is depending on the type of electrodes and conditions applied. Advantageously, SWV allows for quantitative detection, miniaturization (with screen printed electrodes, SPE) and real-time data. Regardless of the use SPE, which tackle some of the main drawback of classical electrodes, for example price, disposable nature, no need for lengthy regeneration procedures, the main condition for a suitable electrochemical sensor is a comprehensive insight in the electrochemical behaviour of the analysed species\textsuperscript{17}. This work focuses on the voltammetric behaviour and oxidation mechanism of cephalosporins antibiotics, a subclass of lactam antibiotics, due to a lack of knowledge of their electrochemical properties. They are used for veterinary purposes and as a replacement for the treatment of penicillin-allergic patients\textsuperscript{18}. Recently, Feier et al. described the electrochemical sensing of cephalosporins at boron doped diamond electrodes (BDD).
Rather than extending the potential window through the use of these expensive BDD electrodes which still required tedious polishing between measurements to attain reproducible voltammograms. We here describe the voltammetric properties of six different (semi-synthetic) cephalosporins using carbon SPE's, starting from unravelling the voltammetric fingerprints of the two main intermediates for semi-synthetic synthesis, namely 7-aminodeacetoxycephalosporanionic acid (7-ADCA) and 7-aminocephalosporanionic acid (7-ACA)\(^{20}\). These two compounds allowed us to explore the influence of the acetoxy sidechain at the C-3 position of the cephalosporin core structure on the electrochemical fingerprint and to optimise and characterise the analytical response of the ß-lactam core.

Secondly, two cephalosporins with redox inactive sidechains, cephalexin and cefacetrile, and their oxidation products will be investigated by means of small scale electrolysis and HPLC-MS/MS analyses. In order to definitely verify that the oxidation process occurs transforming the sulphur group into the corresponding sulfoxide\(^{19}\). Furthermore, cefquinome and cefadroxil, possessing a sidechain at the C-7 position common for many cephalosporins, were electrochemically investigated. Aim is to understand functional groups responsible for the oxidation signal and to explore whether the voltammetric fingerprints can be used for a selective detection of different cephalosporins in one voltammetric sweep using their sidechains and core structure signals to maximise the selectivity.

Figure 1. The chemical structures of the examined semi-synthetic cephalosporins and key intermediates (7-ADCA and 7-ACA). Compounds on the left contain only a methyl group at the C-3 position and compounds on the right contain either an acetoxy group or a bicyclic pyridinium group group at the C-3 position.

**Experimental Section**

**Reagents and materials**

Standards of 7-ADCA, 7-ACA, Cephalexin monohydrate and D-2-phenylglycine (D-PG) were purchased from TCI Europe (Belgium). Standards of Cefquinome sulphate and Cefacetrile sodium were purchased from Sigma-Aldrich (Diegem, Belgium). Standards of D-(−)-4-hydroxyphenylglycine (D-HPG) and thiazoximic acid (THX) were purchased from J&K scientific (Lommel, Belgium) and a standard of Cefadroxil was purchased from Acros Organics (Geel, Belgium). Cephalexin Sulfoxide was purchased from Toronto Research Chemicals (North York, Canada). Carbon ItalSens IS-C Screen Printed Electrodes (SPE) were purchased from PalmSens (Utrecht, The Netherlands) and were used during all electrochemical measurements. All electrochemical measurements were performed using a Metrohm µAutolab III Potentiostat and NOVA 1.11 software.

**Square-wave voltammetry**

Square-wave voltammetry (SWV) was performed to characterize the electrochemical behaviour and to obtain the voltammetric fingerprints of cephalosporins antibiotics. A conditioning potential of -0.1 V was applied for 5 s, followed by a second conditioning step of 5 s at 0 V before a scan from 0.2 V to a final potential of 1.7 V vs. Ag/AgCl external reference electrode was performed. All scans were performed at a frequency of 10 Hz, with an amplitude of 25 mV, and a step potential of 5 mV. The electrolysis was performed to identify possible oxidation product with the aid of LC-MS/MS measurements. A 120 µL drop of 100 µM cephalosporin were casted on the IS-C SPE. Next, different potentials (0.80 V, 0.96 V, 1.00 V, 1.10 V and 1.25 V) were applied versus the internal reference for 60 minutes. The electrolyzed solution was mixed rigorously prior to dilution with ultrapure water to 20 ng/µL (57.6 µM) and then directly injected in the LC-MS/MS.

**Moving average iterative background correction**

A baseline correction method was built in the SWV procedure in the NOVA 1.11 software to automatically correct for the raising background current in order to make the voltammograms easier to interpret. In brief, the method compares the value of a data point Ai to the values of the previous and next data points Ai-1 and Ai+1. If the value of data point Ai is higher than the average of the values of points Ai-1 and Ai+1 (as is the case for an oxidation peak), the average of the values of Ai-1 and Ai+1 will replace the value of Ai to construct the corrected baseline. In all other cases when Ai is lower or the same as the average of Ai-1 and Ai+1, Ai will be the value used for the corrected baseline. This process was performed for each two data points in the voltammogram and repeated until the value of Ai never exceeds the average of the values of Ai-1 and Ai+1 anymore, with a maximum of 1000 iterations. The corrected baseline is now assembled and the background current will be zero. Positive currents are only visible at peaks of oxidation processes.

**Chromatographic analysis**

Mass spectra were recorded using LC coupled to a QTOF-MS mass spectrometer with electrospray ionization (ESI) operating in positive mode. The apparatus consisted of a 1290 Infinity LC (Agilent Technologies, Wilmington, DE, USA) connected to a 6530 Accurate-Mass QTOF-MS (Agilent Technologies) with a heated-ESI source (JetStream ESI). Chromatographic separation was performed on a Kinetex Biphenyl column (150 × 2.1 mm, 2.6 µm), maintained at room temperature, and using a mobile phase composed of 0.04% formic acid in ultrapure water (A) and acetonitrile/ultrapure water (80/20, v/v) with 0.04% formic acid (B), in gradient. The flow rate and the injection volume were set at 0.3 mL/min and 1 µL, respectively. The QTOF-MS instrument was operated in
the 2 GHz (extended dynamic range) mode, which provides a full width at half maximum (FWHM) resolution of approximately 4700 at m/z 118 and 10,000 at m/z 922. Positive polarity ESI mode was used under the following specific conditions: gas temperature 300 °C; gas flow 8 L/min; nebulizer pressure 40 psi; sheath gas temperature 350 °C; sheath gas flow 11 L/min. Capillary and fragmentor voltages were set to 4000 and 135 V, respectively. A reference calibration solution (provided by Agilent Technologies) was continuously sprayed into the ESI source of the QTOF-MS system. The ions selected for recalibrating the mass axis, ensuring the mass accuracy throughout the run were m/z 121.0508 and 922.0097 for positive mode. The QTOF-MS device was acquiring from m/z 50 to 1000 in MS mode. Data-dependent acquisition mode (auto-MS/MS) was applied using two different collision energies (10 and 20 eV) for the fragmentation of the selected parent ions. The maximum number of precursors per MS cycle was set to four with minimal abundance of 2500 counts. In addition, precursor ions were excluded after every spectrum and released after 0.2 min.

**Results and Discussion**

**Voltammetry of cephalosporin antibiotics**

As most organic compounds, cephalosporins possess chemical groups that are prone to oxidation and can be oxidized electrochemically under appropriate conditions. Yet, it is unknown how the two main commonly used intermediates in industrial production (7-ADCA and 7-ACA) and corresponding semisynthetic cephalosporin antibiotics differ from each other in their electrochemical behaviour. The electrochemical fingerprint of 7-ADCA, 7-ACA, and four selected antibiotics - cephalexin, cefadroxil, cefacetrile, and cefquinome (Figure 1) were obtained by recording square-wave voltammograms (SWV) of 50 µM of the compound at a carbon screen printed electrode (SPE) in a 0.1 M phosphate buffer at two pH-values, i.e. 2 and 7.

![Figure 2](image1.png)

Figure 2. Baseline-corrected square-wave voltammetric responses (vs. Ag/AgCl) of 50 µM solutions of 7 ADCA and 7-ACA at bare carbon screen-printed electrodes in 0.1 M phosphate buffer pH 2 (left) and pH 7 (right).

Figure 2 depicts SWV responses for the intermediates dissolved in phosphate buffer pH 2 (left) and pH 7 (right). The background current is corrected in all voltammograms by the moving average algorithm. The oxidations of the intermediates of the cephalosporins (7-ADCA and 7-ACA) occur at high potentials and are of an irreversible nature.

The main difference between the 7-ADCA and 7-ACA voltammograms (Figure 2) for both pH-values is that 7-ADCA exhibits two oxidation signals A (at 1.4 V vs Ag/AgCl at pH 2) and B (at 1.55 V vs Ag/AgCl at pH 2) while 7-ACA showed only one oxidation peak (peak C) at 1.5 V at pH 2. This indicates that the oxidation of 7-ACA is influenced by the presence of the acetoxy sidechain at the C3 position, causing a shift towards a higher potential. Most likely, the second signal of 7-ACA cannot be observed in the given potential window as it is just a small contribution to the increase in current at high potentials (caused by the oxidation of the aqueous electrolyte solution).

The redox processes of both intermediates shift to a more positive potential while changing the pH from 7 to 2. The intensity of peak A is similar at both pH values while the second oxidation peak B intensifies at pH 2. In the case of peak C of 7-ACA, two conclusions can be made: (1) the intensity of the signal decreases almost by half changing the pH from 7 to 2; (2) the small shoulder at 1.5 V at pH 7 is no longer noticeable in the solution at pH 2.

![Figure 3](image2.png)

Figure 3. Baseline-corrected square-wave voltammetric responses (vs. Ag/AgCl) of 50 µM solutions of 7 ADCA-based cephalosporins (solid) and their respective sidechains (dotted) at bare carbon screen-printed electrodes in 0.1 M phosphate buffer pH 2 (left) and pH 7 (right).

Given its structural similarity to 7-ADCA, cephalexin and cefadroxil should also demonstrate two separate oxidative signals. In Figure 3, SWV responses obtained for the 7-ADCA based cephalosporins at a bare carbon SPE phosphate buffers pH 2 (left) and pH 7 (right) are shown. For cephalexin, similar oxidation processes A and B as for 7-ADCA take place in both pH 2 and 7 (Figure 3), however, at a slightly different oxidation potential. At pH 2, signals A and B shift 50 mV towards a lower potential compared to 7-ADCA while in the pH 7-solution the shift in the potential occurs in the opposite direction. The side chain of cephalexin,D-2-phenylglycine (D-PG) (Figure 3) is electrochemically inactive (dotted curve). The oxidation process A and B of cephalexin can therefore be purely related to their 7-ADCA-base structure.

In the case of cefadroxil (basically a cephalexin structure with additional –OH functionality), an additional redox process (D, Figure 3) is observed at 1.12 V at pH 2, and 0.77 V at pH 7. Oxidations of the core structure of cefadroxil (A and B,
Figure 3) drastically decrease in current for both pH-values compared to both cephalexin and 7-ADCA.

The source of the additional redox process (D) can be explained through studying the electrochemistry of the side chains. Thereupon, D-(−)-4-hydroxyphenylglycine (D-HPG) side chain (Figure 3) was submitted to identical SWV analysis at a bare carbon SPE. In Figure 3, SWV responses for D-HPG are overlaid as the dotted curve. Comparing the voltammetric profile of D-HPG to cefadroxil, its electrochemical pattern is significantly different. In essence, the D-HPG signal at 0.81 V in pH 7 overlaps with the additional signal of cefadroxil at 0.77 V (D) as it is well-known that phenolic functionalities show electrochemical activity. However, D-HPG exhibits tailing after the main peak D in pH 7. Both redox processes related to the core structure (A and B) are obviously not observed for the D-HPG side chain in both pH-values. By changing the pH to 2, the redox processes of D-HPG separate into two distinctive peaks at ca. 1.07 V and 1.23 V.

Subsequently, similar experiments were performed with 7-ACA-based cephalosporins, namely cefacetrile and cefquinome (Figure 4). Both antibiotics possess two side chains, one at the primary amine group and a substituent at the C-3 position, either an acetoxyl group (cefacetrile) or a bicyclic pyridinium group (cefquinome).

![Figure 4. Baseline-corrected square-wave voltammetric responses (vs. Ag/AgCl) of 50 µM solutions of 3-substituted cephalosporins (solid) and their respective sidechains (dotted) at bare carbon screen-printed electrodes in 0.1 M phosphate buffer pH 2 (left) and pH 7 (right).](image)

Firstly, the oxidation peak of 7-ACA (C, Figure 4) can be observed plainly for both cefacetrile and cefquinome. The peak potentials shift compared to 7-ACA in both pH-values following the similar pattern as observed for the 7-ADCA based cephalosporins. Cefacetrile does not show any additional signals at lower or higher potentials. Therefore, it seems that its side chain on the primary amine group is electrochemically inactive. In contrast, cefquinome gave a clear additional oxidation peak (E, Figure 4) for both pH-values. It is unlikely that the bicyclic pyridinium group of cefquinome can be responsible for E because it is already positively charged. Therefore, the thiazoximic (THX) group was analysed and the SWV response is overlaid as the dotted curve (Figure 4), showing that the additional signal E of cefquinome is related to the electroactive side chain THX. As almost no shift in potential is observed at pH 7 (at 0.81 V) and at pH 2 (at 0.95 V) the response is identical for cefquinome (solid) and THX (dotted).

These experiments clearly showed that individual cephalosporins have their own unique electrochemical fingerprints, depending on the sidechains present at both the C-3 and C-7 positions. These fingerprints can then be exploited for the development of a quantitative method for the detection of cephalosporins by square wave voltammetry, as the peak current is linearly dependent of the present antibiotic (Figure S1).

### Voltammetric analysis of cephalosporin mixtures

In real life samples, antibiotics will often be present as a mixture. For this reason, a mixture of cephalexin, cefadroxil and cefquinome was analysed in 0.1 M phosphate buffer pH 2 (Figure 5).

![Figure 5. Baseline-corrected square-wave voltammetric responses (vs. int. ref.) of 25 µM triplet mixtures of cephalosporins at bare carbon screen-printed electrodes in 0.1 M phosphate buffer pH 2.](image)

As expected from the previous figures a favourable peak separation is attained in the pH 2 phosphate buffer solution, especially for the oxidation processes of the electro-active side chains cefadroxil, cefquinome (D and E, Figure 5). In pH 2 peak D and E are both detected at 0.84 V and 0.70 V respectively while they would completely overlap in pH 7. Two signals related to the core structure of cephalosporins of cephalexin (A and B, Figure 5) were also both observed separately. The minimal interference with peak A and B of cephalexin and the separation of cefquinome and cefadroxil in pH 2 for 25 µM concentrations thus gives a more reliable and optimal response. The characteristic electrochemical fingerprint of each compound allows to detect them simultaneously and quantitatively without the need for any separation technique in lower concentration range up to 10 µM (Figure S1).

These first-time insights into the electrochemical behaviour of semi-synthetic antibiotics and the two intermediates 7-ADCA and 7-ACA, demonstrated that the various sidechains give rise to different fingerprints. Moreover, the fingerprints of side chains as thiazoximic acid and D-(−)-4-hydroxyphenylglycine, were shown to lead to the additional signals observed at lower potentials of their corresponding antibiotics, respectively cefquinome and cefadroxil. On the other hand the electro-oxidative pathway of the cephalosporin core structure remains unclear.
Cephalosporin core reactions during electrochemical analysis

The first question was whether these oxidations processes for the cephalosporin are related to the oxidation of the sulphur group leading to a sulfoxide derivative. Similar to the suggested for the oxidation of penicillin V. Therefore, cephalexin was chosen as the model compound as a standard of cephalexin sulfoxide was commercially available. From the analysis of cephalexin sulfoxide (Figure 6) it is clear that the first oxidation processes at 1.1 V is absent which could be an indication that indeed cephalexin oxidises to cephalexin sulfoxide at 1.1 V and then oxidises further. However, this has not been confirmed and in literature it has been reported that alkoxylation can take place at the C-2 position because of a stabilised carbocation formed after oxidation of the sulphur group due to the double bound that is not present in penicillins.

Figure 6. Baseline-corrected square-wave voltammetric responses (vs. int. ref.) of 100 µM cephalexin (solid) and cephalexin sulfoxide (dotted) at bare carbon screen-printed electrodes in 0.1 M phosphate buffer pH 2.

LC-QTOFMS analysis on the partially electrolyzed solutions of cephalexin were performed to identify possible oxidation products. Solutions of 100 µM cephalexin were electrolyzed in 0.1 M phosphate at 1.1 V (vs int. ref) for 60 minutes, subsequently the sample was diluted to 20 ng/µL (57.6 µM) with ultrapure water, injected directly afterwards (Figure 7, blue) and after 12 hours (Figure 7, green). The obtained chromatograms are compared to a 20 ng/µL standard of cephalexin (Figure 7, red) and cephalexin sulfoxide (Figure 7, black).

Figure 7. Total ion chromatogram of 20 ng/µL solutions of cephalexin (red), cephalexin sulfoxide (black), directly injected electrolysis sample of cephalexin (1.1 V vs int. ref for 60 min) (blue) and after 12 hours (green).

Firstly, two oxidation products were formed during the first electrochemical oxidation (peak A in Figure 6) were identified, product 1 (P1) at 4.55 min (m/z 318.0925, C₁₆H₁₄N₃O₅S) and product 2 (P2) at 3.65 min (m/z 336.1033, C₁₅H₁₄N₃O₅S) when comparing cephalexin (Figure 7, red) at 4.34 min (m/z 348.1035, C₁₆H₁₄N₃O₅S) and electrolyzed cephalexin (Figure 7, blue). Apparently, the first electrochemical oxidation signal is not related to the suggested formation of cephalexin sulfoxide (m/z 364.0962, C₁₆H₁₄N₃O₅S) but rather suggests a more complicated oxidation pathway as a loss of carbon is observed. This was confirmed by comparing cephalexin sulfoxide (black) to the chromatogram of the partial electrolyzed solution of cephalexin (blue; cephalexin sulfoxide elutes earlier (at 3.23 min) than the P1 and P2.

Secondly, for clarifying the stability and the relation between these two oxidation products the sample of electrolysis was injected again after 12 hours (green). A strong decrease in the intensity of P1 was observed while the abundance of P2 increased, indicating that P1 is formed during the electrochemical oxidation, but not stable and will transform in to P2, most likely though the addition of water (as the mass-difference is 18). As shown in Figure 6, cephalexin exhibits two oxidation signals related to the 7-ADCA base structure. Further electrolysis experiments were performed at different potentials (Figure 8), namely 0.8 V (just before signal A), 0.96 V (at onset oxidation peak A), 1.00 V (at half height oxidation peak A), 1.10 V (at oxidation peak A and before peak B) and 1.25 V (at oxidation peak B). An increase of the amount of both P1 and P2 is observed along oxidation process A. Once oxidation process B starts, the amount of P1 and P2 is negligible meaning that further oxidation/fragmentation takes place. This also strengthened by the chromatogram of the electrolysis at 1.25V as no additional peaks are observed (Figure S2).

Figure 8. Formation of cephalexin oxidation products P1 (m/z 318.0925, C₁₆H₁₄N₃O₅S) (black) and P2 (m/z 336.1033, C₁₅H₁₄N₃O₅S) (red) at different potentials.
Figure 9. Total ion chromatogram of 20 ng/µL solutions of cefacetrile (red), directly injected electrolysis sample of cefacetrile (1.1 V vs int. ref for 60 min) (black).

Lastly, to confirm if the mechanism of oxidation of 7-ACA based cephalosporins is similar, cefacetrile was subjected to small scale electrolysis at 1.25 V (vs int. ref), as oxidation peak C at higher potentials for 7-ACA derived cephalosporins. Similar oxidation products were found for the oxidation cefacetrile (Figure 9 black) as seen for cephalixin. The two products that were identified were product 1’ (P1’) at 4.75 min (m/z 310.0492, C₁₂H₁₀N₂O₃S) and product 2’ (P2’) at 3.61 min (m/z 328.0598, C₁₂H₁₀N₂O₃S) with cefacetrile mainly detected as an ammonium adduct (Figure 9, red) at 4.16 min (m/z 357.0861, C₁₃H₁₂N₂O₅S).

Based on the MS/MS fragmentation patterns of P1/P2 of cephalixin (Figure S3) and P1’/P2’ of cefacetrile (Figure S4), which were almost identical between the two products of cephalexin and cefacetrile, the following mechanism is proposed for the formation of oxidation product P1, which will undergo the addition of another water molecule resulting in product P2 without the need of an electrochemical step. From the experiments above the oxidation of cephalosporins is not driven by sulfoxide formation as is the case for the penicillins. To explain this difference and the observations from these experiments, we put forward the hypothesis that the double bond present in the cephalosporins core structure plays an important role. After a first electrochemical decarboxylation step (E), the addition of water as a chemical step (C) is followed as an intermediate step leading to further oxidation into the keto analogue (E) and in the case of cephalixin forms oxidation product 1. Two oxidation products of both cephalexin and cefacetrile can be correlated to the observed electrochemical experiments (ECE mechanism) through in-depth investigation into the detected accurate masses and a certain level of structural information was found in the fragmentation patterns of the cephalosporins cephalexin, cefacetrile and their respective oxidation products. However, as no standards of these products were synthesised based on the LC-MS/MS measurements it is not possible to distinguish between the nucleophilic attack of water happening at the C2-position (Scheme 1 (A)) or the C4-position (Scheme 1 (B)). Additional measurements are necessary to gain more structural information about the oxidation processes of the core structure of cephalosporins. However a product P3 is detected in small amounts for both cephalixin as cefacetrile which based on the fragmentation pattern indicates the opening of the β-lactam ring of product 2. For the second oxidation step observed for the 7-ADCA based cephalosporins no oxidation products could be identified due to high level of fragmentation.

Scheme 1: Proposed electrochemical oxidation of cephalosporins.

Conclusions

We have clarified the mechanism of the electrochemical oxidation of different cephalosporin antibiotics. Starting from the two main intermediates 7-ADCA and 7-ACA that exhibit different fingerprints (two signals compared to one in the given potential range). These observations were then further applicable to the cephalosporins cephalexin (7-ADCA based) and cefacetrile (7-ACA based) which demonstrated that their sidechains are electrochemically inactive. Moreover, The fingerprints of the most prominent active side chains namely thiazoamic acid and D-(−)-4-hydroxyphenylglycine, were related to the additional signals observed at lower potentials of the corresponding antibiotics (cefquinome and cefadroxil). Thereupon, it was shown that performing electrochemistry in a pH 2 solution provides the best resolution for the investigated antibiotics and allows the maximal usage of these different fingerprints and even allowing us to quantitatively detect them in a triple mixture in concentration up to 10 µM. The use of electrochemical fingerprints displayed a considerable potential for the further development of a fast, reliable and sensitive analysis method for cephalosporins. Lastly, we evidenced through the combination of small scale electrolysis and LC-QTOFMS analysis that the cephalosporins core structure does not oxidises to the suggested corresponding sulfoxide. Moreover, two different oxidation products were clearly detected, identified and there relation clarified. Therefore, allowing us to suggest a new oxidation mechanism for the cephalosporins fitting the experimental results obtained.

ASSOCIATED CONTENT
Supporting Information
The concentration profile of the square-wave voltammetric responses of Cephalexin (2.5-50 μM), Cefquinome (1-50 μM) and Cefadroxil (2.5-50 μM) and the corresponding responses of the triple mixture at bare carbon screen-printed electrodes in 0.1 M phosphate buffer pH 2; Total ion chromatogram of 20 ng/μL 1h electrolysis sample of cephalaxin at 1.1 V vs 1.25 V; Fragmentation patterns of cephalaxin and product 1 (P1) and product 2 (P2) obtained by LC-QTOF analysis combined with small scale electrolysis; Fragmentation patterns of cefacetrile and product 1’ (P1’) and product 2’ (P2’) obtained by LC-QTOF analysis combined with small scale electrolysis; This material is available free of charge via the Internet at http://pubs.acs.org

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Author Contributions

All authors have given approval to the final version of the manuscript.

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