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SPECTROELECTROCHEMISTRY TECHNIQUES APPLIED TO CHEMICAL ANALYSIS

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Que la presente memoria resume el trabajo titulado “*Spectroelectrochemistry techniques applied to chemical analysis*” realizado por Dña. FABIOLA OLMO ALONSO en el Área de Química Analítica (Departamento de Química) y bajo nuestra dirección para optar al Grado de Doctor por la Universidad de Burgos.

Manifestamos nuestra conformidad con el contenido de la memoria y autorizamos su presentación para ser defendida como Tesis Doctoral.

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A mis padres y a mi hermano

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INDEX

WHAT IS THIS THESIS ABOUT?	1
1. SPECTROELECTROCHEMISTRY TECHNIQUES	3
1.1. Spectroelectrochemistry	7
1.1.1. UV/Vis absorption spectroelectrochemistry	9
1.1.2. Photoluminescence spectroelectrochemistry	16
1.1.3. Raman spectroelectrochemistry	19
2. COMBINATION OF SPECTROELECTROCHEMISTRY TECHNIQUES	25
2.1. UV/Vis Bidimensional spectroelectrochemistry (BSEC)	29
2.2. Combination of different spectroscopic techniques in spectroelectrochemistry	35
2.1.1. UV/Vis-PL-Raman spectroelectrochemistry	36
2.1.2. Parallel and Normal UV/Vis and Raman spectroelectrochemistry	37
3. MOLECULES OF BIOLOGICAL INTEREST	39
3.1. Importance of molecules of biological interest	43
3.2. Quinolones and Fluoroquinolones	44
3.2.1. Ofloxacin	46
3.3. Catecholamines	47
3.3.1. Epinephrine	48
3.3.2. Dopamine	49
3.4. Other molecules of biological interest	50
3.4.1. Folic Acid	50
3.4.2. Ascorbic acid	53
3.4.3. Uric acid	54
4. EXPERIMENTAL SECTION	57
4.1. Spectroelectrochemistry (SEC)	61
4.1.1. Electrochemistry	62

4.1.2. UV/Vis absorption SEC	63
4.1.3. Photoluminescence SEC	64
4.1.4. Raman SEC	65
4.1.5. UV/Vis-PL-Raman SEC	66
4.1.6. Parallel UV/Vis-Normal UV/Vis-Raman SEC	67
4.2. Software	67
5. DATA PROCESSING	69
5.1. Time-resolved experiments	73
5.2. Data treatment	74
5.3. Qualitative and quantitative analyses	76
5.3.1. Univariate data treatment	76
5.3.2. Multivariate data treatment	76
6. RESEARCH RESULTS	77
6.1. Normal or parallel configuration in spectroelectrochemistry? Bidimensional spectroelectroanalysis in presence of an antioxidant compound	83
6.1.1. Introduction	83
6.1.2. Materials and methods	85
6.1.3. Results and discussion	87
6.1.4. Conclusions	100
6.2. UV/Vis absorption spectroelectrochemistry of folic acid	103
6.2.1. Introduction	103
6.2.2. Experimental	105
6.2.3. Results and discussion	108
6.2.4. Conclusions	115
6.3. Determination of ofloxacin in urine using UV/Vis absorption spectroelectrochemistry	117
6.3.1. Introduction	117
6.3.2. Experimental Section	120

6.3.3. Results and discussion	122
6.3.4. Conclusions.....	138
6.4. Derivative UV/Vis spectroelectrochemistry in a thin-layer regime: deconvolution and simultaneous quantification of ascorbic acid, dopamine and uric acid.....	139
6.4.1. Introduction.....	139
6.4.2. Materials and methods	142
6.4.3. Results and discussion	144
6.4.4. Conclusions.....	157
6.5. Triple Spectroelectrochemistry. Simultaneous UV/Vis absorption, photoluminescence and Raman Spectroelectrochemistry.....	159
6.5.1. Introduction.....	159
6.5.2. Experimental Section.....	160
6.5.3. Results and discussion	164
6.5.4. Conclusions.....	171
6.6. Triple Spectroelectrochemistry. Simultaneous UV/Vis absorption in Normal and Parallel Configuration and Raman Spectroelectrochemistry.....	173
6.6.1. Introduction.....	173
6.6.2. Experimental Section.....	173
6.6.3. Results and discussion	176
6.6.4. Conclusions and future perspectives.....	179
7. FINAL CONCLUSIONS.....	181
8. FUTURE PERSPECTIVES.....	185
9. REFERENCES	189
10. ABBREVIATIONS.....	231

WHAT IS THIS THESIS ABOUT?

The significant advances in technology and materials for spectroscopy during the last decades have allowed a significant improvement of Spectroelectrochemistry (SEC). Detectors, gratings, light sources, optical fibers and computers provide much better and faster optical responses, allowing us to obtain much more reproducible responses and with much more significant information. In this Thesis, the clear improvement of the quality of the optical responses has been used to obtain SEC measurements for analytical purposes. From the early days, where only obtaining an optical response of an electrochemical process was a significant advance to understand the process taking place at the electrode/solution, to nowadays, where the optical responses can be obtained with very high reproducibility, SEC has grown a lot, passing from a characterization technique to a reliable and promising analytical technique. Sometimes, an additional problem in the study of the electrode process is the complexity of obtaining fully correlated responses when different characterization techniques are used. In this Thesis, up to three different independent spectroscopies (UV/Vis absorption, Photoluminescence and Raman) have been concomitantly used to obtain a full picture of the processes taking place at the electrode/solution interface.

The general objective of this Thesis is to demonstrate the capabilities of SEC for analysis, showing the attractive responses that can be used to solve very complex analytical problems.

The specific objectives of this Thesis are:

- ✓ To show that a correct selection of the optical configuration is mandatory to obtain reliable results.
- ✓ To demonstrate that the development of good SEC cells makes this technique a very interesting option for quantitative analysis.
- ✓ To obtain high quality SEC data to determine compounds of biological interest in presence of high interfering compounds and in complex matrices.
- ✓ To demonstrate that the trilinear character of SEC data is very useful to deconvolve the electrode process using the most appropriate data transformation and the most suitable chemometric tools.

- ✓ To show that the combination of SEC techniques provides a full-picture of the processes taking place in the electrode/solution interface.

With this purpose, this work is divided in 8 chapters. The first chapter describes the SEC techniques used along this Thesis. In the second one, a revision of the combination of SEC techniques is done. The third chapter introduces the molecules studied along this work. Experimental and data processing details are provided in the fourth and fifth chapter, respectively. The sixth one shows the main results obtained in this work. Section 6.1 shows the importance of selecting the best optical configuration to obtain the most reliable results. In Section 6.2 folic acid is determined in a complex matrix. Ofloxacin, an antibiotic usually used for urinary tract infections, is determined in urine using UV/Vis absorption spectroelectrochemistry in Section 6.3. Also, three different molecules of biological interest, ascorbic acid, dopamine and uric acid, are determined using derivative UV/Vis spectroelectrochemistry in a thin-layer regime, showing the high capability of SEC trilinear data to resolve complex mixtures, Section 6.4.

Finally, as a proof of concept, the possibility of combining three spectroscopies is demonstrated using UV/Vis absorption in parallel configuration, Photoluminescence and Raman scattering in Section 6.5 and UV/Vis absorption in normal and parallel configuration and Raman scattering in Section 6.6.

Therefore, this Thesis sets the basis for developing analytical methods based on SEC as a powerful tool for applications in the fields of pharmaceutical, biological and environmental analysis.

1. SPECTROELECTROCHEMISTRY TECHNIQUES

Summary

This first chapter will focus on the description of the fundamental theoretical basis of the main instrumental analysis technique used in this doctoral thesis, which is spectroelectrochemistry (SEC). Briefly, SEC is a powerful technique resulting from the combination of classical electrochemistry and spectroscopy. Several advantages can be obtained from this technique, since two responses of different nature are simultaneously recorded in a single experiment. More work in this technique is mandatory because certain shortcomings related mainly to instrumentation and set-ups devices have to be improved. Moreover, several combinations derived from different electrochemical and spectroscopic techniques are possible. Three SEC techniques will be applied throughout this doctoral thesis: UV/Vis absorption SEC, photoluminescence SEC and Raman SEC.

The key objectives of this chapter are as follows:

- To describe the basics of SEC, detailing different aspects such as its development, the importance of this technique, its strengths and weaknesses and its different applications.
- To introduce UV/Vis absorption SEC, which is the predominant technique in this doctoral thesis. In addition, the advances that this technique has undergone, its applications and the experimental devices and optical configurations used to carry out experiments with this technique will be discussed in depth.
- To describe the basis of photoluminescence SEC, its applications and experimental set-ups.
- To introduce Raman SEC, its development and its most significant applications. In addition, the most recurrent Raman signal amplification methods will be described, focusing in particular on the method applied in this work, which is known as SERS (Surface-Enhanced Raman Scattering).

Resumen

Este primer capítulo se centrará en la descripción de los fundamentos teóricos de la técnica de análisis instrumental protagonista de esta tesis doctoral, que es la espectroelectroquímica (SEC). En resumen, la SEC es una poderosa técnica fruto de la combinación de dos técnicas clásicas, la Electroquímica y la Espectroscopia. Varias ventajas se obtienen con esta técnica, ya que en un mismo experimento se registran simultáneamente dos respuestas de distinta naturaleza. Por otro lado, es necesario mejorar ciertas deficiencias relacionadas principalmente con la instrumentación y los dispositivos espectroelectroquímicos. Además, es posible realizar diversas combinaciones derivadas de las diferentes técnicas electroquímicas y espectroscópicas. A lo largo de esta tesis doctoral se usarán tres técnicas SEC: SEC de absorción en el UV/Vis, SEC de fotoluminiscencia y SEC Raman.

Los principales objetivos de este capítulo son los siguientes:

- Describir las bases de la SEC, detallando distintos aspectos como su desarrollo, la importancia de esta técnica, sus puntos fuertes y débiles y sus diferentes aplicaciones.
- Introducir la SEC de absorción en el UV/Vis, que es la técnica predominante en esta tesis doctoral. Además, se profundizará en los avances que ha experimentado esta técnica, en sus aplicaciones y en los dispositivos experimentales y las configuraciones ópticas empleadas para llevar a cabo experimentos con esta técnica.
- Describir las bases de la SEC de fotoluminiscencia, sus aplicaciones y montajes experimentales.
- Introducir la SEC Raman, su desarrollo y aplicaciones más destacadas. Además, se describirán los métodos de amplificación de la señal Raman más utilizados, centrándonos especialmente en el método usado en este trabajo, que es SERS (Surface-Enhanced Raman Scattering).

1.1. Spectroelectrochemistry

The proposal of using optically transparent electrodes (OTE's) based on semiconducting metal oxides, with the aim of studying electrode reactions, by the researchers Mantell and Zaromb,¹ Cooper² and Kuwana,³ gave rise to the birth of spectroelectrochemistry (SEC). It was one of these researchers, Ted Kuwana, who developed in 1964 the first OTE to measure the absorption changes of an electrochemical process.³ In addition, Kuwana also developed the first photoluminescence SEC set-up in 1963⁴ with the objective of observing the spectrum of luminol when this molecule was electrochemically oxidized. Since these historical events, numerous advances and improvements of this technique have taken place,⁵ as will be shown in this chapter. But first, a description of SEC will be given.

SEC, as its name suggests, is a multi-response technique that combines electrochemistry with spectroscopy. Therefore, SEC provides simultaneously in a single experiment two signals of different nature and completely independent from a chemical system subjected to an oxidation/reduction process. That is to say, by means of this technique, the electrochemical information and the spectroscopic evolution of an electron transfer process are obtained simultaneously, which allows to study a chemical system from two different points of view.^{6,7} This powerful technique has a great future prospect due to the advantages obtained when the best qualities of each of these techniques are combined.^{7,8}

Electrochemistry by itself is very useful in the field of electroanalysis for the study of electroactive species, since it reports the processes that take place on the electrode surface. It provides information quickly and efficiently about the redox behavior of electroactive species, by recording the variation of the current intensity or of the potential.⁹ Electrochemical techniques are characterized by their sensitivity, accuracy and high precision using low cost and easy portable equipment,^{10,11} and they have been widely used in the fields of chemistry, physics and biology for many studies.¹² Nevertheless, this technique has some limitations when it is used to study complex systems, in which two or more compounds are adsorbed on the electrode surface. This limitation is due to the fact that the simultaneous study of complex matrices formed by several analytes usually causes an overlapping of the electrochemical signal, which makes it difficult to differentiate each one of them. At this point, the combination of electrochemistry with a spectroscopic technique could handle this limitation due to the complementary

information that spectroscopy provides about the system under study. Whereas electrochemistry provides information on kinetic and thermodynamic processes, spectroscopy provides information on molecular electronic/vibrational processes.^{8,13} It is uncommon and almost impossible to find different compounds exhibiting the same electrochemical and spectroscopic behavior. So, it is very likely that compounds that are highly interfering and have similar electrochemical responses can be identified thanks to the spectroscopic response, which is one of the most powerful advantages of SEC in chemical analysis.

SEC is a quite versatile technique, since, as can be inferred, several different electrochemical techniques can be chosen, and different spectral regions can be explored. Accordingly, a wide range of combinations can be applied, depending on the system under study.⁷ Furthermore, the dynamic nature of SEC allows the recording of time-resolved spectroscopic information that is essential for the understanding of electrochemical processes.⁷ In other words, SEC allows performing operando experiments, where thanks to the spectroscopic signal it is possible to characterize and identify products and intermediates in an electrochemical process. In this sense, *in-situ* spectroscopic information about the electrogenerated species is obtained at any time, under potential control.^{7,8}

All these advantages makes SEC a powerful multi-response technique, which has been widely used for the study of reaction mechanisms,^{14–17} conductive polymers,^{18,19} carbon materials,^{20–22} supercapacitors,^{23–25} solar cells,^{26,27} memory devices,²⁸ for the follow-up of the synthesis and characterization of nanoparticles^{29–31} and in other fields, such as in electrocatalysis^{32–35} or in the characterization of metallic complexes.^{36–39} Without overlooking the importance of SEC in quantitative analysis,^{6,40–43} where the study, determination and quantification of compounds of biological interest are acquiring a great importance.

It is a fact that SEC is a technique that provides many advantages over the application of electrochemistry or spectroscopy separately, but to get the most out of it, it is also essential to carefully choose the most appropriate spectroscopic technique and set-up for each situation. Taking into consideration that spectroscopy techniques are based on the interaction of electromagnetic radiation with matter, which may involve processes of absorption, emission or scattering of light, several spectroscopic techniques are known,

which leads to different SEC techniques.^{7,8} Among these the most common are the following:

- **UV/Vis absorption SEC.** It provides electronic information about a system involved in a redox process.
- **Photoluminescence (PL) SEC.** It is applicable to the study of systems involving luminescent species and is considered one of the most sensitive and selective technique.⁴⁴
- **Raman SEC.** It is a vibrational technique widely used to study species that are adsorb on the electrode and to detect changes in the chemical composition of the electrode.⁴⁵
- **Infrared (IR) SEC.** It is also a vibrational technique that allows the study of molecules adsorbed on substrates.
- **Nuclear magnetic resonance (NMR) SEC.** It is used to record the structural changes of the components involved in an electrochemical reaction. In this way, the ability to record the NMR spectra *in-situ* allows the reaction pathway of the electrochemical oxidation to be elucidated.^{46,47}

In addition, other techniques such as electron paramagnetic resonance (EPR)^{23,48,49} and X-ray diffraction^{50,51} have also been coupled to SEC. Among the just mentioned SEC techniques, UV/Vis absorption SEC, photoluminescence (PL) SEC and Raman SEC are the most used from a quantitative and qualitative point of view. In this thesis, UV/Vis SEC is going to be the main one, although its coupling with these two other spectroscopic techniques (PL and Raman) will also be highlighted. Therefore, the following sub-Sections will focus on the description of these three multi-response techniques (UV/Vis SEC, PL-SEC and Raman SEC).

1.1.1. UV/Vis absorption spectroelectrochemistry

As mentioned above, due to the importance of UV/Vis SEC in this work, we will start by describing this technique, which arises from the combination of electrochemistry with UV/Vis absorption spectroscopy. In absorptometry measurements, the light beam strikes a system with an initial intensity (I_0) and leaves it with another intensity (I). If the analytes of the chemical system absorb part of the initial intensity, the transmitted intensity (I) will be lower than the incident intensity (I_0). The relationship between both intensities is

known as transmittance (T) and this phenomenon is based on the Lambert-Beer law (Equation 1-1) which is used for quantitative purposes.⁶

$$A = -\log(I/I_0) = -\log T = \varepsilon_\lambda \cdot b \cdot C \quad \text{Equation 1-1}$$

where A is the absorbance, ε_λ ($\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) is the molar absorptivity coefficient at a specific wavelength, b (cm) is the optical pathway, C ($\text{mol} \cdot \text{L}^{-1}$) is the concentration of the absorbent specie, and T is the transmittance.

Absorbance yields usually a positive value when the light is compared with a solution without the absorbing analyte, but it can take negative values when the initial light is sampled in presence of the analyte (reference spectrum) and the analyte is transformed during an experiment, as usually takes place in SEC.

UV/Vis SEC provides in a single experiment the variation of the absorbance of a system involved in a process of oxidation-reduction.^{30,52,53} In its beginnings, the experiments were performed under conditions close to equilibrium and it was mainly used to study reaction mechanisms and to determine electrochemical parameters (number of electrons exchanged in the electrode process,⁵⁴ electron-transfer rate,⁵⁵ diffusion coefficients,^{56,57} and formal potentials).⁵⁴ This technique can also be implemented to characterize new materials and perform electrocatalytic studies *in-situ* and *operando*. In these early stages, quantitative analysis studies are scarcely found. It was not until the end of the 20th century and the beginning of the 21st century when most analytical applications were developed, coinciding with the development and improvement of the experimental set-ups. Nowadays, UV/Vis SEC is becoming increasingly important in this field of quantitative analysis, since the facility to continuously record the electrochemical signal, at the same time as recording spectra, provides the information needed to determine the components of a complex mixture, as well as the information required to explain the electron transfer processes. Moreover, UV/Vis SEC can be applied to quantify a wide variety of compounds and it is considered an outstanding technique to troubleshoot complex matrices without carrying out any previous pre-treatment of the sample. This is feasible because it is unlikely to find two compounds with the same electrochemical behavior and the same spectra, despite possible overlaps. That is, even if the compounds have the same electrochemical behavior and their spectra are overlapping, UV/Vis SEC is a very powerful technique to resolve such complex matrices. This quantitative application will acquire great prominence in this doctoral thesis, as will be demonstrated in the Chapter

6, where UV/Vis SEC is used to quantify some analytes of pharmacological interest in a real matrix, such as are folic acid (Section 6.2) and ofloxacin (Section 6.3). Furthermore, it should be noted that in some situations it is necessary to use multivariate statistical tools to successfully separate the analytes present in the mixture, as will be demonstrated in the Section 6.4, where the derivative UV/Vis coupled with PARAFAC make possible the quantification in a complex matrix of three highly interfering analytes (dopamine, ascorbic acid and uric acid).

Another interesting application of UV/Vis SEC can be found in the preparation of biosensors. For example, a method to prepare sensors consists on the electrodeposition of nanoparticles (NPs). The electrodeposition process and the modification of the electrode surface can be followed *in-situ* by UV/Vis SEC. Information on whether the electrode has been successfully modified is obtained immediately, saving the time it would take to analyze the surface with high resolution microscopy techniques. Analysis with these microscopic techniques requires the use of expensive instrumentation, which is not affordable for many researchers. In addition, electrodeposition processes are not reproducible and the NPs can change with the time.³⁰ Thus, *ex-situ* characterization does not guarantee that the NPs used for determination are the same as those used as a sensor. For this reason, UV/Vis SEC is a good alternative to obtain this information, as it has been demonstrated in previous investigations of our research group with the publication of an article where it was demonstrated that UV/Vis SEC provides reliable information on the gold NPs electrodeposited on a Pt electrode.³¹ Subsequently, a similar study was carried out in which this technique was applied to follow *in-situ* the electrodeposition of Ag NPs on a Pt screen-printed electrode (SPE). This modified electrode was then used as a sensor for the detection of hydrogen peroxide.³⁰ In summary, UV/Vis SEC is above all an optimal technique to follow the electrosynthesis of NPs exhibiting a characteristic plasmonic band such as gold or silver. It provides *in-situ* spectral information that allows to identify the type of NPs deposited on the electrode quickly. This low-cost method compared to high-resolution microscopy techniques, such as Scanning Electron Microscopy (SEM), also guarantees the successful modification of the electrode.

In an UV/Vis SEC experiment, the light beam can interrogate the working electrode and the adjacent solution to it in different ways, thus two optical arrangements known as normal configuration and parallel configuration are distinguished.⁷ The following sub-Sections are focused on explaining these configurations.

1.1.1.1. *Normal Configuration*

In normal arrangement, the light beam samples the electrochemical system following a perpendicular direction to the working electrode (WE) surface (Figure 1-1), registering spectral changes that take place both in the solution and at the WE surface. Two normal modes can be distinguished: normal transmission (Figure 1-1a) and normal reflection (Figure 1-1b).

- In **normal transmission**, light passes through the WE making the use of optically transparent electrodes (OTEs) mandatory. These OTEs are materials with good optical transparency and electrical conductivity, which allow the light beam to pass through the electrode.^{58,59} Initially, indium tin oxide (ITO) films were the most widely material used as OTE.⁶⁰ However, the limitations of the indium sources and of the narrow range of potentials that can be applied in this type of materials, have led to seek for other alternatives, among which are metallic meshes, carbon nanotubes electrodes or thin metallic films.⁶¹ Unfortunately, meshes were not the best options. Diffusion around the mesh is not easy and some thin noble metals films are not very good conductors, requiring a layer of material to improve their conductivity, which results in a decrease in transparency. For these reasons, carbon nanomaterials, such as carbon nanotubes and graphene, are considered as the best option for OTEs. These materials allow working in a very broad range of potentials, and they have outstanding electrical and optical properties, in besides being transparent materials with high conductivity.^{62,63} It should be pointed out that the optical path-length coincides with the thickness of the diffusion layer, in the order of micrometers.
- In **normal reflection** arrangement, as its name suggests, the light beam is reflected on the surface electrode, thus the diffusion layer is sampled twice, achieving a better sensitivity than the obtained with OTEs.⁷ Although, it was not widely used in its early days, the development of set-ups in which optical probes were used to carry out the light beam has made it more successful than normal transmission. In this mode, a better sensitivity is achieved an almost any material can be used as a working electrode, being gold, platinum, and carbon the most common.⁷ In addition, screen-printed electrodes (SPEs) have gained considerable prominence and are being extensively used for this type of experiments. SPEs have the advantage of being electrodes that require a small sample volume,

guarantee reproducibility between measurements and are disposable.⁶⁻⁸ There are situations where an electrode is needed for each measurement, since in some systems the electrogenerated product is adsorbed on the surface of the electrode, as occurs during the oxidation of dopamine at pH=7.^{6,64} SPEs are very suitable for studying this type of systems since the use of an SPE for each experiment guarantees reproducibility.

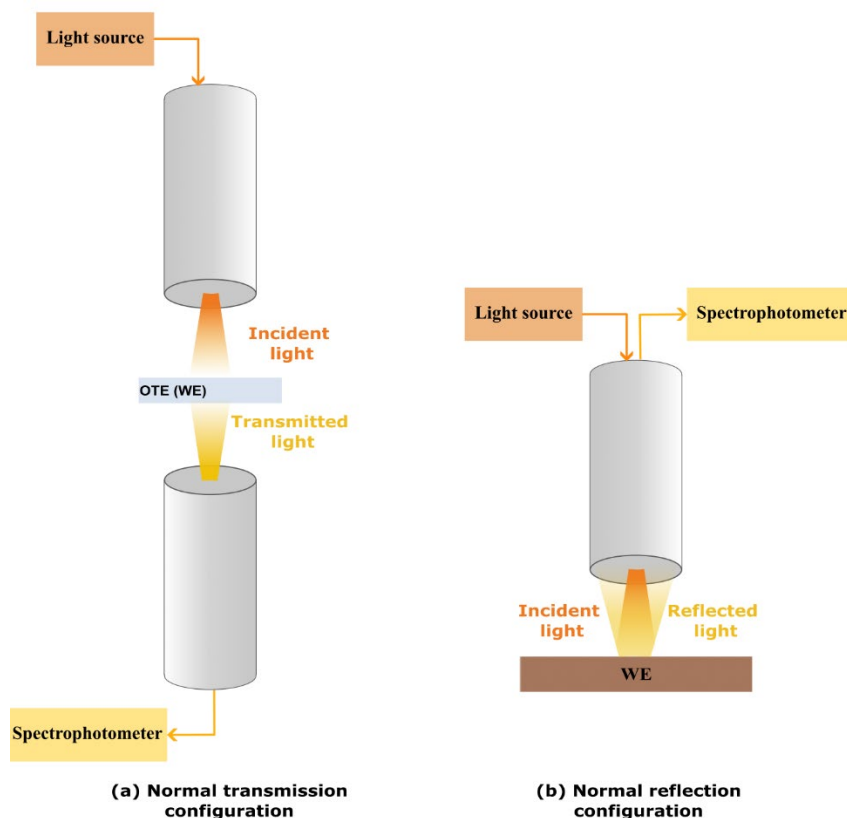


Figure 1-1. Schematic representation of the normal configuration UV/Vis SEC. (a) Normal transmission configuration, (b) Normal reflection configuration.

1.1.1.2. *Parallel Configuration*

In the parallel configuration the light beam follows a parallel direction to the electrode surface and only the first micrometers of the solution closest to the working electrode are sampled. In this case, with the spectroscopic response it is not possible to track the changes occurring on the surface electrode. Hence, the spectral changes recorded are related to the diffusion layer.^{65,66} A schematic view of this configuration is depicted in Figure 1-2, which shows how the light beam coming from the light source interrogates the chemical process in an arrangement parallel to the working electrode. The spectral

changes are collected in the spectrometer, which is synchronized with the potentiostat.⁴² This mode usually improves the sensitivity to soluble compounds with respect to the normal arrangement, since the optical pathway is longer than the diffusion layer, as can be as longer as the length of the WE, which leads to a higher sensitivity. Therefore, using the parallel arrangement is possible to achieve lower detection limits.⁴² However, the experimental set-up for this configuration has historically been more difficult to implement because of the difficulty of aligning the light beams. This drawback is being overcome using optical fibers.

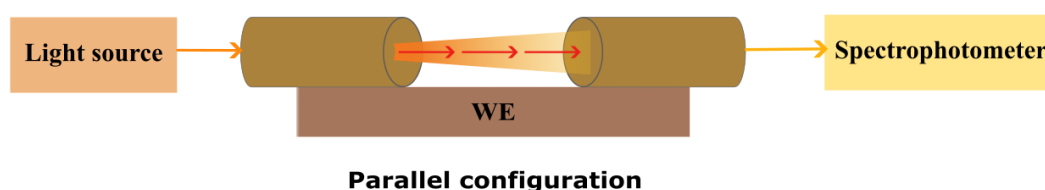


Figure 1-2. Schematic representation of the parallel configuration UV/Vis SEC.

Parallel SEC allows performing experiments in both semi-infinite and thin-layer regime, depending on the set-up used. On the one hand, the use of bulk cells allows working in semi-infinite diffusion conditions, which is more convenient to study the mechanism of a reaction in a similar way to the classic electrochemical experiments. On the other, by using thin-layer cells we work in a steady-state which could be very useful in the quantification field, where total electrolysis of the analyte occurs because in this regime the solution is confined in a small volume. Nevertheless, the use of separators is required to control the thin-layer thickness. These cells usually are limited by the high ohmic drop if the three electrode system is not well-designed.^{66,67} Over the years, different thin-layer cells have been developed, outstandingly those proposed by Heineman et. al,^{68,69} which consist of a transparent inert wall and are designed for OTEs. In addition, other approaches based on thin spectrophotometric cuvettes^{70,71} and disk electrodes^{57,67,72} have also been developed.

Recent advances based on SPEs have been demonstrated to be much easier to implement. In this sense, to perform measurements in the semi-infinite regime the alignment of two optical fibers on the working electrode (Figure 1-3a) is proposed. However, this experimental set-up, based on attaching the optical fibers to the electrode, is not useful for systems where the electrochemically generated product is adsorbed on the surface

electrode, because it will cause the modification of the electrode surface and the electrode fouling. Any modification of the electrode surface requires the electrode to be rendered unusable and therefore for each of the measurements a new electrode or a polished electrode has to be used.⁴² Moreover, in this case, it would also be unfeasible to change the fibers to another WE, since the reproducibility within the set of experiments would be absolutely lost, because the distance between the fibers may change, affecting the optical pathway.⁴² For this reason, a new set-up based on the fixing of the optical fibers on a quartz plate was proposed, (Figure 1-3b), which was then placed on the WE.⁵² This second set-up is quite simple and easy to use, but in this case is mandatory to carry out measurements in thin-layer regime. It should also be noted that, despite the advantages of using optical fibers, there is a drawback due to the cladding of these fibers, which is usually around 20-30 μm thick. This means that these tens of micrometers of the solution closest to the working electrode are never interrogated.

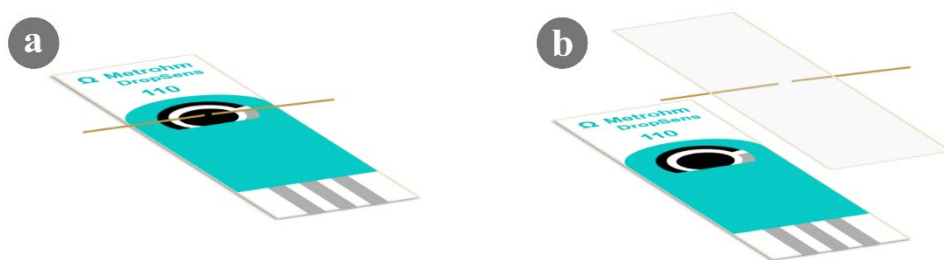


Figure 1-3. UV/Vis parallel set-ups based on the alignment of the fibers (a) on the surface electrode and (b) on a quartz place.

Considering that working in semi-infinite regime is more common in electrochemistry and taking into account that these conditions are more suitable to understand and explain electrochemical processes because most of the electrochemistry models are based on this approach, this doctoral thesis will address the challenge of developing a new cell that allows us to perform UV/Vis SEC in parallel configuration and in semi-infinite diffusion regime, being able to change the electrode without modifying the optical pathway. This cell will be described in Section 6.3.

To sum up, parallel configuration is more convenient for monitoring those processes in which the products generated on the electrode process are soluble compounds that diffuse away from the working electrode surface, since the optical path-length will be longer than the one of normal configuration. On the one hand, parallel arrangement allows us the

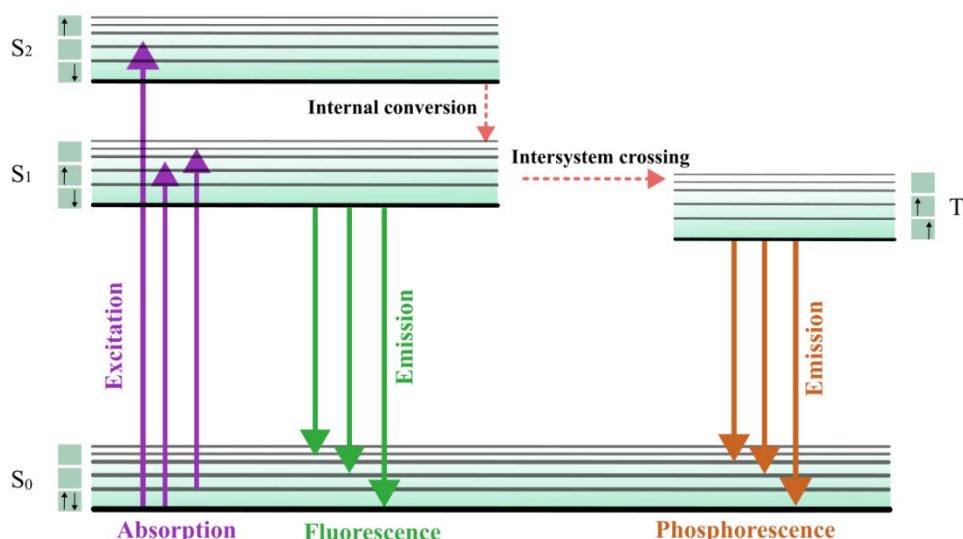
detection of lower analyte concentrations, being more convenient to perform quantitative analysis, since its great sensitivity could facilitate the quantification in real matrices. On the other hand, this configuration does not allow to register the spectral changes that take place on the electrode surface, therefore it will not be useful for monitoring chemical compounds or materials which are adsorbed on the surface electrode, such as for example polymerization processes, in which the use of the normal configuration is mandatory. Hence, the importance of selecting the correct UV/Vis SEC set-up is critical to extract the most representative picture of the electrode process. Moreover, the combination of the two configurations in one experiment, which is denoted as bidimensional spectroelectrochemistry,⁶⁶ is the best solution to understand the electrode process.

1.1.2. Photoluminescence spectroelectrochemistry

This Section is focused on the description of another SEC technique used during this doctoral thesis, which combines PL with electrochemistry. But, before emphasizing the characteristics of this technique, as well as the devices that allow us to perform experiments using PL-SEC, a brief review of the theoretical basis of PL will be presented.

PL is a photon emission process that occurs for particular molecules from an electronic excited state. To observe the PL phenomenon, the excitation of the molecule from the lowest vibrational level of the ground state to another vibrational level in the electronic excited state must first occur. After this excitation, the molecule is relaxed and returns to the ground state with a photon emission. Depending on the energy of the electronic excited state, two different types of PL processes can be distinguished: fluorescence and phosphorescence.^{73,74} The difference between the two PL processes is represented in the Jablonski diagram shown in Figure 1-4.

- **Fluorescence** emission occurs when the molecule passes from a singlet electronic excited state to another vibrational level in the electronic fundamental state. This process occurs rapidly, which implies that the fluorescence lifetime is in the order of nanoseconds.⁷³⁻⁷⁵
- **Phosphorescence** emission takes place when the molecule decays from a triplet electronic excited state to another vibrational level in the electronic fundamental state. This transition involves a change of multiplicity, which is the reason for the longer lifetime of phosphorescence, being of the order of microseconds to seconds, or even much longer.⁷³⁻⁷⁵

Figure 1-4. Jablonski diagram.⁷³

Chemical structure of the compounds is fundamental to be fluorescent. Usually aromatic compounds or those which contain conjugated double bonds are fluorescent compounds, but not all organic compounds show fluorescence.⁷⁵ In addition, the photon emission process can be accompanied by non-radiative processes that decreases the fluorescence intensity, which is known as fluorescence quenching, and it is caused by molecular interactions between the fluorophore and quenchers. Molecular oxygen is one of the best-known quenchers and sometimes the system requires the prior removal of the oxygen present in the sample to obtain adequate fluorescence yields.⁷³ There are many processes which can affect the fluorescence as for example, photobleaching, pH, temperature, concentration, solvents, i.e.^{73,75–77}

PL-SEC register the emission spectra changes of fluorescent compounds during an electrode process.⁷⁸ There are different possibilities in PL-SEC, from the generation of a fluorescent compound to the removal of it, or the generation or removal of quenchers in solution. Thus, by oxidation/reduction of the reagents, changes in the spectra occur. In the meantime, oxidation/reduction of other compounds present in the sample can enhance or quench the fluorescence. So, both responses can be used to identify and quantify the fluorescent species present in the sample. This technique allows recording both the changes upon the electrode surface and in the diffusion layer.

Good alignment and focusing of the incident and collected beams in the same position is mandatory to obtain good PL measurements and, therefore, to reach good detection limits.

Possible interference with ambient light must also be taken into account.⁷ So, one of the main challenges of PL-SEC was the development of systems that would help to avoid interference from fluorescent molecules located in the bulk solution. By removing background fluorescence, the signal-to-noise ratio and resolution will be improved. To this end, several strategies have been proposed, as cited below:

- **Epifluorescence microscopy with a thin-layer cell.** In epifluorescence microscopy a parallel beam of light passes straight up through the sample, which maximizes the amount of illumination. It is a powerful technique characterized by high sensibility and high microscopic resolution. In addition, working in a thin-layer regime yields a higher photoluminescence signal, since most of the reactant is transformed.⁷⁹
- **Confocal microscopy.** It has a pinhole detector aperture to filter out light from outside the focal plane of the objective lens. In this sense, a clean PL signal is registered, since this response comes only from the focal plane.⁸⁰ It has been successfully used for quantitative analysis in cells and tissues.⁷⁶
- **Total internal reflection fluorescence (TIRF).** The excitation of the fluorophore is enabled in a thin region of a sample, usually less than 200 nm thick. In this method the excitation light is totally reflected inside a transparent solid at its interface solution, generating an electromagnetic field (an evanescent wave) at the interface. For this purpose, the excitation incident angle is set to a value greater than the critical angle, achieving total reflection of the incident light beam.^{77,81}

These strategies require expensive and tedious instrumentation, but it must be recognized that reliable PL-SEC measurements can also be obtained without a perfect focus. Nevertheless, the development of a simple and reproducible PL-SEC set-up is a challenge, and here again we are faced with one of the major problems of SEC, the lack of commercial devices that allow this type of experiments. The simplest way to perform PL-SEC measurements is the one based on the normal reflection configuration explained in the previous Section for UV/Vis SEC. However, a side configuration is a better choice, since this approach avoids the influence of reflected radiation from the light source.

The selectivity and sensitivity of PL-SEC is much better than the one of UV/Vis SEC, as changes in emission intensity are more easily detected than changes in absorbance. This strength of PL-SEC together with the electrofluorochromism phenomenon, whereby the fluorescence of molecules can be converted electrochemically, has made this technique

very attractive for analytical purposes.⁸² In this regard, the development of electrochromic devices (ECDs) has acquired great importance. Organic conducting polymers, and especially polyoxometalates (POMs), are promising candidates for the construction of these devices.⁸³

1.1.2.1. Side Configuration

In PL-SEC the optimal arrangement is the side configuration since it allows avoiding the influence of reflected radiation from the light source. In this approach, both the emission and the collected fibers are focused on the electrode with an angle of less than 90° , usually between 45° and 60° , being the illumination and collection fibers parallel between them and as close as possible, as it is shown in the schematic view of Figure 1-5.

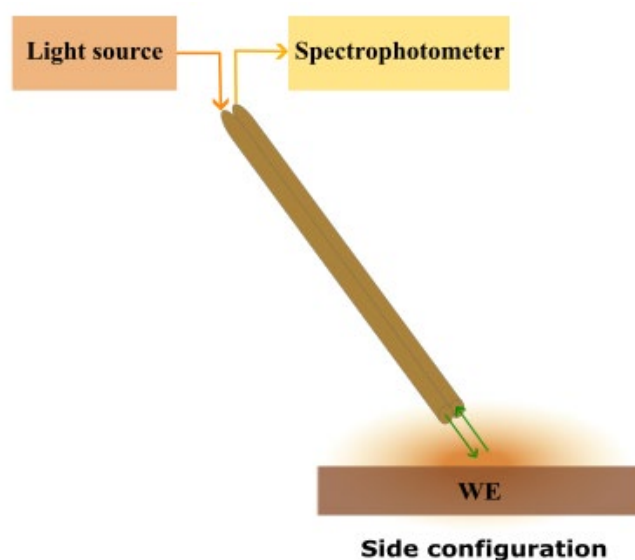


Figure 1-5. Schematic representation of side configuration in PL-SEC.

1.1.3. Raman spectroelectrochemistry

Lastly, Raman SEC is a technique that combines Raman spectroscopy with electrochemistry. Following the same explanation protocol than UV/Vis SEC and PL-SEC, a very brief description of Raman spectroscopy will be presented, before focusing on Raman SEC.

Raman spectroscopy is named after its discoverer, C.V. Raman, and Indian researcher who reported this effect in 1928 along with his coworker and student K.S. Krishnan.⁸⁴

Two years later, in 1930, C.V. Raman was awarded the Nobel Prize in physics for this breakthrough.

Raman spectroscopy is a scattering technique based on the inelastic scattering of the incident light due to its interaction with vibrating chemical structures.⁴⁵ For this phenomenon to take place, the sample must be illuminated with a monochromatic laser beam, and after its interaction with matter, scattered light is originated in all directions. Most of this scattered light has the same frequency as the incident radiation, which constitutes Rayleigh scattering, which is an elastic phenomenon. In turn, a small fraction of the scattered light has a frequency different from that of the incident light, giving rise to an inelastic process denoted as Raman scattering. The latter phenomenon is possible because the electrons of the molecule involved in the process does not return to the initial vibrational level, returning either to a higher or lower energy level than the initial one. Accordingly, two Raman scattering processes can be distinguished: Raman Stokes and Raman Anti-Stokes. In Raman Stokes scattering, the scattered photon is less energetic and has a longer wavelength than the incident one. Whilst in Anti-Stokes Raman, the scattered photon has more energy than the incident one, because the struck molecule has transferred part of its vibrational energy to this photon. In the first one, Raman Stokes scattering, the excited electron of the molecule is placed in the fundamental vibrational state, while in the Anti-Stokes scattering the excited electron of the molecule is located in an excited vibrational state. As a consequence, Raman Stokes scattering is a much more probable phenomenon, therefore, Stokes bands are more intensity than the Anti-Stokes ones.^{45,85,86} The simplified energy diagrams of all these processes are depicted in Figure 1-6.

Raman spectroscopy is frequently compared to IR spectroscopy because both techniques provide information about the vibrational models of a molecule. However, there are noticeable differences between the two techniques. Vibrational modes which implies a change in the polarizability of the molecule are active in Raman, whereas, vibrational modes which imply a change in the dipole moment of the molecule are active in IR. Therefore, in Raman scattering there is no water interference, being possible to record Raman spectra in aqueous media, which is not feasible for IR.⁸⁵ Therefore, Raman can be more easily combined with electrochemistry where most of the studies are performed in aqueous solution. It is also worth mentioned that Raman spectroscopy is considered one of the most powerful techniques for qualitative analysis, as Raman spectra provide real

fingerprints of the molecules. However, despite all these advantages, Raman scattering is a very poor phenomenon in intensity for quantitative purposes, since the probability of occurrence of this process is truly small. It has been reported that out of every 10^3 photons that show Rayleigh scattering, only one undergoes Raman scattering.⁸⁷ Consequently, the Raman signal obtained is too weak to be used for quantitative analysis.

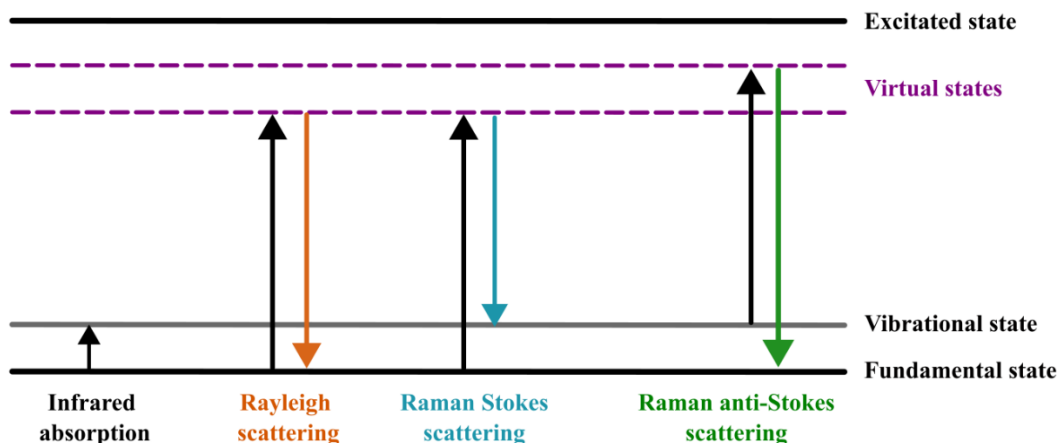


Figure 1-6. Energy diagrams of Rayleigh scattering, Raman Stokes scattering and Raman Anti-Stokes scattering.

In the early years, Raman spectroscopy was an underdeveloped technique. It was not until the 1960s that this technique began to gain importance, which concurred with the development of the laser's sources capable of emitting high power monochromatic light. However, the improvement in the light source is not enough to improve the lack of sensitivity.⁸⁸ In the mid-1970s, a new phenomenon termed *Surface-Enhanced Raman Scattering* (SERS) was discovered, which brought about a major revolution in the Raman field. From this moment on, Raman became a sophisticated vibrational spectroscopic technique widely used in materials, chemical and life sciences.^{8,89}

1.1.3.1. *Surface-Enhanced Raman Scattering (SERS)*

SERS was the first documented phenomenon capable of increasing Raman intensity. It was discovered in 1974 by M. Fleischman and co-workers (P.J. Hendra and A-J. McQuillan) when they detected an unusual enhancement in the Raman intensity of pyridine upon roughening a silver surface electrode.⁹⁰ They considered that the roughening of the surface leads to an increase in the number of species adsorbed in the surface area, which explains the high-quality Raman spectra obtained. This fact was

subsequently studied by R. P. Van Duyne and D.L. Jeanmaire⁸⁸ and J.A. Creighton,⁹¹ who proved that the roughness of the surface is not enough to achieve high enhancement of the Raman spectra. Nowadays, there is full consensus on the factors contributing to this enhancement, which are grouped under two main mechanisms, which take place simultaneously: Electromagnetic and Chemical.⁹²

In the Electromagnetic mechanism (EM), the interaction between the electric field of the light and the surface plasmon is responsible of the amplification of the Raman signal. Thus, the metallic nanostructures present on the surface of the metallic electrode undergo plasmonic resonance, which induces an enhancement of the incident electric field, and consequently of the Raman intensity. This is the most prevalent effect, and it has been estimated that the EM is able to increase the Raman signal in a factor between 10^6 and 10^9 .⁹² The chemical mechanism (CM) implies a chemical interaction between the molecule and the substrate, which usually involves a charge transfer (CT) between both. It has been estimated that CM is capable of increasing the Raman signal between 10^1 and 10^3 times. Although theoretical studies have shown that CM can cause a Raman signal enhancement of 10^7 orders of magnitude.⁹³

SERS substrates

Since its discovery, SERS has become increasingly important for analytical purposes.⁹² The high sensitivity and the non-destructive nature of SERS makes this technique one of the most promising analytical tools for the near future.^{86,92} However, despite the efforts made, the drawbacks related to its lack of reproducibility have not yet been solved, because the generation of reproducible SERS substrates is not an easy task. This irreproducibility is mainly because SERS substrates present heterogeneous areas where the Raman signal is highly amplified, denoted as hot spots.⁷

The properties of the substrate play a fundamental role in the SERS phenomenon. Several methods to generate such substrates are described in the literature, among which metallic nanostructures and semiconductor/dielectric substrates stand out. In this doctoral thesis, the emphasis will be made on metallic nanostructures, since nanoparticles (NPs) are going to be used to generate SERS substrates. Moreover, this type of SERS substrates is widely used for analytical purposes.⁹⁴⁻⁹⁸ The metallic NPs more common used to generate SERS substrate are coinage metals, such as gold, silver and copper, as they are materials which exhibit plasmonic properties.⁸⁵

NPs can be synthesized chemically or electrochemically.^{85,92} The application of electrochemical oxidation-reduction cycles (ORC) was one of the first strategies to generate roughened substrates. Different parameters, such as the electrochemical protocol applied, the metal surface and the electrolytic medium must be considered to obtain sensitive and reproducible substrates. Nevertheless, with this protocol, the morphology of the nanostructures cannot be easily controlled, making the chemical method a more suitable protocol for the generation of NPs with a well-defined shape and size. The chemical synthesis of NPs procedures consists of the chemical reduction of the metal ion with a reducing agent, such as citrate or sodium borohydride. This protocol allows controlling the size and shape of the generated NPs.²⁹ In this second case, the combination of SERS with electrochemistry acquires great prominence, since electrochemistry is used to adsorb the analyte on the plasmonic surface, giving rise to an increase in the analytical signal. As has been demonstrated by Brosseau et al. who have used Au and Ag modified electrodes to determine uric acid in synthetic urine. In such paper, uric acid is electrochemically adsorbed on the modified electrode, demonstrating that electrochemistry is essential to increase Raman intensity.⁹⁹ EC-SERS also plays a key role when it comes to detecting intermediate species.^{8,85}

In EC-SERS, the supporting electrolyte must be free of Raman active species or have vibrational modes perfectly distinguishable from those of the target molecule. The most commonly used electrolytes used are sulfates, perchlorates and phosphates.⁸⁵ Indeed, EC-SERS is well suited for detecting biological samples, since typical biological solutions and synthetic bodily fluids are perfect supporting electrolytes for EC-SERS. For instance, levofloxacin has been detected in synthetic urine by EC-SERS.¹⁰⁰ Aside from the interest of EC-SERS in the medicine^{101,102} and pharmacology areas,¹⁰³ it is also gaining great interest in the fields of environment,^{104,105} food contaminants¹⁰⁶ and catalysis.^{89,107,108} Furthermore, SERS is also used for trace^{106,109} and ultra-trace analysis¹¹⁰ due to its high sensitivity; and its non-destructive character and selectivity also makes this technique a powerful tool for the characterization of different materials.¹¹¹ Apart from this, time-resolved Raman SEC is also very useful not only to follow the modification of the electrode surface, but also where these changes are taking place. In addition, this technique provides also information about possible molecules adsorbed on the electrode and their transformation.⁷

2. COMBINATION OF SPECTROELECTROCHEMISTRY TECHNIQUES

Summary

In the previous Chapter, three main SEC techniques (UV/Vis SEC, PL-SEC and Raman SEC) have been explained. The large amount of information provided by a SEC experiment is a sheer fact. And without overlooking this, advances in SEC have led to experiments where not only is it possible to obtain two signals simultaneously, but also the coupling of two spectroscopic techniques has allowed the simultaneous recording of three signals completely independent of each other. The first approaches in which two spectroscopic and one electrochemical signal were obtained were achieved with a technique designated as UV/Vis Bidimensional SEC (BSEC). Different BSEC devices and experiments are reported in literature, as shown below. In addition to the different BSEC approaches and the advancement that this technique has undergone over the years, recently, different spectroscopic techniques have been coupled to electrochemistry. Therefore, the different studies reported so far because of the coupling of two spectroscopic techniques together with electrochemistry are collected, showing the benefits derived from it. And finally, different experimental devices are described where, this time, the results obtained come from the coupling of three spectroscopic signals and an electrochemical one.

Therefore, the main objectives of this chapter are as outlined below:

- To describe UV/Vis BSEC, its emergence, progress, experimental set-ups, and advantages of this technique.
- To describe the different spectroscopic techniques that have been combined so far in a SEC experiment.
- To propose other spectroscopic combinations and the development of the appropriate experimental set-ups that allow the recording of this type of experiments.

Resumen

En el capítulo anterior se han explicado las tres principales técnicas SEC (SEC de absorción en el UV/Vis, SEC de PL y SEC Raman). Es un hecho evidente, la gran cantidad de información que proporciona un experimento de SEC. Y sin pasar esto por alto, los avances en SEC han desembocado en experimentos en los que no sólo es posible obtener dos señales simultáneamente, sino que el acoplamiento de dos técnicas espectroscópicas ha permitido el registro simultáneo de tres señales completamente independientes entre sí. Las primeras aproximaciones en las que se obtuvieron dos señales espectroscópicas y una electroquímica se lograron con una técnica denominada SEC Bidimensional UV/Vis (BSEC). En la literatura se han descrito diferentes dispositivos y experimentos BSEC, como se muestra a continuación. Además de los diferentes enfoques BSEC y del avance que esta técnica ha experimentado a lo largo de los años, recientemente se han acoplado técnicas espectroscópicas de diferente naturaleza a la electroquímica. Por lo tanto, se recogen los diferentes estudios reportados hasta el momento debido al acoplamiento de dos técnicas espectroscópicas junto con la electroquímica, mostrando los beneficios derivados de ello. Y, por último, se describen diferentes dispositivos experimentales donde, en esta Tesis, los resultados obtenidos provienen del acoplamiento de tres señales espectroscópicas y una electroquímica.

Por tanto, los principales objetivos de este capítulo son los que se exponen a continuación:

- Describir la técnica conocida como UV/Vis BSEC, su aparición, progreso, montajes experimentales, y ventajas de esta técnica.
- Describir las diferentes técnicas espectroscópicas que se han combinado hasta ahora en un experimento de SEC.
- Proponer otras combinaciones espectroscópicas y el desarrollo de los montajes experimentales adecuados que permitan registrar este tipo de experimentos.

2.1. UV/Vis Bidimensional spectroelectrochemistry (BSEC)

Bidimensional SEC (BSEC) is a powerful multi-response technique resulting of the combination of the two optical arrangements described in the previous chapter (normal configuration and parallel configuration).⁶⁶ So, some considerable advantages can be expected over performing experiments using one of the optical arrays, since it provides three signals in a single experiment (one electrochemical and two spectroscopic).⁶⁶ In BSEC two beams of light are incident on the solution under study: one of them follows a perpendicular direction to the electrode surface, whereas the other one passes through the solution following a direction parallel to the electrode surface,⁶⁶ as is shown in Figure 2-1. Through the combination of the two configurations, changes both on the electrode surface and in the bulk solution are recorded simultaneously. This made BSEC a very useful technique for tracking diffusive and adsorptive processes.⁵⁸ Moreover, it provides really valuable information in the study of electro-polymerization processes, since this technique provides information about the different products generated during the electrolysis processes, being able to detect the products adsorbed on the electrode surface and the products that diffuse to the solution.^{58,66}

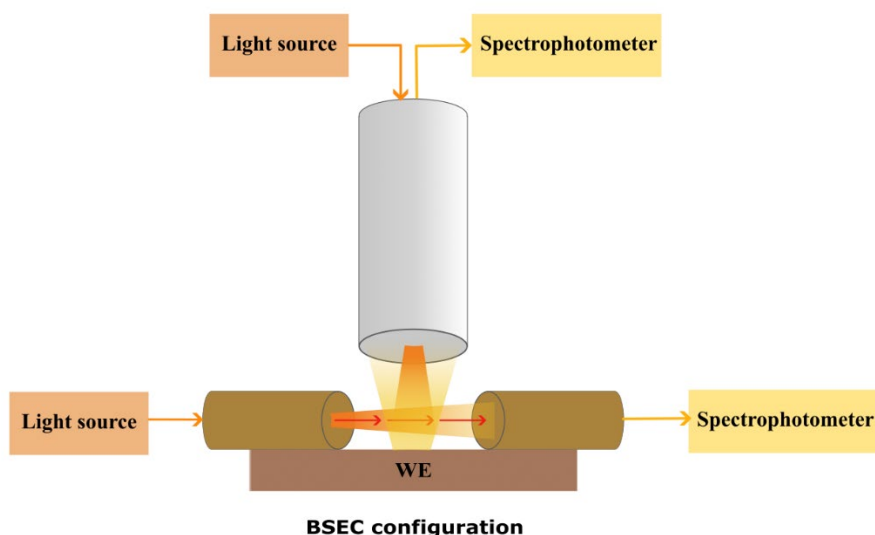


Figure 2-1. Schematic representation of bidimensional configuration in SEC.

This technique emerged in 2001 in our research group, demonstrating that BSEC provides us very valuable information, since depending on the configuration used, certain differences are found in the voltabsorptograms.⁶⁶ BSEC provides information about the

electrode process, being a powerful tool to explain whether the system is defined by a simple electron-transfer or if there are other coupled processes. For example, an adsorption of the products on the electrode surface could be taking place at the same time, and it can be easily identified thanks to this combination.⁷

The emergence of BSEC brought with it the necessary creation of a home-made BSEC cell, such as the one designed in our research group (Figure 2-2) in this same year (2001).⁶⁶ This proposal cell was based on spectrophotometric cuvettes, in which a flat OTE allows the passage of two light beams simultaneously. One of them parallel to the electrode surface, while the other passes through the solution following a perpendicular trajectory respect to the electrode surface. This cell can be used with different types of OTE and can be easily adapted to the system under study. Its versatility, low cost and simplicity of assembly and cleaning have propitiated its use in subsequent years for the study of diverse electropolymerization processes, as well as for the monitoring of polymer degradation processes.

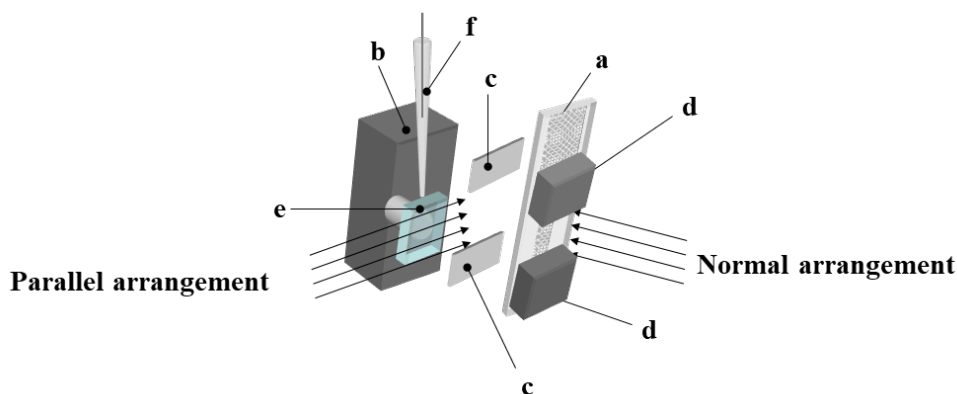


Figure 2-2. Schematic representation of the thin-layer BSEC cell. (a) Gold-sputtered WE; (b) epoxy resin piece; (c) spacers; (d) epoxy resin brackets; (e) circular window; (f) reference electrode. *Reproduced with permission from ref.⁶⁶ Copyright 2001 Analytical Chemistry.*

For instance, in 2001 the electropolymerization mechanism of 4,4'-bis(butylthio)-2,2'-bithiophene was followed by BSEC, concluding that this polymer is generated in two steps: nucleation and growth. Furthermore, this study states that the polymer remains deposited on the surface electrode and that oligomeric soluble species diffuse into the solution.¹¹² The same experimental set-up was applied a year later to follow the electropolymerization process of 4,4'-bis(2-methylbutylthio)-2,2'-bithiophene

(MBTBT), where the formation of the oligomeric species is observed before the polymer deposition on the electrode surface starts.¹¹³

In 2004, the appropriateness of BSEC to elucidate the reaction mechanisms in which chromophores are involved was demonstrated. For this purpose soluble Prussian Blue was electrogenerated from the oxidation/reduction of the $\text{Fe}(\text{CN})_6^{3-} / \text{Fe}(\text{CN})_6^{4-}$ system. This study has contributed to explain that Prussian Blue can be generated by a chemical process that takes place simultaneously with the electron transfer reaction, and that the concentration of the $\text{Fe}(\text{CN})_6^{3-} / \text{Fe}(\text{CN})_6^{4-}$ system has an influence on the generation rate of this soluble Prussian Blue.¹¹⁴

Besides the suitability of this technique to study the mechanisms of polymerization, it is also worth mentioning the possibility of studying the degradation of polymeric films by BSEC. The simultaneous acquisition of three different signals has made it possible to identify soluble and insoluble species, as well as electroactive and inert species implicated in the degradation of polymer films. An example of this is found in a study performed in 2006 by our group, in which the stability during *p*- and *n*-doping of the 4,4'-bis(butylthio)-2,2'-bithiophene polymer was investigated. In this research, UV/Vis BSEC has allowed to detect the soluble degradation products and the potential window in which this degradation occurs.¹¹⁵ Moreover, in the same year, the degradation of polyaniline films was also followed by BSEC where different types of degradation are detected, depending on the applied potential.¹¹⁶

A new BSEC cell was proposed in 2006, with the aim of incorporating a reflection probe that allows the enhancement of the normal signal. This new home-made cell (Figure 2-3) was based on the previous one and was also designed with the aim of being able to insert it into a spectrophotometric cuvette. This cell shares the strengths of the previous one, being an easy to assemble and clean device, with versatility and low cost. The main difference, which in turn constitutes a considerable advantage, is that this cell allows the incorporation of solid WEs, which entails an improvement in the electrical conductivity. In addition, the nature and geometry of the WE can be tailored and several materials such as Au, Pt, Hg or glassy carbon can be used as WEs. This cell was used to follow the compounds released by a polymer film, after having been previously validated with a standard system (*o*-tolidine).⁶⁷

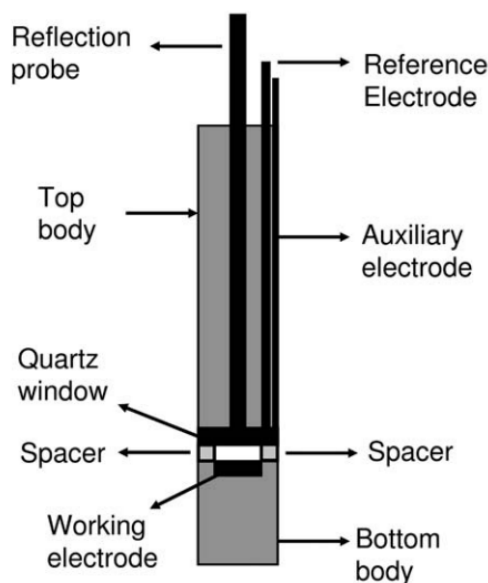


Figure 2-3. Schematic representation of the thin-layer bidimensional SEC cell. *Reproduced with permission from ref. ⁶⁷ Copyright 2006 Electroanalytical Chemistry.*

However, despite all the efforts that have been targeted on the development of BSEC, it has been impossible to find commercial BSEC cells that could facilitate the progress of BSEC as a technique. Up to this point, the cell models described were based on a spectrophotometric cuvette. It was not until 2015 when the first BSEC cell that was not based on a cuvette was developed, also by our research group. This new design was based on optical fibers and also required the use of OTEs, as the light beam would pass through the electrode.⁵⁸ In this model SWCNTs-OTEs were used due to their transparency and good conductivity. Figure 2-4 shows the detailed schematic view of this cell. As can be seen, two optical fibers are aligned following a parallel direction to the working surface electrode; and in turn an optical fiber probe is focused following a perpendicular direction to the working electrode.⁵⁸

A further noteworthy advance took place in 2017, with the development of a cell (Figure 2-5) for the study of an *o*-vanillin-copper (II) complex. This set-up, unlike those shown so far, does not require the use of OTEs, as the normal arrangement is based on a normal reflection configuration. Thus, a glassy carbon disk will be used as the WE, replacing the use of OTEs, which, at this stage, was deemed a significant advance in SEC.¹⁵ Moreover, this new cell uses a piezoelectric positioner to move an external slit in order to fix with high precision the position of the parallel beam respect to the electrode surface.

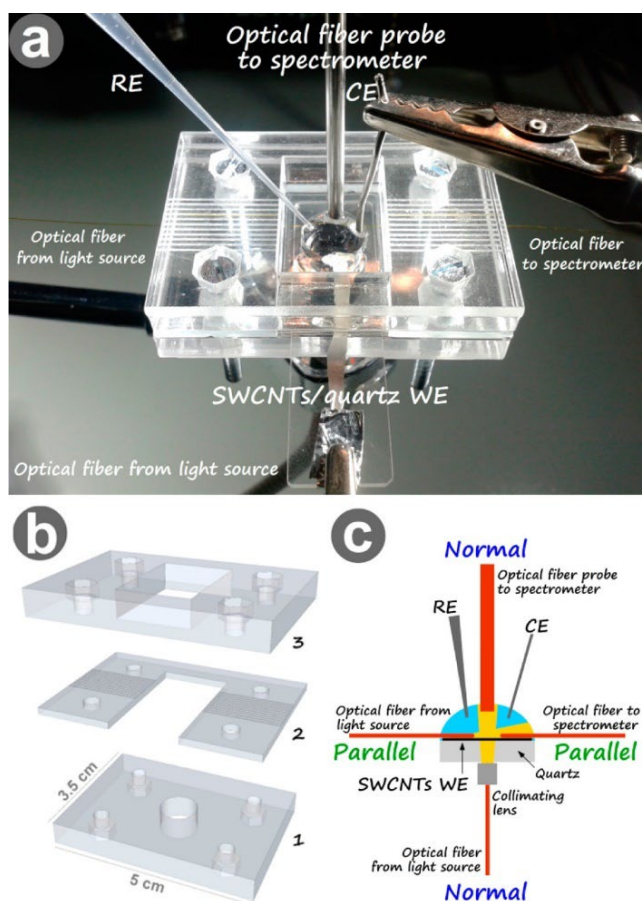


Figure 2-4. (a) BSEC cell for SWCNTs-OTEs, (b) schematic of the disassembled cell, and (c) detailed schematic view. *Reproduced with permission from ref.⁵⁸ Copyright 2015 Analytical Chemistry.*

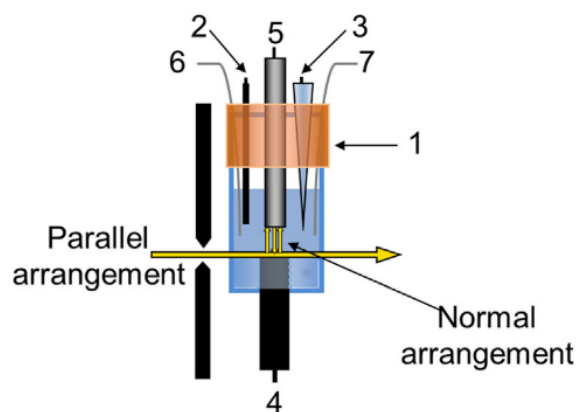


Figure 2-5. Schematic representation of the BSEC cell. (1) Suba-Seal septa, (2) counter electrode; (3) reference electrode; (4) WE; (5) reflection probe; (6) N₂ inlet; (7) N₂ outlet. *Reproduced with permission from ref.¹⁵ Copyright 2017 Electrochimica Acta.*

A variation of BSEC, was proposed in 2018, in which the two spectroscopic responses are recorded in a normal configuration, as depicted in Figure 2-6. This is of great interest for sampling different regions of the same electrode surface. In this work, one of these regions had been modified with carbon nanotubes, while the other remains unmodified. Dopamine oxidation in the two different regions of the electrode was studied concomitantly.¹¹⁷ This strategy is very interesting to study different catalysts under the exactly same experimental conditions.

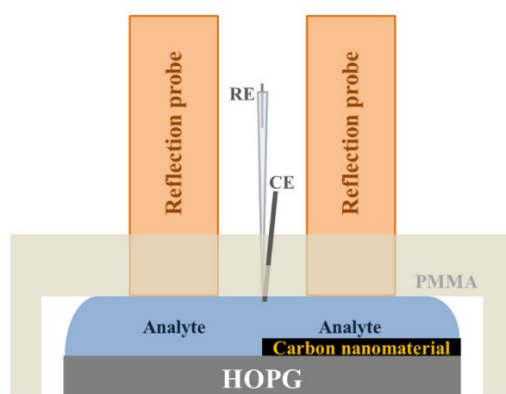


Figure 2-6. Schematic representation of the spectroelectrochemistry cell. *Reproduced with permission from ref.¹¹⁷ Copyright 2018 Electrochemistry Communications.*

All these works highlight the advantages of BSEC, where the combination of the best qualities of both configurations leads to obtaining a huge amount of information in less time, laying the groundwork for novel experiments in which simultaneous measurement is a crucial factor. However, the problems arising from the experimental set-up, which result in a lack of available cells to perform experiments under these conditions, have not been fully solved. The assemblies described to date require either the use of OTEs or a system of three external electrodes. In other terms, although the developments known so far can be considered a starting point for future BSEC applications, further innovation and development of more easily adaptable set-ups is still needed.

Gathering all this information, the design of a new home-made BSEC cell that allows to simplify the assembly and the incorporation of screen-printed electrodes (SPEs) is proposed in this doctoral thesis. A description of such cell is given in Section 6.1. In addition, it has been used to prove the different information that could be extracted from each arrangement, demonstrating that the way a system is sampled plays a key role in SEC.

2.2. Combination of different spectroscopic techniques in spectroelectrochemistry

Hitherto, emphasis was placed on BSEC, a technique that shows that different information can be obtained depending on the optical configuration used with the same spectroscopy technique, demonstrating the advantages of sampling a system simultaneously from two different points of view. But what about the combination of different spectroscopic techniques?

The fusion of spectroscopic techniques to perform SEC experiments can be a very powerful and key tool to analyze the behavior of a chemical system, due to the different type of information that each spectroscopic technique can provide. Moreover, this aspect has already been demonstrated with several investigations carried out in our research group. Several studies demonstrate the potential of performing experiments combining two different spectroscopic techniques to follow the systems simultaneously.

The first one alludes to the simultaneous combination of UV/Vis absorption in the long optical pathway and Raman scattering.⁶⁵ This pioneer work in the field of combining two different spectroscopic techniques constituted a breakthrough, highlighting the multi-response, real-time, *in-situ* and versatile nature of SEC. In this paper, the study of dopamine in acidic media and the electropolymerization process of EDOT were followed by UV/Vis and Raman SEC. Six years later, in 2022, UV/Vis, this time in normal reflection configuration, and Raman were again combined for the detection of crystal violet during *in-situ* formation of Ag NPs.¹¹⁸ In 2023, UV/Vis in parallel configuration and Raman scattering, and PL and Raman were combined to perform SEC experiments in order to clarify the origin of Surface Oxidation-Enhanced Raman Scattering (SOERS).¹¹⁹ The most recent advance resulting from the combination of techniques has been presented by J.V. Perales et al., in 2023, who have proposed the combination of parallel UV/Vis absorption SEC and scanning electrochemical microscopy (SECM) to assess the antioxidant capacity of *o*-vanillin.¹²⁰

In summary, several investigations on the combination of techniques have been developed at recent times. As far as we concerned, in all cases, two techniques have been merged together with electrochemistry. These types of combinations could help us in the identification of the chemical processes occurring in an electrochemical reaction and in

the development of protocols for the quantification of molecules with great interest in different areas, especially in the environmental, medical, and pharmacological fields, due to the wealth of information that is obtained.

At this point, the following question arises: Why not to combine three spectroscopic techniques? This aspect will be addressed in the following two sub-Sections, because in the course of this doctoral thesis two new combinations are proposed, denoted as: UV/Vis-PL-Raman SEC and Parallel UV/VIS-Normal UV/Vis-Raman SEC.

2.1.1. UV/Vis-PL-Raman spectroelectrochemistry

In this work an ambitious, novel, and challenging proposal will be addressed, which consists of combining three spectroscopic techniques, with the aim of performing UV/Vis-PL-Raman SEC. It is evident that a wealth variety of information will be obtained with the fusion of all these techniques, since a single experiment will provide four different signals (the electrochemical response, the one related to UV/Vis absorption spectra, the one obtained from Raman scattering and the one related to fluorescent emission spectra). UV/Vis absorption spectroscopy provides information on the compounds present or electrogenerated, as well as on the possible reactions between the existing compounds. Raman scattering provides information on the changes in the vibrational modes of the bonds and on the interaction between the molecules and the substrates responsible for the amplification signal. Finally, if the system consists of fluorescent species, PL is useful to follow the disappearance of the initial species and the formation of the products, and it also allows the identification of intermediate products.

However, certain difficulties arise from the experimental set-up, since no commercial cell is available to carry out this type of experiments. For this reason, the first stage is going to focus on the construction of a home-made cell easy to use and applicable for the recording of UV/Vis-PL-Raman SEC, as intended in this doctoral thesis (Section 6.5). This cell is expected to increase the number of UV/Vis-PL-Raman SEC applications and to take advantage of its many benefits.

Besides, it must be considered that SEC experiments are performed in a small space, as a single 50 μ L drop of the solution to be studied is usually sufficient. For this reason, the optimal arrangement of each technique must be selected to be able to condense all these techniques. Figure 2-3 show a schematic view of how the three spectroscopic techniques

are coupled. UV/Vis measurements were performed aligning two optical fibers following a parallel direction to the surface electrode (Letters A and B in Figure 2-3); PL was performed aligning two optical fibers in the side configuration (Letters C and D in Figure 2-3); and lastly, the Raman probe is incorporated perpendicularly to the working electrode (Letter E in Figure 2-3).

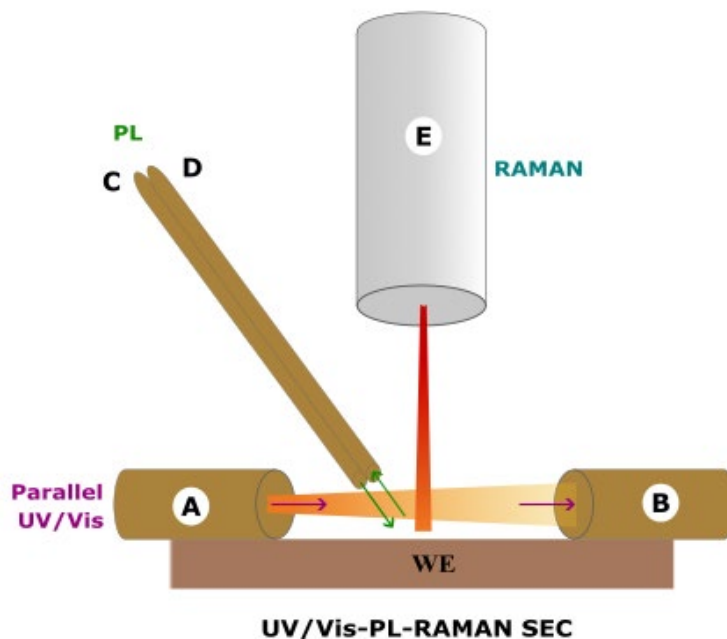


Figure 2-7. Schematic view of how UV/Vis, PL and Raman are coupled to perform UV/Vis-PL-Raman SEC.

In the best case, UV/Vis-PL-Raman SEC could allow in the future the quantification of an analyte with four different signals. In this sense, self-validation of the obtained results can be achieved. Also, the fusion of all these techniques is very useful for those studies, which due to their irreproducibility of the chemical system cannot be carried out with each of the techniques separately, as is the case of monitoring the chemical deposition of nanoparticles.

2.1.2. Parallel and Normal UV/Vis and Raman spectroelectrochemistry

From the set-up just described for carrying out SEC experiments combining UV/Vis, PL and Raman, as well as the cell developed by S. Hernández et al.,¹¹⁸ the necessary modifications are proposed to develop a device that allows performing Raman and BSEC

experiments simultaneously, as it is represented in Figure 2-4. It is not straightforward to combine Raman and Normal UV/Vis, since a SEC experiment takes place in a small space. For this reason, the UV/Vis absorption signal will be recorded in reflection, but in this case the optical fibers are arranged following a side configuration (Letters C and D in Figure 2-4). This configuration is not only suitable to perform PL experiments, but it also presents a remarkable advantage when different spectroscopic techniques must be coupled simultaneously to study the surface, as previously reported S. Hernández et al.¹¹⁸ In turn, two optical fibers will be aligned on a SPE to record the parallel UV/Vis results (Letters A and B in Figure 2-4), and lastly the Raman Probe (Letter E in Figure 2-4) is incorporated perpendicular to the surface electrode.

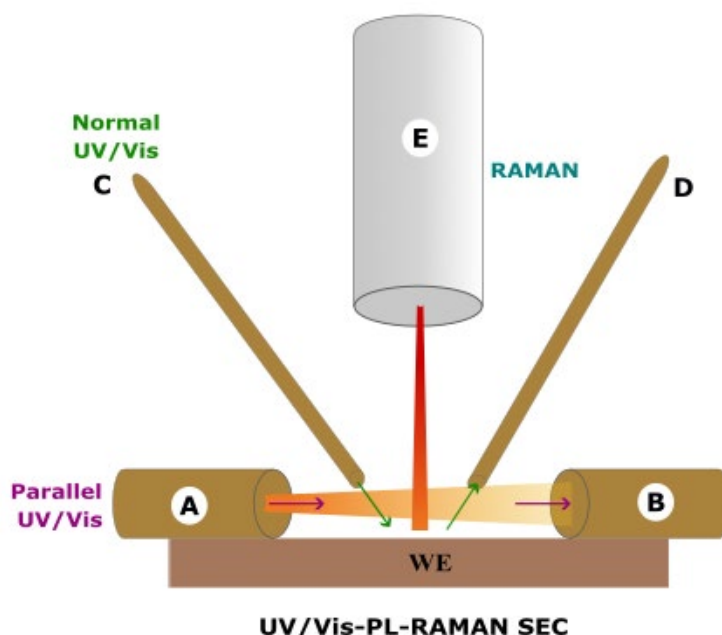


Figure 2-8. Schematic view of how Parallel UV/Vis, Normal UV/Vis and Raman are coupled to perform Parallel UV/Vis, Normal UV/Vis and Raman SEC.

3. MOLECULES OF BIOLOGICAL INTEREST

Summary

Compounds of biological interest play an essential role in the metabolism of organisms, being protagonists of most of the processes that take place in ecosystems. However, despite the essentiality of most of these compounds and their benefits, it is of great interest and importance the development of optimal working protocols that allow the quantification of these compounds in real biological media or in pharmaceutical samples. Control of the amount of drug administered, as well as the ability to quantify these compounds in the ecosystem is essential to avoid the adverse effects that they can generate both in our own organism and in the surrounding habitat. SEC stands out as a technique that is constantly growing and is becoming increasingly important in the field of quantification of compounds of biological interest. Thanks to this technique and the development of different working protocols, it is possible to carry out the spectroelectrochemical determination and quantification of this type of compounds. Specifically, this chapter is devoted to provide information on the functions of several pharmaceutical compounds studied during this thesis and which will appear later in the research results chapter, such as ofloxacin, epinephrine and folic acid.

Therefore, the main objectives of this chapter are:

- To demonstrate the importance of developing working protocols that allow the determination and quantification of compounds of biological interest in real media.
- To describe the compounds of biological interest analyzed in this work (fluoroquinolones, neurotransmitters, folic acid, ascorbic acid and uric acid).
- To compile the most widely used methods of analysis to date, highlighting the advantages of SEC over these methods.

Resumen

Los compuestos de interés biológico juegan un papel esencial en el metabolismo de los seres vivos, siendo los protagonistas de la mayoría de los procesos que tienen lugar en los ecosistemas. Sin embargo, a pesar de la esencialidad de la mayor parte este tipo de compuestos y de sus beneficios, es de gran interés e importancia el desarrollo de procedimientos de trabajo óptimos que permitan la cuantificación de dichos compuestos en medios biológicos reales o en muestras farmacéuticas. Un control de la cantidad de medicamento suministrado, así como el poder cuantificar en el ecosistema este tipo de compuestos es esencial para evitar los efectos adversos que estos puedan generar bien en nuestro propio organismo o en el hábitat que nos rodea. Se destaca la SEC como una técnica que se encuentra en constante crecimiento y que está adquiriendo cada vez más importancia en este ámbito de la cuantificación de compuestos de interés biológico. Gracias a esta técnica y al desarrollo de diferentes protocolos de trabajo es posible llevar a cabo la determinación y cuantificación espectroelectroquímica de este tipo de compuestos. En concreto, este capítulo está dedicado a proporcionar información sobre las funciones de varios compuestos farmacéuticos estudiados durante esta tesis y que aparecerán más adelante en el capítulo de resultados, como el ofloxacino, la epinefrina y el ácido fólico.

Por tanto, los principales objetivos de este capítulo son:

- Demostrar la importancia del desarrollo de protocolos de trabajo que permitan determinar y cuantificar compuestos de interés biológico en medios reales.
- Describir los compuestos de interés biológico analizados en este trabajo (fluoroquinolonas, neurotransmisores, ácido fólico, ácido ascórbico y ácido úrico).
- Recoger los métodos de análisis más empleados hasta el momento, destacando las ventajas de la SEC frente a estos métodos.

3.1. Importance of molecules of biological interest

Recently, in literature many molecules of biological interest have been studied by SEC, such as adrenaline,^{121–123} dopamine,^{6,65,117,123,124} levodopa,^{125,126} hydroquinone,^{127,128} catechol,¹²⁹ ascorbic acid^{42,124} and uric acid.^{43,124} These molecules are widely used for pharmaceutical purposes, i.e., as drugs.

Drugs are chemical compounds used to cure diseases or alleviate symptoms. Determining which amount of the dose administered for each drug is assimilated by the patient is fundamental to the subsequent process of developing new drugs. Therefore, it is essential to be able to quantify this type of analytes in patient samples, such as urine or plasma. Furthermore, comprehending the biological activity of drugs is essential to evaluate their efficacy and safety. On the other hand, the determination of drugs in aqueous media, such as in rivers, lakes, and aquifers, is also of great importance in the environmental contamination field. However, it is not a straightforward task, because the target compounds are usually present in complex matrices where the presence of interferents can hinder the quantification of the analyte of interest. Different methods for the determination and quantification of analytes of interest can be found in literature, such as liquid chromatography, mass spectrometry and other spectroscopic techniques. However, given the complexity of these techniques, which require expensive equipment and long analysis times, the development of electrochemical sensors has become increasingly important. Nevertheless, here comes into play the importance of the principal technique of this thesis: SEC, in particular the already described UV/Vis SEC.

UV/Vis SEC has been widely developed in our laboratories, obtaining different working protocols for the spectroelectrochemical determination and quantification of different analytes in real samples. Examples of this are shown along this thesis, in which several molecules of interest have been studied. Especially, protocols will be developed for the quantification of a group of molecules belonging to the fluoroquinolone (FQ) family. In addition, folic acid in a pharmaceutical sample and epinephrine in the presence of an interfering compound, such as ascorbic acid, will also be quantified. Therefore, the great potential of UV/vis SEC as an analytical technique will be demonstrated through the possibility of detecting these analytes in the presence of interfering species, without the need to implement any tedious sample pretreatment.

Finally, considering that this doctoral thesis evolves around the combination of different spectroscopic techniques and that different experimental set-ups will be developed for the performance of this type of experiments, a simultaneous and in-situ monitoring of a FQ through four different signals (three spectroscopic and one electrochemical response) will also be conducted.

As just mentioned, different protocols will be developed and optimized to quantify compounds with pharmacological interest, but especially the focus will be on FQs, so the next sub-Section will be devoted to describing this type of drugs, with special attention to ofloxacin.

3.2. Quinolones and Fluoroquinolones

Quinolones and fluoroquinolones (FQs) are a group of synthetic antibiotics whose basic structure (Figure 3-1) consists of two rings, which have a nitrogen in position 1, a carboxyl group in position 3 and a carbonyl group in position 4. The incorporation of a fluorine in position 6 gives rise to the well-known FQs.¹³⁰

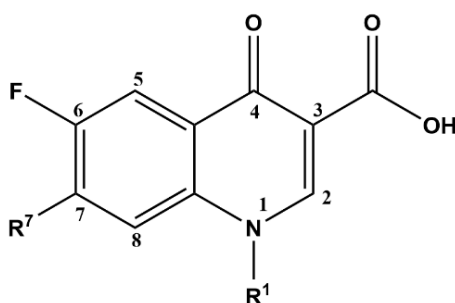


Figure 3-1. Chemical structure of a fluoroquinolone.¹³¹

This group of antibiotics has bactericidal capacity, i.e. they cause bacterial death by inhibiting bacterial deoxyribonucleic acid (DNA) synthesis through the formation of a quinolone-enzyme-DNA complex.^{132,133} They are widely used for the treatment of respiratory infections, thanks to their good tolerability profile and broad antibacterial spectrum.¹³² They are also used to promote livestock growth and in aquaculture.¹³⁴ FQs are classified according to their spectrum of activity into 1st, 2nd, 3rd, and 4th generation FQs.^{130,131,135,136}

- **1st generation:** This group only includes quinolones that do not contain the fluorine group. They are rarely used, as they are only effective against

enterobacteria and some Gram-negative bacteria. They have no activity against Gram-positive or other atypical pathogens. An example is nalidixic acid, which was the first quinolone used as an antibacterial agent in the 1960s. This group also includes oxolinic acid and pипemidic acid.

- **2nd generation:** It includes FQs, i.e., quinolones that have a fluorine at position 6. This fluorine increases the potency and antibacterial spectrum of FQs with respect to quinolones since it facilitates the penetration of the molecule into the tissues. In addition, the presence of a piperacinic or methyl-piperacinic group at position 7 also increases the efficacy. They are active against Gram-negative bacteria and have a longer half-life than the previous one. 2nd generation FQs include ofloxacin, norfloxacin, enoxacin, lomefloxacin and ciprofloxacin.
- **3rd generation:** They have a higher efficacy against Gram-negative and Gram-positive bacteria. Levofloxacin, sparfloxacin and temafloxacin belong to this group.
- **4th generation:** They preserve the characteristics of the 3rd generation FQs, but also, they are effective against anaerobic and atypical microorganisms. Moxifloxacin, gatifloxacin, trovafloxacin and clinafloxacin are examples of 4th generation FQs.

The first quinolones synthesized and used in pharmacology were 1st generation quinolones. Nevertheless, the need to cover a wider bactericidal spectrum led to the inevitable appearance of 2nd and 3rd generation quinolones, which are currently the most widely used.

These types of drugs, characterized by their great potential to deal with various bacterial infections, are, in turn, under surveillance because their widespread use has led to the rapid emergence of bactericidal resistances. In addition, this has resulted in other ecological consequences. For example, FQs have been detected in wastewater treatment plants, surface water and other environmental matrices, which may have repercussions on living organisms, since the continuous release of FQs into the aquatic environment promotes antibiotic resistance.¹³²

Moreover, the World Health Organization (WHO) and the United States Institute of Medicine have already been concerned about the prevalence of trace amounts of pharmaceutical compounds in the environment. FQ antibiotics can enter the environment

through untreated wastewater, human and animal excreta, or by direct discharge from aquaculture. An assessment of the environmental risks related to the percentage not metabolized and disposed to the different environmental matrices is essential.^{132,134}

3.2.1. Ofloxacin

Among the variety of FQs reported, we decide to study ofloxacin (OFL). This 2nd generation FQ, whose chemical structure is represented in Figure 3-2, is commonly used for the treatment of respiratory, digestive and urinary tract infections.^{137–139}

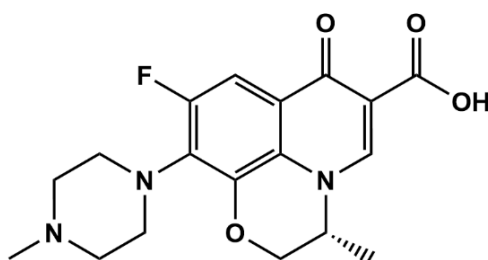


Figure 3-2. Chemical structure of OFL.¹³⁶

This molecule is formed by ionizable amino and carboxylic groups, so depending on the pH of the medium it can be found in different structural forms. Accordingly, OFL has two dissociation constants: pK_{a1} from 5.89 to 6.05 and pK_{a2} from 7.9 to 8.2.^{133,137} Therefore, for acidic conditions, i.e., when the pH of the medium is lower than its first pK_a , OFL is positively charged, since the amino group at 4-position of the piperazine has a positive charge. When the pH value of the medium has an intermediate value between pK_{a1} and pK_{a2} the carboxylic group loses a proton and the OFL is present in its zwitterion form (uncharged). Finally, for pH values above the pK_{a2} , deprotonation of the amino-functional group occurs, so that the OFL is negatively charged.^{137,138,140}

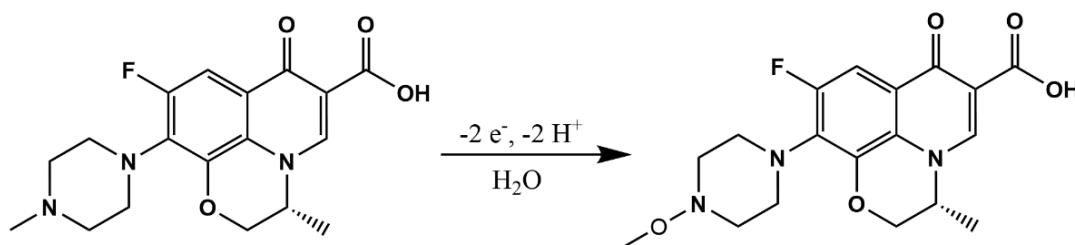


Figure 3-3. Oxidation mechanism of OFL.¹⁴¹

The electrochemical oxidation process of OFL has been widely reported in the literature. As depicted in Figure 3-3, it is an irreversible process where the transfer of two electrons and two protons takes place.^{137,141–144} This process is controlled by adsorption.¹⁴⁵

About 70 % of ingested OFL is released into the environment, which has led to the recent detection of significant concentrations of OFL in aquatic and terrestrial environments. Studying the behavior of OFL and developing analytical protocols that allow its quantification in these environments is essential to prevent the problems that these effluents can cause, such as the famous antibiotic resistance.^{137,138,146,147} Until now, among the most commonly used methods to detect OFL are liquid chromatography,^{148–151} mass spectrophotometry,^{152–154} UV/Vis spectrometry^{155,156} and fluorescence.^{157–159} These techniques are time-consuming and require the use of very expensive equipment, which has led to the development of electrochemical sensors suitable for the study and detection of this type of molecules. Nonetheless, in many cases, prior optimization work or even the use of modified electrodes is required.^{137,143–145,160,161} For this reason, in this work, UV/Vis SEC is proposed to study the behavior of OFL, in order to subsequently develop a working protocol that allows the quantification of OFL in real media. In addition to undergoing changes in the absorption spectrum during its oxidative process,¹⁶² this compound also undergoes changes in the fluorescence spectrum. Therefore, its study by PL-SEC is also of great interest, since the information provided by the two techniques can be of great help in understanding the behavior of this molecule. Besides, being able to combine or select different techniques helps in the development of working protocols that allow its quantification in different complex matrices. It is also noteworthy that given the two pK_a values, spectral changes are observed as a function of the pH of the solution in both the UV/Vis and PL spectra. For this reason, a previous study of this molecule at different pH is necessary in order to develop and optimize an analysis protocol.

3.3. Catecholamines

Catecholamines are important neurotransmitters, i.e. biomolecules whose main function is the transmission of information from neurons to other neurons, glands, or cells. The basic structure of catecholamines consists of a benzene ring with two hydroxyl groups, an intermediate chain and a terminal amine group.¹⁶³ Catecholamines play an essential role in the regulation of physiological processes as a neurotransmitter and hormone. They act as transporters in the nervous system and participate in the constriction of blood

vessels, as well as controlling tissue metabolism. For this reason, they have been used in medicine for the treatment of heart attacks, bronchial asthma, and cardiac surgery.^{121,164,165} Epinephrine, dopamine and noradrenaline are the three major catecholamines.¹⁶³ Since this doctoral thesis will encompass the study of epinephrine and dopamine, the following sub-Sections are devoted to these two neurotransmitters.

3.3.1. Epinephrine

The epinephrine (EP), also called adrenaline, and chemically known 4-[(1R)-1-Hydroxy-2-(methylamino)ethyl]-1,2-benzenediol, whose chemical structure is shown in Figure 3-4, is a hormone biosynthesized in the adrenal medulla and sympathetic nerve endings and secreted by the adrenal gland.^{122,164} This notably important neurotransmitter is produced in large proportions in the brain and is necessary for message transfer in the central nervous system. Low levels of EP have been found in patients with Parkinson's disease.¹⁶⁶ Pharmaceutical samples of EP generally contains antioxidants, such as sodium metabisulfite, with the main objective of minimizing the analyte oxidation, since exposure of EP to air favors its spontaneous oxidation.¹⁶⁵

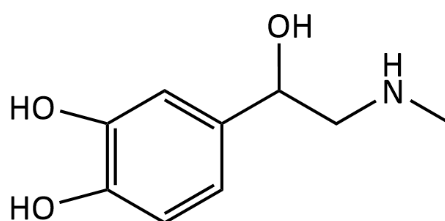


Figure 3-4. Chemical structure of epinephrine.¹⁶⁵

In addition, it has been proven that EP oxidation products are toxic to neurons. Its oxidation mechanism has been studied by spectrophotometric and electrochemical methods, showing that it depends on the pH of the solution.¹⁶⁷ Its oxidation mechanism in acidic media is represented in the Figure 3-5, where it is observed that EP is reversibly oxidized to form epinephrinequinone (EPQ) with the transfer of two electrons and two protons.

On the other hand, it has been shown that the oxidation mechanism of EP in basic medium is different. In this case, the first step also consists of the oxidation of EP to form EPQ. Secondly, EPQ undergoes intramolecular cyclization via 1,4-Michael addition, forming leucochrome, which is subsequently oxidized to adrenochrome by a two-electron-proton transfer.^{168–171}

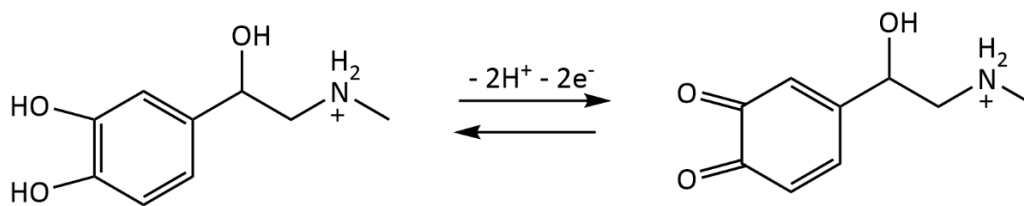


Figure 3-5. Epinephrine oxidation mechanism in acidic media.^{165,167}

Several methods have been implemented to determine EP, such as liquid chromatography,¹⁷² liquid chromatography coupled to mass spectrometry,^{173,174} spectrophotometry,¹⁷⁵ fluorimetry,^{176,177} chemiluminescence^{178,179} and capillary electrophoresis.^{180,181} However, most of them are sophisticated methods that require expensive instrumentation and longer analysis times. More recently, electrochemical sensors have also been developed for the sensitive and selective detection of EP.^{182–185} Nevertheless, in this work, EP will be quantified in the presence of an interfering and antioxidant compound by UV/Vis SEC.

3.3.2. Dopamine

Another well-known catecholamine is dopamine (DA), chemically named 4-(2-aminoethyl)benzene-1,2-diol, whose chemical structure is represented in Figure 3-6.¹⁸⁶ This chemical substance, which is mainly produced in the cerebral cortex, plays an essential role in the mammalian central system. DA controls various biological functions, such as cognitive processes, emotions, muscle movements. It is also involved in the endocrine and cardiovascular systems. Analogous to EP, several pathologies such as Parkinson's disease and schizophrenia are related to the levels of this neurotransmitter in the human body. Low levels of DA have been proven to be related to Parkinson's disease, whereas high levels of this neurotransmitter are associated with schizophrenia.^{6,187,188}

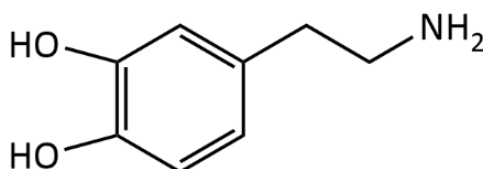


Figure 3-6. Chemical structure of dopamine.¹⁸⁶

The oxidation mechanism of DA also depends on the pH of the medium, being very similar to that explained for EP. Its oxidation in acidic medium generates

dopaminequinone (DAQ) in a reversible process involving the transfer of two protons and two electrons, as shown in Figure 3-7.^{171,186}

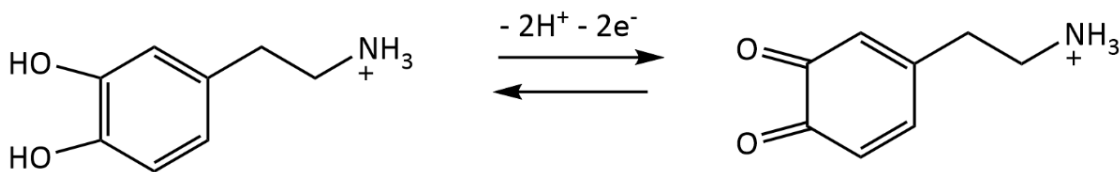


Figure 3-7. Dopamine oxidation mechanism in acidic media.^{171,186}

On the other hand, the oxidation mechanism of DA in basic medium involves the formation of leucodopaminechrome, which is subsequently oxidized to dopaminechrome by a two-electron-proton transfer. The latter compound is then transformed into 5,6-dihydroxyindole, which in turn is oxidized to 5,6-indolquinone, a compound that polymerizes.^{6,171}

The methods mentioned in the previous Section for the determination of EP, are also applicable for dopamine. Therefore, studies by liquid chromatography,^{189,190} liquid chromatography coupled to mass spectrometry,^{191,192} fluorimetry,^{193,194} chemiluminescence¹⁹⁵ and capillary electrophoresis¹⁹⁶ have been described in literature to quantify DA. However, in a similar way, the expensive instrumentation of these techniques and the longer analysis times have led to the use of electrochemical sensors.^{197–199} Considering that DA is an intrinsically redox species and both it and its oxidation products absorb in the UV/Vis region,^{6,187,188} in this work UV/Vis SEC is proposed as a more suitable alternative for the study and quantification of this neurotransmitter.

3.4. Other molecules of biological interest

3.4.1. Folic Acid

Folic acid (FA), commonly called vitamin B9 and also designated by the IUPAC as (2S)-2-[[4-[(2-amino-4-oxo-1H-pteridin-6-yl)methylamino]benzoyl]amino] pentanedioic acid,²⁰⁰ is a water-soluble vitamin of the group B, and it is the natural form of folate.^{201,202} FA has a low solubility in water, which increases slightly in acidic or basic media, being more stable in alkaline media.²⁰² This vitamin consists of three main parts, as it is shown in Figure 3-8, where the core of the molecule is a heterocyclic pterin structure joined by

a methylene bridge to p-aminobenzoic acid (PABA), which in turn is joined by a peptide bond to L-glutamic acid (Glu).^{201–203}

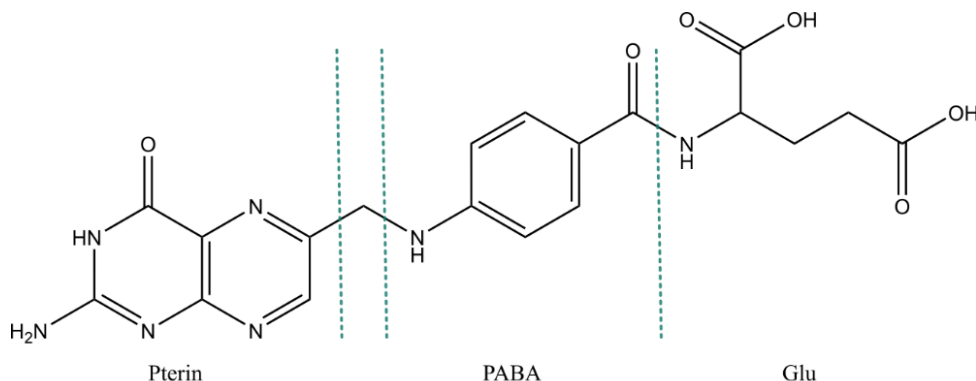


Figure 3-8. Chemical structure of folic acid.^{200,202,203}

FA is usually found in fruits and green leafy vegetables sources in its reduced forms,²⁰⁴ that is, as 7,8-dihydrofolate (DHF) or 5,6,7,8-tetrahydrofolate (THF), whose chemical structures are depicted in Figure 3-9.^{200,205} For instance, THF is essential in many biosynthetic pathways, such as in the purine and pyrimidine biosynthesis.

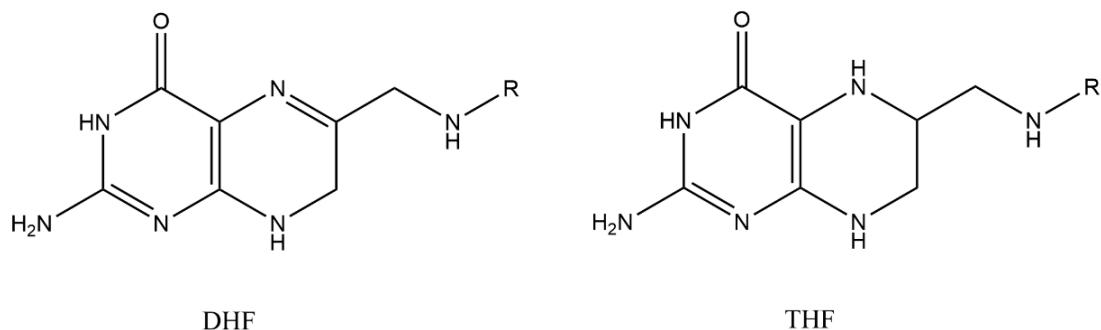


Figure 3-9. Chemical structure DHF and THF.²⁰⁰

FA is inactive in the human body, so the liver must convert it into the main active form at the cellular level: 5-methyltetrahydrofolate (5-MTHF). Besides being generated in the liver, this active folate can also be absorbed directly from food. 5-MTHF is involved in several metabolic processes, such as in the synthesis of glycine from serine, in the synthesis of DNA and Ribonucleic Acid (RNA) and in the regulation of homocysteine levels.²⁰²

This compound of great biological interest, plays an essential role in numerous biochemical processes and it is fundamental during pregnancy to prevent neuronal tube defects (NTDs). Regarding the first characteristic, FA is involved in the formation and maturation of blood cells and in cell multiplication. It is also implicated in the regulation of gene activity, in the synthesis of purines and pyrimidines and in the metabolism of amino acids, alongside vitamin B12.^{200,206} Several concerns about the supplementation of FA are worth mentioning; for example, an excess of FA may conceal megaloblastic anemia due to vitamin B12 deficiency.^{200,206} In other cases, the supplementation of FA may reduce immune function in post-menopausal women and increase the cancer risk. Just as low FA intake may promote tumorigenesis, high dietary intake of folate may also promote tumor growth.²⁰² The other fundamental role of FA is based on the crucial need for pregnant women to maintain the levels of this compound in the proper range, which in human blood serum ranges from 2 to 15 ng/mL.²⁰¹ This is essential to prevent neuronal defects, such as spina bifida or anencephaly, as well as other complications that may arise during gestation.²⁰⁶ Therefore, the WHO recommend FA supplementation to prevent NTDs. But, on the other hand, this supplementation must be under surveillance, being of vital importance to control the dose administered to avoid DNA methylation changes in the offspring, thus guaranteeing their health.²⁰⁷

Considering that both deficiency and excess of this vitamin can induce serious health problems, it is of great importance to develop analytical techniques that allow the study and quantification of FA in real samples. Many methods, including spectrophotometry,^{208,209} electrophoresis,^{210–212} liquid chromatography,^{213,214} liquid chromatography coupled with mass spectroscopy,^{215–217} fluorescence,^{165,218} and chemiluminescence,^{219,220} among others, have been developed for FA determination. However, these techniques require expensive instrumentation, sample pre-treatment and are time-consuming. For this motive, electrochemical techniques have been gaining importance, due to their lower cost, simplicity and relatively short analysis times.^{221,222} Electrochemical detection is often accompanied by the development of modified electrodes^{223,224} with the aim of developing methods with high selectivity and good sensitivity, which allow the determination of FA in complex matrices where the presence of interferents may hinder it. Consequently, the use of SEC is considered a robust approach to perform FA analysis on a real matrix.

3.4.2. Ascorbic acid

Ascorbic acid (AA) or vitamin C, Figure 3-10, is an essential vitamin in the human diet. It comes mainly from fresh fruits and vegetables and can be found in the mammalian brain along with neurotransmitters. This aspect makes ascorbic acid an important neurotransmitter interfering compound, as will be demonstrated in the results chapter.^{166,225}

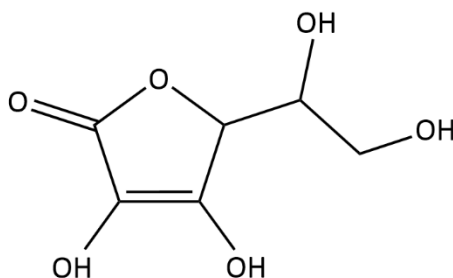


Figure 3-10. Chemical structure of ascorbic acid.²²⁶

AA plays an essential role in the regulation of metabolic processes in the human body, participating in the processes of cell division, gene expression and in the activation of biological defense mechanisms.²²⁷ Its deficiency is associated with scurvy.⁴²

The oxidation of ascorbic acid in solution results in the formation of dehydro-L-ascorbic acid (DHA), which is an unstable compound, that is quickly hydrolyzed to form an electro-inactive compound known as 2,3-dicheto-L-gulonic acid. This oxidation mechanism of AA is depicted in the Figure 3-11.²²⁶

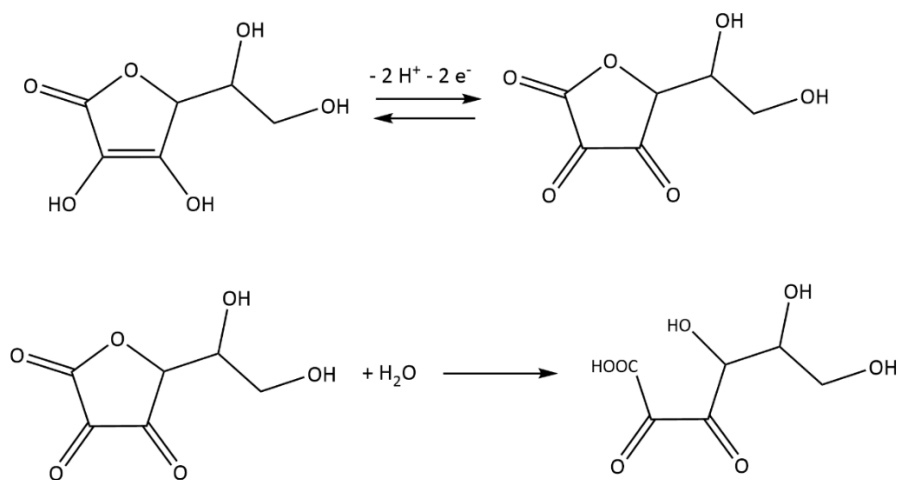


Figure 3-11. Oxidation mechanism of ascorbic acid.²²⁶

Due to its antioxidant activity, it is a compound that has been studied to a large extent, mainly by electrochemical methods.^{166,225} However, studies have also been reported where AA has been quantified by spectrophotometric methods,^{228,229} colorimetry,^{230,231} and liquid chromatography,^{232,233} among others.

3.4.3. Uric acid

Uric acid (UA), whose chemical structure is shown in Figure 3-12, is the final product of purine metabolism and is an important natural antioxidant in the human body. It is eliminated from the biological fluids of the human body through urine, feces, and sweat.^{225,234}

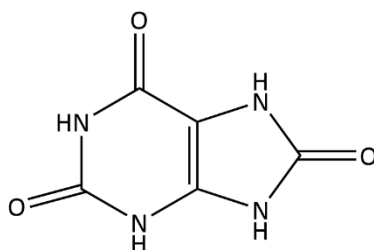
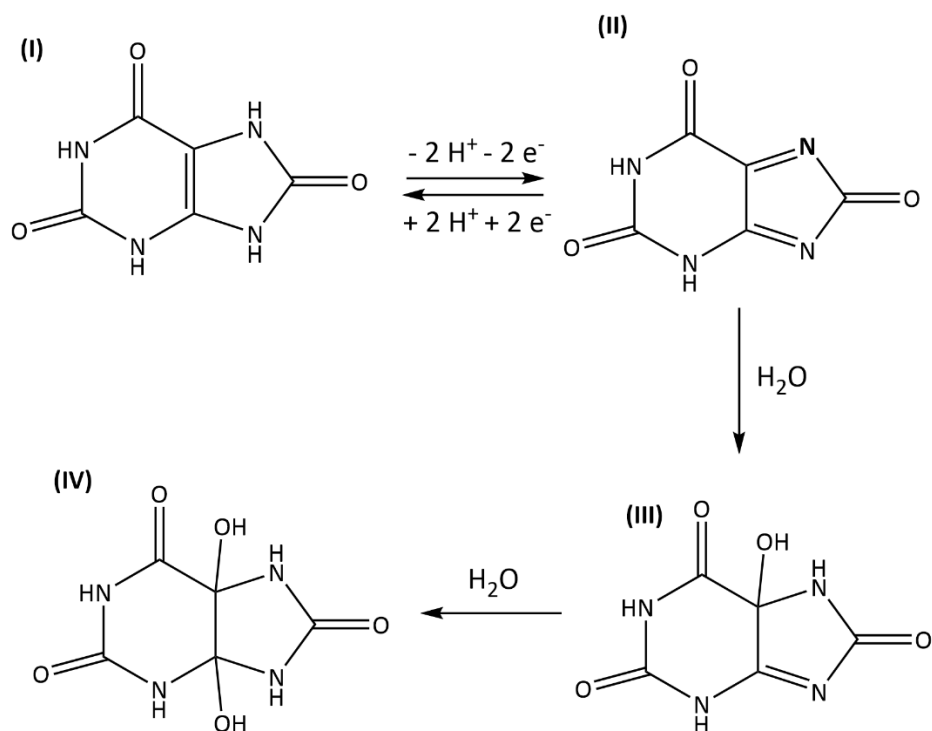


Figure 3-12. Chemical structure of uric acid.²³⁵

The correlation of high or low levels of UA in serum with certain disorders has been ascertained, establishing the normal range of UA in blood to be between 3.5 y 7.2 mg/dL.²³⁴ The elevated amount of UA in the blood, known as hyperuricemia, may contribute to the development of certain diseases such as gout, anemia or gouty nephropathy.^{225,234} On the other hand, low levels of UA have been linked to an increased risk of neurodegenerative diseases, including Parkinson's disease.²³⁴

The electrochemical oxidation process of UA (I) involves the formation of a di-imine specie (II) in a process in which two electrons and two protons are transferred, as depicted in Figure 3-13. This di-imine is unstable in aqueous solution, so it is transformed into an imine-alcohol (III), which by hydration leads to the formation of uric acid-4,5-diol (IV).^{235,236}

Electrochemical sensors have acquired great importance in the field of UA quantification,²³⁷⁻²⁴⁰ due to the advantages they present over other more tedious and expensive methods, such as liquid chromatography,^{241,242} colorimetry^{243,244} or spectrophotometry.²⁴⁵

Figure 3-13. Oxidation mechanism of uric acid.^{235,236}

4. EXPERIMENTAL SECTION

Summary

Throughout this Doctoral Thesis different SEC techniques have been used, so that, although the experimental set-up in all of them is similar, depending on the applied technique certain nuances must be considered. Experimental set-ups in which only one spectroscopic technique is coupled to electrochemistry have already been widely used in the group and described in the literature. However, the coupling of more techniques entails the necessary development of different experimental cells. Therefore, the main objectives of this chapter are as follows:

- To describe the experimental set-ups each of the techniques separately. Starting with how an electrochemical experiment should be carried out, and continuing with the three SEC techniques used in this work (UV/Vis SEC, PL-SEC and Raman SEC).
- To show and describe the specific experimental devices for the different combinations proposed in this work.

Resumen

En el desarrollo de esta Tesis Doctoral han sido utilizadas distintas técnicas SEC, por lo que, aunque el montaje experimental en todas ellas es similar, dependiendo de la técnica usada se deben tener en cuenta ciertos matices. Los dispositivos experimentales en los que solo una técnica espectroscópica es acoplada a la electroquímica, ya han sido ampliamente utilizados en el grupo y descritos en literatura. Sin embargo, el acople de más técnicas lleva consigo el necesario desarrollo de diferentes celdas específicas. Con todo ello, los objetivos principales de este capítulo son los siguientes:

- Describir los montajes experimentales propios de cada una de las técnicas por separado. Empezando por cómo se debe llevar a cabo un experimento de espectroelectroquímica, para luego proceder a la descripción de las tres técnicas SEC principales en este trabajo (SEC de UV/Vis, SEC de fotoluminiscencia y SEC Raman).
- Mostrar y describir los dispositivos experimentales propios de las distintas combinaciones propuestas en este trabajo.

4.1. Spectroelectrochemistry (SEC)

In the early days performing SEC experiments was not an easy task, since maintaining synchronization between the two signals was a great challenge. In the beginning, the solution to this drawback resided in monitoring the spectroscopic response using very long acquisition times and selecting a fixed potential or current. In dynamic studies, however, it is mandatory to ensure that the electrochemical and spectroscopic changes occur at the same time, requiring the use of a trigger which synchronizes the two signals. Nowadays, as SEC has gained importance, there are commercial devices that can guarantee this synchronization, allowing SEC experiments to be conducted in a simple, fast, and reproducible way. In this Thesis a customized version of the commercial equipment provided by Metrohm-DropSens is used. Specifically, two types of modified SPELEC are employed: a Metrohm-DropSens UV/Vis SPELEC (Figure 4-2a) and a Metrohm-DropSens RAMAN SPELEC (Figure 4-2b). These devices include the corresponding light source, the spectrophotometer and the potentiostat which are full-synchronized. In the UV/Vis SPELEC the light source is a deuterium-halogen lamp, while in the Raman SPELEC the light source is a laser. The UV/Vis SPELEC is employed for performing UV/Vis SEC and PL-SEC. For performing PL-SEC, an external monochromatic LED is used as light source, since the sample must be excited to record the corresponding emission spectrum.

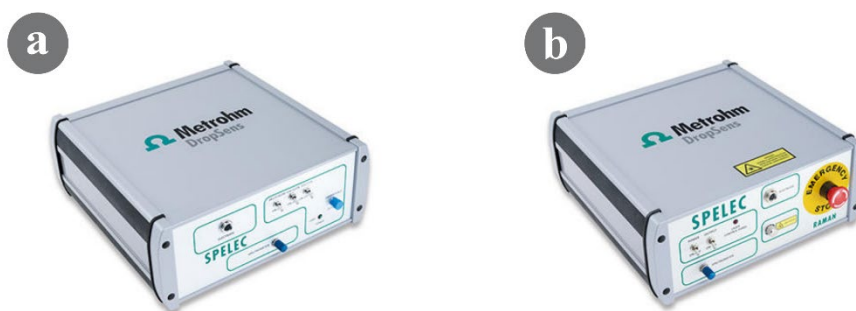


Figure 4-1. Spectroelectrochemistry instruments (a) Metrohm-DropSens SPELEC, (b) Metrohm-DropSens RAMAN SPELEC.

Using the commercially available SPELEC, synchronization of potentiostat and spectrometer is guaranteed. In addition, both the potentiostat and the spectrometer are connected to the same computer, which is used for collecting the data. Along with the main problem of synchronization, which has been solved with the commercial equipment

described here, there was also the aforementioned challenge of aligning the optical fibers, while maintaining reproducibility between the measurements. To save this second obstacle, several SEC cells tailored to the requirements of each study have been developed, which will be described in the Results and Discussion chapter.

4.1.1. Electrochemistry

Electrochemistry measurements were recorded using commercial screen-printed electrodes (SPEs) supplied by Metrohm-DropSens, as shown in Figure 4-2. These SPEs consist of a three-electrode system: a 4 mm diameter disc working electrode (WE) where occurs the redox reaction; a counter electrode (CE) where takes place the complementary redox reaction; and a pseudo-reference electrode (RE) that maintains a constant potential if the electrolytic medium is not changed. Two different types of SPE have been used during this doctoral thesis: carbon SPE (C-SPE, DRP-110, Metrohm-DropSens) (Figure 4-2) whose WE and CE are both made of carbon; and gold SPE (Au-SPE, DRP-220-AT, Metrohm-DropSens) (Figure 4-2) whose WE and CE are gold. Both of them contain a silver RE.

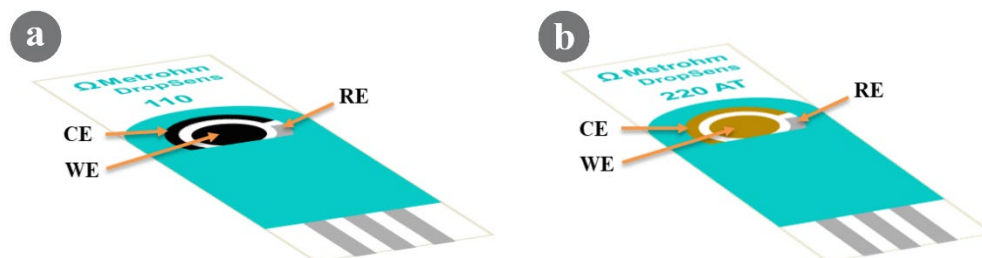


Figure 4-2. SPE electrodes (a) Carbon SPE, (b) Gold SPE.

Several electrochemical techniques are known, however during this work only voltammetry, chronoamperometry and multiamperometric detection will be used.

- In voltammetry, the variation of the current with respect to potential/time is recorded. That is, the potential difference between the WE and the RE changes during the course of the experiment at a defined scan rate.
- In chronoamperometry, a constant potential is applied and the change in the current between the WE and the CE with respect to time is tracked.
- In multiamperometric detection, as in the chronoamperometry, the current variation with respect to time is recorded when a constant potential is applied.

This technique is based on various chronoamperometries, i.e. different potentials are applied during a given time, which may be of great interest when different compounds with different oxidation potentials are present in the same sample. For example, this technique makes it possible to separate the oxidation processes.

In order to carry out the electrochemical part of a SEC experiment, i.e. to be able to apply the desired potential(s) to the sample, the use of a potentiostat, which is directly connected to the three-electrode system, is required.

4.1.2. UV/Vis absorption SEC

In UV/Vis spectroscopy, a light beam irradiates the sample, so a light source, which can be a deuterium lamp for ultraviolet radiation or a halogen lamp for visible radiation, is used. The reflected light is collected into a spectrophotometer.

Optical fibers are used to transport the light beam from the light source to the SEC cell, and from the cell to the spectrometer. As outlined in Section 1.1.1, UV/Vis SEC can be performed by selecting different configurations. Thus, depending on the selected arrangement, different optical fibers will be used. In the parallel configuration, two bare optical fibers (Ocean Optics) of 100 μm diameter are aligned faced to face on the WE, fixing them with nail polish. Whereas, to conduct UV/Vis SEC in normal configuration a reflection probe consisting of a central collection optical fiber that is surrounded by 6 illumination optical fibers (FCR-7UV200-2-2.5X100-ME-SR, Avantes) is employed (Figure 4-3a).

In addition, for BSEC experiments two light sources are not necessary, since the light from one source can be bifurcated to the probe that records the optical changes in normal configuration and to the bare optical fibers that sample the system following a direction parallel to the WE. In this case, a bifurcated optical fiber (QBIF600-UV-VIS, Ocean Optics) is required (Figure 4-3b). However, BSEC requires two spectrometers, and the electrochemistry will be controlled by one of the two signals, i.e., a unique potentiostat is used to apply the potential due to the correct synchronization of both instruments.

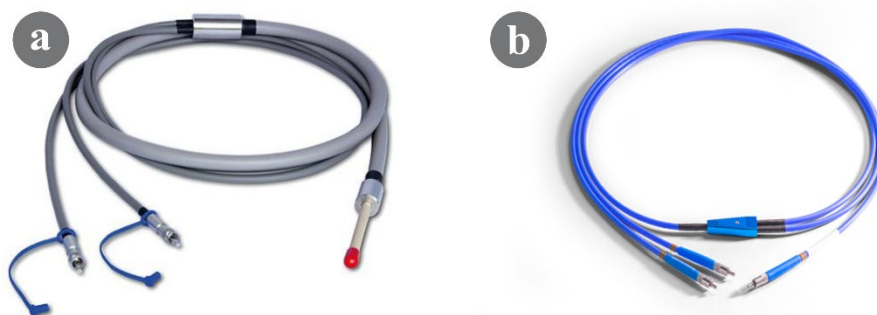


Figure 4-3. (a) Reflection UV/Vis probe and (b) bifurcated optical fiber.

4.1.3. Photoluminescence SEC

The equipment used to perform PL-SEC is very similar to the one described for UV/Vis SEC. The main difference is that in PL-SEC the light source is a light emitting diode (LED), which provides monochromatic light at a certain wavelength, causing the excitation of the sample. The wavelength of the LED used must be selected according to the analyte. That is to say, it is mandatory to consider the spectral section in which the analyte to be studied absorbs, in order to select a LED whose wavelength coincides with one of the absorption bands of the UV/Vis spectrum, causing the excitation of the sample. In this way, the emission spectrum is recorded at higher wavelengths than the excitation one. Spectroscopic information is gathered in the spectrometer, which is synchronized with the potentiostat, as it was explained for UV/vis SEC. Thus, both electrochemical and spectroscopic data are collected in the same computer.

As in UV/Vis experiments, optical fibers are used in PL-SEC to transport the preceding light beam from the LED source to the sample and from the sample to the spectrometer. In this case, different optical bare fibers of 200, 400 and 600 μm (Ocean Optics) are used. These fibers are fixed to a SEC cell in a side configuration, i.e. the incident light beam is focused on the WE with at an angle of less than 90° . This configuration has some advantages over the normal configuration since the recorded analytical signal will be less influenced by the reflected radiation. This configuration was selected over the normal configuration because the fluorescence probe can be incorporated more easily, facilitating the incorporation of both set-ups on a small surface.¹¹⁸

4.1.4. Raman SEC

In Raman, the light source used will be a laser. The light is transmitted to the sample and then collected in the spectrometer by a Raman probe (DRP-RAMANPROBE, Metrohm-DropSens) (Figure 4-4) which is focused perpendicular to the electrode surface, without coming into contact with the solution



Figure 4-4. Raman probe.

In Raman SEC it is important to keep in mind some crucial considerations. For instance, to choose the appropriate laser, the nature of the substrate must be considered, since the laser frequency interacts with the plasmonic substrate.^{246,247} There are different laser sources, being 532 nm, 633 nm and 785 nm the most used. Among them, 633 nm and 785 nm are quite convenient to excite SERS activity for Au, Ag and Cu, and in addition, the 785 nm one provides lower luminescence effect. Thus, in this work all Raman results are recorded with a 785.18 nm laser, since as it is a low-energy laser, a higher power can be implemented, without damaging the sample. In addition, by working with this wavelength possible interferences caused by fluorescent emissions of the analyte can be minimized or even avoided. Raman intensity is proportional to the laser power, but even so, a proper laser power must be carefully selected to avoid thermal decomposition of the sample.

Depending on the position of the laser beam, two optical configurations can be used to illuminate the sample, namely top and bottom illumination,⁸⁵ the former being the most typical and the one that will be used throughout this work. A Raman probe with its own filter that cuts-off the laser wavelength, allowing radiation with longer wavelengths to pass through, is going to be employed in this case.

The Raman spectrometer contains three main parts: the entrance slit to the monochromator, the grating and the detector, and it should be optimized for the wavelength range of the laser. The spectra resolution will be affected by the combination of the slit and the grating.

Thus, a proper recording of the Raman signal requires a suitable combination of each of its components (laser source, Raman probe and spectrometer), in parallel, it is necessary the coupling of the potentiostat and the SEC cell. It should be noted that all components must be correctly synchronized to obtain reliable results, and this is possible thanks to the commercial instruments that our research group has developed in collaboration with Metrohm-DropSens.

4.1.5. UV/Vis-PL-Raman SEC

SEC experiments, in which UV/Vis, PL and Raman are combined simultaneously, are performed using three customized SPELEC instruments (Metrohm-DropSens) perfectly synchronized with each other, as it is represented in the Schematic view of Figure 4-5. In this case, the potentiostat of one of the SPELEC instruments will be responsible for controlling the potential and the correct synchronization between the three instruments allows us to obtain time-resolved spectra.

When performing UV/Vis-Raman-PL-SEC, signals coming from the laser or the LED can interfere with the other optical responses, being essential to use appropriate optical filters to remove the contribution of the light reflected by the LED and to prevent the spectrometers from being saturated by the light from both the LED and the laser used. For this purpose, a high-pass filter is coupled to the spectrometer that collects the UV/Vis absorption response, avoiding its saturation with the light coming from the LED. This type of filter can be adapted to each situation, since it only allows the passage of those signals whose wavelengths are in the selected range, attenuating the signals outside this spectral range. Depending on the system under study, the high-pass filter can be adapted, allowing the pass of those wavelengths where the absorption spectrum is observed and attenuating the wavelength of the LED. In addition, it is also necessary to attenuate the signal from the laser source that could interfere with the fluorescence response. For this purpose, a notch-filter will be connected to the spectrometer which collects the fluorescence spectra. This notch-filter has the task of minimizing the light coming from the laser source. Both filters are placed between the bare optical fibers that collect the

light beam reflected by the sample and between another commercial fiber (Ocean Optics, QP450-1-XSR and Ocean Optics, QP600-025-VIS) that is connected to each spectrometer.

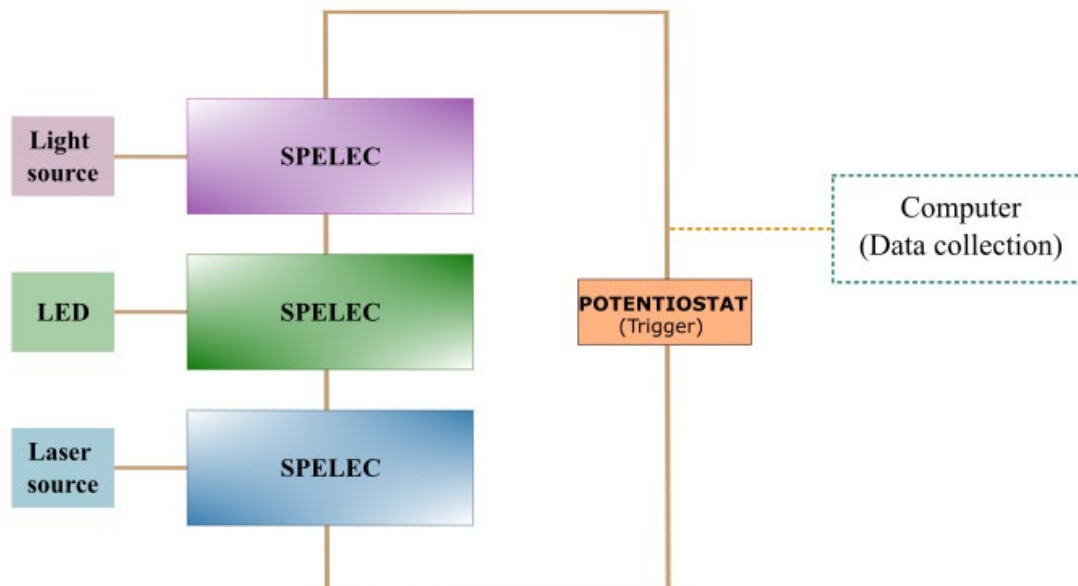


Figure 4-5. Schematic view of how the different SPELECS are synchronized. The Raman-SPELEC is the one that controls the electrochemistry and all three are connected to each other. In addition, these three SPELECS are connected to the same computer, where the data are recorded.

4.1.6. Parallel UV/Vis-Normal UV/Vis-Raman SEC

The combination of two UV/Vis and one Raman responses requires an experimental set-up equivalent to the one detailed in the previous Section. So, the Raman SPELEC will control the potential and all SPELECS instruments are interconnected and perfectly synchronized. The main difference resides in the fact that, as in the BSEC measurements, only one light source is required for the two UV/Vis SPELEC and therefore a bifurcated fiber will split the light beam to sample the system in the two optical configurations, as detailed above.

4.2. Software

An advanced software, DropView SPELEC from Metrohm-DropSens, has been used during this Thesis to perform SEC experiments. Different versions of this software have been employed developed by Metrohm-DropSens between 2019 and 2022. DropView

SPELEC is an indispensable resource for recording SEC signals and has the advantage of being a well-developed software, which allows the proper synchronization between all signals. In addition, it also allows to perform a first and quick analysis of the SEC data, extracting in a simple way the most important information related to the experiments.

5. DATA PROCESSING

Summary

As previously mentioned, SEC experiments have been carried out with the software DropView SPELEC (Metrohm-DropSens). Although from the signals obtained with this software a first glimpse about the behavior of the system under study can be deduced, a proper processing of the data is essential for a thriving interpretation of the recorded signals. This chapter describes the tools used to analyze and interpret the data, including both univariate and multivariate analysis. Given the complexity involved, it is not possible to tackle this with the DropView software. Therefore, the use of other mathematical programs is mandatory, among which Matlab stands out.

Therefore, this chapter covers the following main objective:

- To describe the basics of data processing, including the most relevant terms and software used.

Resumen

Como se ha mencionado anteriormente, los experimentos SEC se han llevado a cabo con el software DropView SPELEC (Metrohm-DropSens). A pesar de que a partir de las señales obtenidas con este software puede deducirse una primera información sobre el comportamiento del sistema objeto de estudio, es esencial realizar un correcto procesamiento de los datos para obtener una interpretación mejorada de las señales registradas. En este capítulo se describen las herramientas utilizadas para analizar e interpretar los datos, lo que incluye tanto el análisis univariante como el multivariante. Esto no puede abordarse con el software DropView, dada la complejidad que entraña. Por lo tanto, es obligatorio el uso de otros programas matemáticos, entre los que destaca Matlab.

Por lo tanto, este capítulo cubre el siguiente objetivo principal:

- Describir los fundamentos del tratamiento de datos, incluyendo los términos más relevantes y el software utilizado.

5.1. Time-resolved experiments

Time-resolved UV/Vis SEC experiments provide a trilinear data matrix in which there is a correlation between the analyte concentration, the spectroscopic signal (absorbance variation) and the electrochemical response (potential/time), yielding a large data cube (Figure 5-1). This data cube contains the absorbance values collected when a scan potential is applied. From this data cube surfaces at a fixed concentration (green area in Figure 5-1), at a fixed wavelength (violet area in Figure 5-1), and at a fixed potential (yellow area in Figure 5-1), can be extracted. Several representations can also be obtained from each surface. For instance, green area shows the individual spectra at a fixed potential and concentration and the derivative spectra with respect to the wavelength. In the violet zone, individual voltabsorptograms or derivative voltabsorptograms at a fixed wavelength and concentration are depicted. It should also be emphasized that these signals can be smoothed to remove the random noise. Lastly, this data cube provides the possibility to perform individual measurements at a fixed wavelength and potential by applying univariate or multivariate tools. Therefore, in a UV/Vis SEC measurement a matrix of n potentials $\times$ m absorbance variation is registered for each experiment. Given the size of the optical and electrochemical data, a single SEC experiment contains thousands of points and in some cases, millions of points.

Accordingly, the large amount of information obtained in a SEC experiment implies the unavoidable use of appropriate software tools to facilitate the data processing and interpretation. Indeed, this data acquisition is even more complex in SEC experiments where two or more spectroscopic techniques are combined. For this reason, the data obtained were subsequently processed with Matlab to extract as much information as possible. Matlab has a wide library of toolboxes, but in this doctoral thesis the package of functions used has been developed by our research group and by Rasmus Bro et. Al.²⁴⁸ This software allows us to perform the data analysis, and carry out a quantitative analysis, it also allows us to perform multivariate analysis, being a powerful tool when solving complex matrices that include highly interfering analytes.

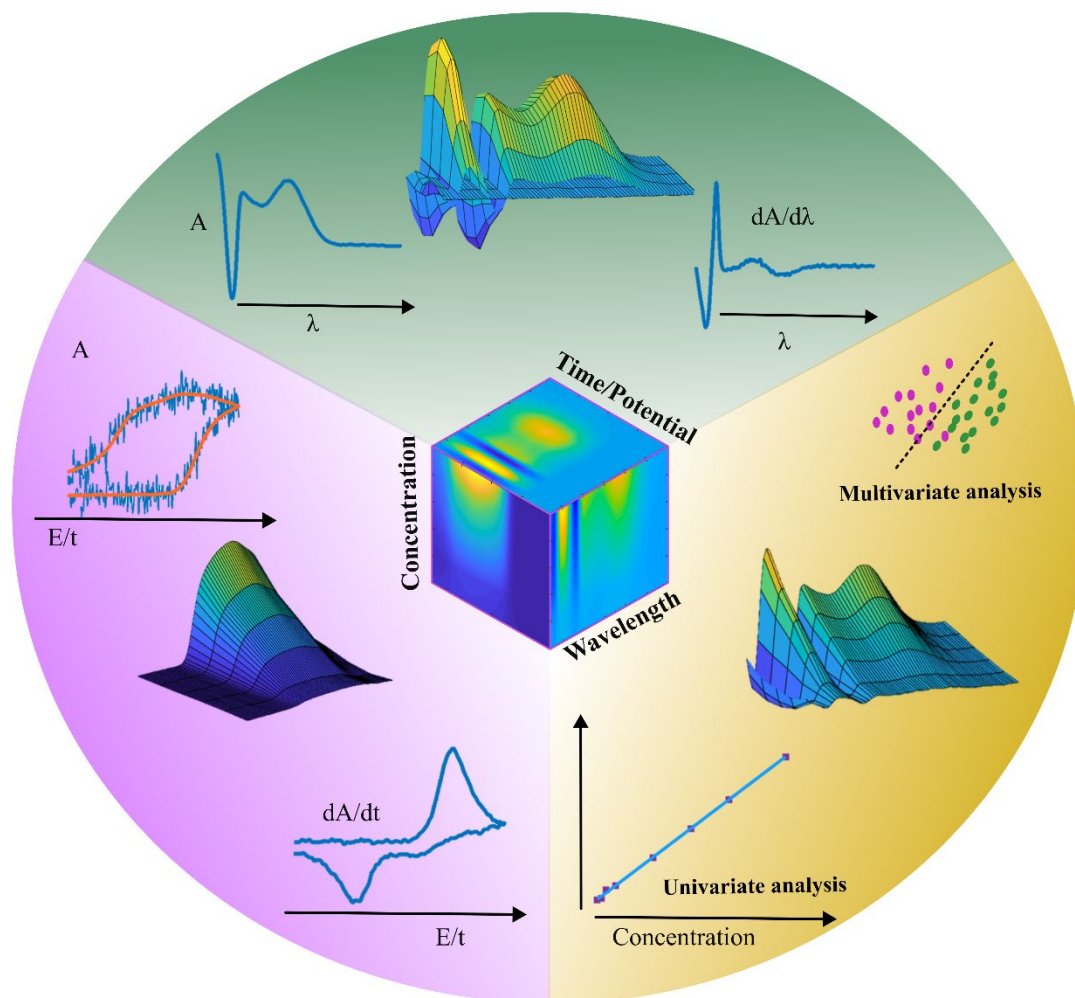


Figure 5-1. UV/Vis SEC data cube with the corresponding data obtained during an experiment.

5.2. Data treatment

As briefly mentioned in the previous Section, the data obtained in a UV/Vis SEC experiment can be processed in several ways. A great deal of information can be obtained from the representation of the absorbance evolution at a selected wavelength with respect to potential/time. This graph is named voltabsorptogram. The representation of the derivative of the absorbance with respect the time (derivative voltabsorptogram) together with the voltammogram is of great help in explaining what type of process is associated to the spectral change, since by comparison of both signals it is possible to identify whether the absorbance variation is due to an electron transfer, an adsorption or an electrodeposition process.²⁴⁹ The evolution of the derivative absorbance signal with

respect to potential or time, has been applied to follow redox reactions²⁵⁰ and to characterize electrochromic films.²⁵¹

Another signal that can also reveal a wealth of information comes from plotting the derivative of the absorbance with respect to the wavelength. This signal can be very useful when solving complex mixtures in which highly interferent compounds coexist, since this representation allows a better discrimination of all the absorptometric changes recorded during the electrochemical experiment. In other words, the derivative signal respect to the wavelength makes it possible to discriminate the contribution of each molecule, facilitating quantification.

In some cases, spectral signals must be filtered, since they are accompanied by random noise. In SEC, filtering can be applied to data registered in two dimensions (wavelength and time or potential) simultaneously, which allows improving the elimination of this noise, obtaining cleaner signals. For instance, the most widely used filter in analytical chemistry is the Savitzky-Golay (SG) smoothing algorithm, which is applied in the time domain.²⁵² In addition, other mathematical methodologies including the Fourier filter or the wavelet transform also provide high-quality signals.^{252,253} The size of the filtering window depends on the amount of points recorded, being really important to check that the signal has not been over-filtered. For this purpose, the user must compare the original signal with the filtered one in order to verify that the spectral information of the filtered signal has not been substantially modified.

An analogous interpretation and data processing can be applied for PL and Raman SEC. In a PL-SEC experiment, the representation of the variation of the optical signal at a selected wavelength with respect to potential/time, is denoted as voltaFluorogram. Whereas, in a Raman experiment the analogous representation of the evolution of a determined Raman band with respect to the potential, is termed voltaRamagram. In these cases, valuable information can also be obtained from the representation of the derivative. Moreover, Raman signals often required an additional pretreatment, since EC-SERS spectra are often accompanied by a background signal due to photoluminescence phenomena, making it necessary to apply a baseline correction.²⁵⁴ For this purpose, it is essential to select the points of the spectra where Raman scattering is not observed in order to build a mathematical model based on a polynomial curve. This baseline adjustment is also carried out with Matlab.

5.3. Qualitative and quantitative analyses

Once the data obtained with SPELEC have been exported and preprocessed with the specific mathematical software Matlab, a subsequent qualitative and quantitative analysis can also be performed with this software. In this Doctoral Thesis both univariate and multivariate analyses will be performed, selecting the most appropriate for each situation. It should be noted that although most of the data have been processed with Matlab, another mathematical software, R (version 3.6.1) has also been used to generate univariate calibration models, predict the analyte concentration in the test sample and to assess the limits of detection of the method.

5.3.1. Univariate data treatment

In a UV/Vis SEC experiment it is common to use the signal at a given time or potential and at a fixed wavelength to detect the analyte of interest. Considering that the selected wavelength corresponds to a characteristic band of the target molecule under study. Thus, in many cases this information is enough to detect reagents, products and intermediates. Moreover, it is also very useful in quantitative analysis, where the representation of this signal versus the concentration allows the quantification of the analyte in a real sample, using a very simple mathematical model.

5.3.2. Multivariate data treatment

Sometimes, in complex systems with multiple analytes or different interfering compounds, univariate analysis is not enough and multivariate data analysis is required. The trilinear character of UV/Vis SEC experiments allows the use of powerful statistical tools such as Parallel Factor Analysis (PARAFAC) in order to quantify a specific analyte. PARAFAC allows building a mathematical model without introducing any extra information and provides the factors who contribute to the spectroscopic response, being of great help to solve complex matrices formed by highly interfering components. The main obstacle when using PARAFAC lies in the selection of the number of components, which has been solved with the introduction of the *core consistency diagnostic* (CORCONDIA). This term was suggested by Ramus Bro²⁵⁵ and is used to determine the right number of components. Thus, a CORCONDIA value close to 100% implies an adequate model. The N-way toolbox developed by Rasmus Bro²⁵⁶ has been used in this work.

6. RESEARCH RESULTS

Summary

This chapter details the results obtained during the development of this Doctoral Thesis, where the methodology described so far has been used. This chapter is divided into several Sections:

- In the first Section we proceed to study a system by BSEC, demonstrating how the configuration selected plays an essential role in spectroelectroanalysis, due to the different information obtained depending on the sampled region. This study demonstrates how epinephrine can be determined in the presence of a highly interfering compound using exclusively the signal obtained by the parallel configuration. It shows, thereby, that depending on the study it is crucial to select the most appropriate optical configuration.
- The second Section demonstrates the power of UV/Vis absorption SEC as a powerful tool to quantify folic acid (vitamin B9) in a pharmaceutical tablet. After optimizing the method, it is observed how only the spectroscopic signal is useful for such quantification. However, without electrochemistry this would not be possible, since it is necessary to selectively oxidize the folic acid.
- In the third Section, UV/Vis absorption SEC is again the main protagonist. By means of this technique, ofloxacin, an important antibiotic, can be quantified in a complex matrix, such as a real urine sample. Despite the large number of interfering compounds that can hinder such quantification, a working protocol has been developed which, together with the appropriate multivariate tools, has allowed the quantification of ofloxacin without the need to carry out any pretreatment of the urine sample.
- In the fourth Section, UV/Vis SEC in parallel configuration and in a thin-layer regime is used to simultaneously determine three highly interfering compounds: dopamine, ascorbic acid and uric acid. To sort out this challenging system, it is essential to make use of the suitable chemometric tools. The derivative of absorbance together with PARAFAC permits the separation of the contribution of each of these molecules and thereby their quantification.
- The fifth Section proposes the design of a cell that allows simultaneous measurements by coupling the three spectroscopic techniques described in this work (UV/Vis, PL and Raman) to electrochemistry. All these signals are of great value in the study of electrode processes, as well as in the field of quantification,

where in the best scenario quantification can be achieved using four different signals. First, the designed cell is validated using a well-known reference system such as $\text{Ru}(\text{bpy})_3^{2+}$. Subsequently, it is demonstrated how ofloxacin can also be studied by applying this design.

- Lastly, the sixth Section proposes the design of a cell that allows SEC experiments coupling UV/Vis absorption spectroscopy in both configurations together with Raman spectroscopy. As future research we would like to apply this design to the monitoring and study of systems of interest.

Resumen

Este capítulo detalla los resultados obtenidos durante el desarrollo de esta Tesis Doctoral, donde se ha utilizado la metodología descrita hasta el momento. Este capítulo se divide en varias secciones:

- En la primera sección se procede al estudio de un sistema mediante SEC bidimensional en el UV/Vis, demostrando como la configuración seleccionada juega un papel esencial en espectroelectroanálisis, debido a la diferente información obtenida en función de la zona del sistema muestreada. En este estudio se demuestra cómo se puede determinar epinefrina en presencia de un compuesto altamente interferente utilizando únicamente la señal obtenida mediante la configuración paralela. Se demuestra, por tanto, que en función del interés del estudio es muy importante seleccionar la configuración óptica adecuada.
- En la segunda sección se demuestra el poder de la SEC de absorción en el UV/Vis como una potente herramienta para cuantificar ácido fólico (vitamina B9) en un comprimido farmacéutico. Tras la optimización del método, se observa como únicamente la señal espectroscópica es válida para dicha cuantificación. Sin embargo, sin la electroquímica esto no sería posible, ya que esta es esencial para la oxidación selectiva del ácido fólico.
- En la tercera sección vuelve a aparecer la SEC de absorción en el UV/Vis como principal protagonista. Gracias a esta técnica se consigue cuantificar ofloxacino, un importante antibiótico, en una matriz compleja, como es una muestra real de orina. A pesar del gran número de compuestos interferentes que pueden dificultar dicha cuantificación, se ha desarrollado un protocolo de trabajo que, junto con las herramientas multivariantes adecuadas ha permitido la cuantificación de ofloxacino sin la necesidad de llevar a cabo ningún pretratamiento previo de la muestra de orina.
- En la cuarta sección se emplea la SEC de absorción en configuración paralela y en régimen de capa fina para determinar de manera simultánea tres compuestos altamente interferentes entre sí: dopamina, ácido ascórbico y ácido úrico. Para resolver este complejo sistema, se requiere el uso necesario de las herramientas quimiométricas adecuadas. La derivada de la absorbancia junto con PARAFAC

permiten la separación de la contribución de cada una de estas moléculas, y por ende su cuantificación.

- En la quinta sección se propone el diseño de una celda que permita llevar a cabo medidas simultáneas acoplando las tres técnicas espectroscópicas descritas en este trabajo (UV/Vis, PL y Raman) a la electroquímica. Todas estas señales son de gran ayuda en el estudio de procesos, así como en el campo de la cuantificación, donde en el mejor de los casos se puede lograr la cuantificación utilizando cuatro señales distintas. En primer lugar, se procede a validar la celda diseñada utilizando un sistema de referencia bien conocido, como es $\text{Ru}(\text{bpy})_3^{2+}$. Posteriormente, se demuestra como también el ofloxacino puede ser estudiado aplicando este diseño.
- Por último, en la sexta sección se propone el diseño de una celda que permita llevar a cabo medidas SEC simultáneas acoplando la espectroscopía de absorción UV/Vis en ambas configuraciones junto con la espectroscopía Raman. Esta celda se ha validado utilizando la *o*-tolidina como sistema de referencia. Como investigaciones futuras se pretenda aplicar este diseño para el seguimiento y estudio de sistemas de interés.

6.1. Normal or parallel configuration in spectroelectrochemistry? Bidimensional spectroelectroanalysis in presence of an antioxidant compound.

This sub-Section will cover the study of a system by BSEC, demonstrating that the way a chemical system is sampled plays a key role in spectroelectroanalysis. For this purpose, epinephrine will be detected in the presence of an antioxidant and interfering compound, ascorbic acid in this case. The differences between the two optical configurations, related to the region of the solution sampled, will be shown. Briefly, parallel configuration provides the best analytical results, since the interfering effect of the ascorbic acid is minimized when only the first micrometers of the solution adjacent to the electrode surface are sampled. So, after developing an analytical method in which the conditions of the experiment are properly optimized, it is demonstrated how epinephrine can be perfectly detected using the parallel configuration, which is not feasible with the normal one. This has been published in *Journal of Electroanalytical Chemistry*, 2023, 935, 117333.²⁵⁷

6.1.1. Introduction

Spectroelectrochemistry (SEC) is a technique that provides the electrochemical information and the spectroscopic evolution of an electron-transfer process. In this way, signals of different nature are obtained concurrently from a chemical system undergoing an oxidation-reduction process.^{7,8,258} Normal and parallel configuration are the two main optical arrangements for UV/Vis molecular absorption SEC.^{58,66,259–261} On the one hand, in normal configuration, the light beam follows a perpendicular trajectory to the electrode surface, providing information related to changes that occurs both in the solution and at the electrode surface.^{6,117,259,262} This arrangement is characterized by its easy assembly, but its optical pathway is limited to the thickness of the diffusion layer. On the other hand, in parallel configuration (long optical pathway arrangement), the light beam samples only the solution adjacent to the working electrode in parallel direction with respect to its surface, including only information about the spectral changes that take place in the solution closer to the electrode surface.^{52,124} The main advantage of this configuration is the longer optical path length compared to the one of the normal configuration, which is

associated with higher sensitivity and lower detection limits for soluble compounds.^{259,262,263} The main disadvantage could be the assembly due to the difficulties in the alignment of the light beam coming from the light source and going to the detector. However, this drawback can be easily solved by using optical fibers that can be attached to the electrode surface.⁵⁸

In 2001, bidimensional SEC (BSEC), the simultaneous combination of normal and parallel arrangements, was developed.⁶⁶ BSEC provides simultaneously one electrochemical and two spectroscopic signals.^{58,66} A light beam samples the system in perpendicular direction to the working electrode surface, meanwhile, a parallel light beam with respect to the electrode surface passes through the solution. Thus, BSEC can distinguish in a single experiment which processes take place in the solution and which ones on the electrode surface during an electrochemical reaction.^{58,66,259,262} Although the use of UV/Vis absorption SEC is proving to be of great utility in quantitative analysis,^{6,123,124,264–267} BSEC has never been used for this purpose so far, although it has been widely used for the study of reaction mechanisms and the characterization of conductive polymers.^{113,114} In all these works, BSEC has proven to be a very powerful tool that guarantees that all the information obtained is related to the same electrochemical process.

This great potential of BSEC has been exploited in this work to demonstrate an additional advantage of parallel configuration over the normal one, even over the electrochemical response. This advantage is of great utility in quantification of compounds in presence of interfering and antioxidant species. For this purpose, commercial screen-printed electrodes (SPEs) and a fiber-optic based BSEC cell are used to detect the concentration of epinephrine (EP) in presence of ascorbic acid (AA). EP, also known as adrenaline, is a neurotransmitter that plays a fundamental role in human metabolism, regulating physiological processes and acting as chemical messenger.^{121,171,268,269} Low levels of EP have been found in patients with Parkinson's disease and can result in different mental and physical problems such as anxiety, depression or migraine headaches. EP has been widely studied with electrochemical techniques,^{270–272} but scarcely with SEC, although EP and its oxidation products have well-differentiated spectra.^{121,122,273} For its part, AA is considered an essential vitamin in the human diet that can be found in the brain in presence of EP. It has an antioxidant character and it can be considered as a direct interfering species of EP when detected electrochemically.^{166,272,274–277} This type of

interference has been previously avoided combining SEC with multivariate analysis.^{123,124} In this work, we avoid the interference of AA in the determination of EP using simple experiments, without any multivariate analysis, and employing the advantages of electrochemistry of controlling the generation of the oxidized compounds.

To demonstrate experimentally the capabilities of BSEC for the analysis of mixtures with interfering species that have antioxidant properties, the main objective of this work is the quantification of EP in the presence of AA, allowing us to select the best configuration for quantitative purposes thanks to the large amount of information provided by BSEC. So, this BSEC study, which had never been carried out before, allows us to distinguish the different results obtained using each configuration and to select the best arrangement for this kind of systems, all in a single experiment.

6.1.2. Materials and methods

6.1.2.1. *Reagents and materials*

L(-)-Epinephrine (EP, 99 %, Acros Organics), ascorbic acid (AA, L(+)-Ascorbic acid, ACS reagent, Acros Organics) and perchloric acid (HClO₄, 60 %, Panreac) were used as received without further purification.

Commercial SPEs (DRP-110, Metrohm-DropSens), which included a three-electrode configuration printed on the same support, were used to perform all the experiments. Each SPE has a 4 mm diameter disk screen-printed carbon working electrode (WE), a carbon counter electrode (CE) and a silver pseudo-reference electrode (RE).

In all experiments, cyclic voltammograms (CVs) corresponding to a first scan, starting at a potential where no electrochemical reaction takes place, are shown. All CVs are represented respect to a silver pseudo-reference electrode. For comparison with experiments in literature, a difference of +0.588 V respect to a reversible hydrogen electrode (RHE) is measured using the same supporting electrolyte composition, Figure 6-1.

All solutions were daily prepared using high-quality water (18.2 MΩ cm resistivity at 25 °C, Milli-Q Direct 8, Millipore). All experiments were performed at room temperature.

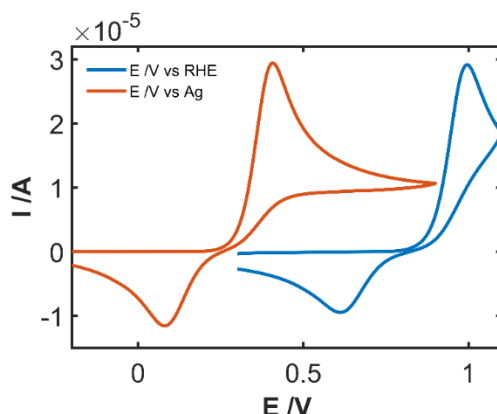


Figure 6-1. CVs of $2 \cdot 10^{-3}$ M EP in 0.1 M HClO_4 between -0.20 V and +0.90 V at $0.01 \text{ V} \cdot \text{s}^{-1}$ using a silver pseudo-reference electrode, orange line, and a reversible hydrogen electrode (RHE), blue line.

6.1.2.2. Instrumentation

Two customized SPELEC instruments (Metrohm-DropSens), controlled by DropView SPELEC software (Metrohm-DropSens), were used for the BSEC experiments.

6.1.2.3. Experimental set-up

A bifurcated optical fiber (QBIF600-UV/VIS, Ocean Optics) was connected to the light source. A scheme of the BSEC cell used to perform the experiments is shown in Figure 6-2.

As can be observed, a reflection probe which consists of 6 illumination optical fibers and a central collection optical fiber was connected to obtain the optical response in normal configuration (FCR-7UV200-2-2.5X100-ME-SR, Avantes). The reflection probe was placed at 1.8 mm from the WE using a clamp. In order to obtain the optical response in parallel arrangement, two bare optical fibers (100 μm in diameter, Ocean Optics) were aligned face to face on the WE at a distance of 0.2 mm. One of the optical fibers was connected to the light source and the other one to the spectrometer. To perform the corresponding measurements, a solution drop (100 μL) was placed on the electrode, covering the three-electrode system and the ends of the reflection probe and the bare optical fibers. In all experiments the initial solution was taken as reference spectrum.

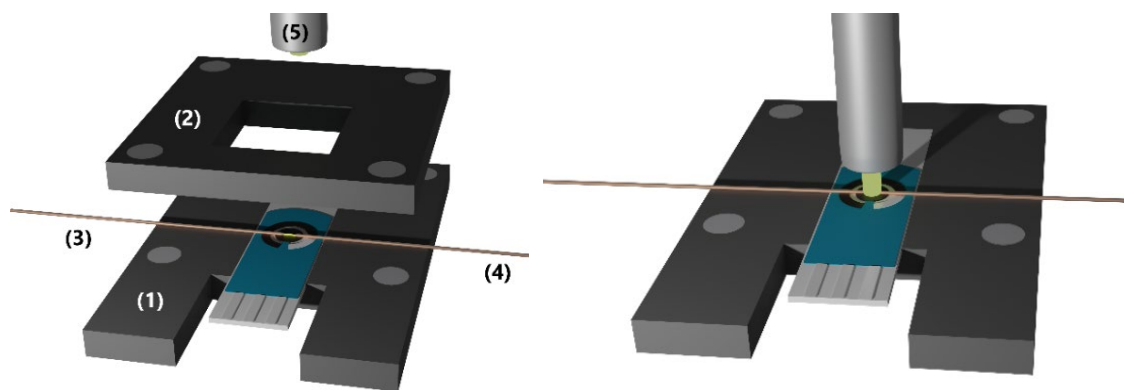


Figure 6-2. Schematic view of the BSEC cell (a) disassembled and (b) assembled; upper body has been removed to clearly observe the position of the optical fibers in the assembled cell. (1) Lower body for supporting the SPE (2) upper body to fix the electrode (3) illumination optical fiber in parallel configuration (4) collection optical fiber in parallel configuration and (5) reflection probe for normal configuration measurements.

6.1.3. Results and discussion

6.1.3.1. BSEC of EP using cyclic voltammetry

The BSEC behavior of EP in acidic medium is shown in Figure 6-3. This figure is related to a solution of $2 \cdot 10^{-3}$ M EP in 0.1 M HClO_4 . As can be observed, the cyclic voltammogram (CV) between -0.20 V and +0.90 V at $0.01 \text{ V} \cdot \text{s}^{-1}$ shows a quasi-reversible behavior (Figure 6-3a) with an oxidation peak at +0.28 V and a cathodic one peaking at +0.15 V. At this acidic pH, the oxidation mechanism corresponds to the oxidation of EP to epinephrinequinone (EPQ).^{121,167,171,269} This oxidation process in acidic media is related to the transfer of two electrons and two protons, with the generation of EPQ, that is regenerated in the backward scan, being the formal potential of +0.215 V. The optical responses in parallel and normal configuration are plotted in Figure 6-3b and Figure 6-3c, respectively. In the two configurations, an absorption band that evolves along the experiment can be observed centered at 385 nm, corresponding to the oxidation of EP to the appropriate quinone.

Figure 6-3d displays the cyclic voltabsorptograms (CVAs) at 385 nm for the two optical configurations. In parallel arrangement, a quasi-stationary stage is reached at the end of the forward scan. At this point, the absorbance achieves an almost constant value (around 0.37 a.u.) because the optical fibers only sampled approximately the first 120 μm closest to the working electrode surface (diameter of the optical fibers attached to the electrode

surface, 100 μm , covered by a cladding). However, all the diffusion layer is sampled in normal configuration, and the absorbance continues to increase in the cathodic scan until the potential is low enough to reduce the EPQ generated. It should be remarked that, in both CVAs, absorbance starts to increase at the same potential, around +0.20 V due to the oxidation of EP to EPQ. In the same way, absorbance also starts to decrease at the same potential in the backward scan due to the reduction of EPQ to EP, around +0.19 V. As it is explained in the introduction Section, the main advantage of BSEC is that it provides in a single experiment one electrochemical and two spectroscopic signals simultaneously, all of them related to a single system, in this case the oxidation of EP.

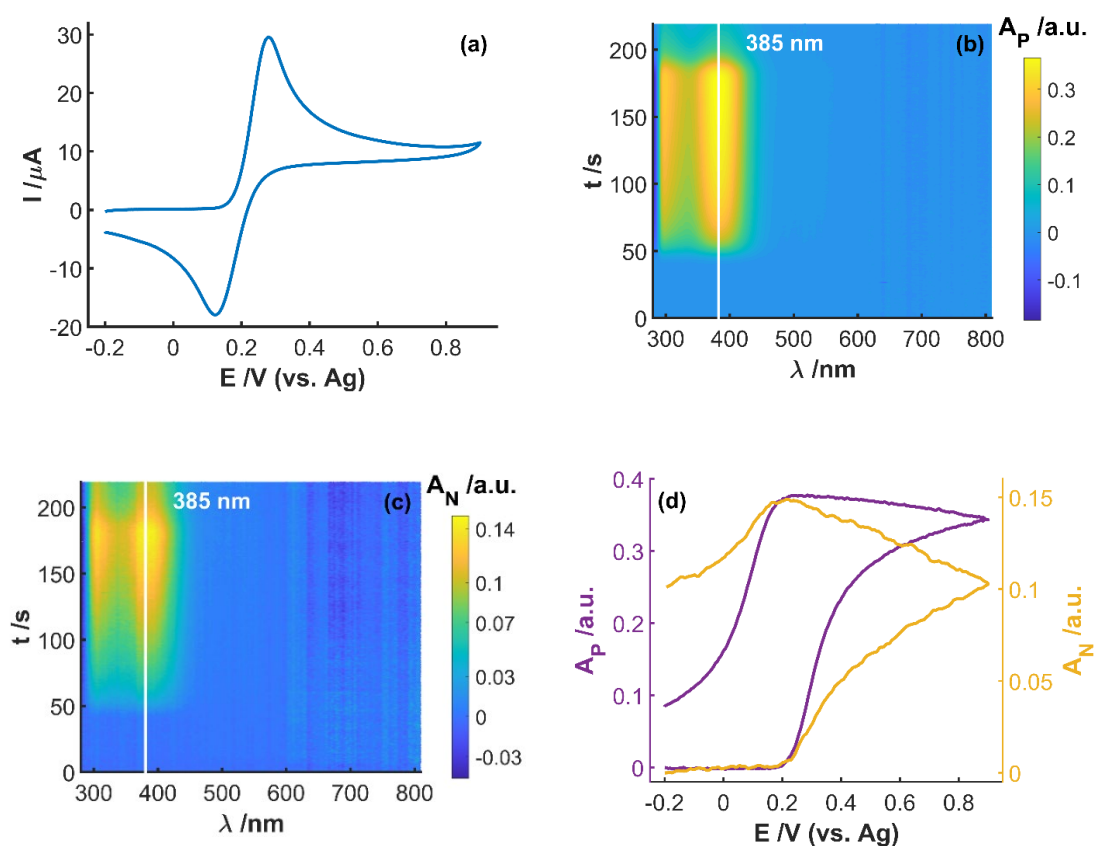


Figure 6-3. BSEC experiment of $2 \cdot 10^{-3}$ M EP in 0.1 M HClO_4 between -0.20 V and +0.90 V at $0.01 \text{ V} \cdot \text{s}^{-1}$.¹ (a) CV. Contour plot of the optical responses in (b) parallel and (c) normal configurations and (d) CVAs at 385 nm for the two optical configurations.

This multi-response technique avoids problems of data correlation when consecutive experiments are performed, due to the difficulty of achieving exactly the same experimental conditions. Figure 6-3 also reveals that the absorbance signal in parallel configuration is remarkably higher than that obtained in normal arrangement (0.37 a.u.

vs. 0.15 a.u.), due to the longer optical path length (2 mm) in parallel configuration vs. the thickness of the diffusion layer in normal arrangement.

An acidic pH has been selected to study EP because at a higher pH the product generated during the oxidation process can polymerize, yielding an insoluble product deposited on the electrode surface.^{278,279} In that case, BSEC is a very useful analytical technique because the adsorbed products are easily detected in normal direction, providing different spectra in the two optical configurations, normal and parallel.^{66,112} Acidic medium allows us to work using the same electrode for all the experiments.

BSEC experiments can be useful to extract valuable information about the process taking place at the electrode surface, including the diffusion coefficients or the thickness of the diffusion layer. For this purpose, suitable models have been described in literature^{57,259} which consider a precise definition of the position of the light beam interrogating the diffusion layer.

In a very simple approach, a very rough estimation of the thickness of the diffusion layer can be obtained from the ratio of the optical signal in normal and parallel configuration, knowing the distance between the two optical fibers in parallel arrangement. The ratio between the absorbance in the two configurations is proportional to the ratio between the optical pathways, and therefore, the approximate diffusion layer thickness can be calculated from Equation 6-1.

$$w(E) = \frac{A_N(E)d}{2A_P(E)} \quad \text{Equation 6-1}$$

where $A_N(E)$ is the absorbance in normal configuration at a specific wavelength and potential, $A_P(E)$ is the absorbance in parallel configuration at the same wavelength and potential, d is the optical pathway in parallel direction (distance between the two optical fibers) and $w(E)$ is the thickness of the diffusion layer at a specific potential, which is sampled twice in a near-normal reflection experiment.

Figure 6-4 shows the evolution of the thickness of the diffusion layer with potential. For example, in the vertex potential, +0.90 V, at 385 nm a thickness of 308 μm is calculated, reaching a value of 370 μm at +0.40 V in the backward scan, and finally a value of 390 μm at +0.20 V in the backward scan. In spite of being an approximation, these values are in good agreement with the expected value which can be calculated from the approximation $w = \sqrt{\pi Dt}$.

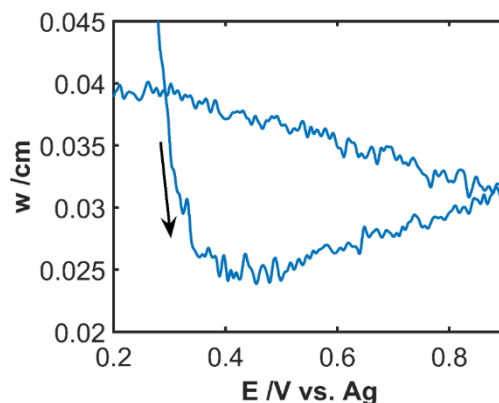


Figure 6-4. Evolution of the thickness of the diffusion layer with potential calculated from BSEC experiment performing a cyclic voltammetry of $2 \cdot 10^{-3}$ M EP in 0.1 M HClO_4 between -0.20 V and +0.90 V at $0.01 \text{ V} \cdot \text{s}^{-1}$.

6.1.3.2. BSEC of mixtures of EP and AA using cyclic voltammetry

After this initial spectroelectrochemical study of EP oxidation, BSEC experiments selecting cyclic voltammetry as electrochemical technique were carried out to compare the behaviors of EP, AA and a mixture of both compounds, all prepared in acidic medium (0.1 M HClO_4). In Figure 6-5 are plotted the CVs and the corresponding CVAs at the characteristic wavelength of EPQ, 385 nm.

The CVs in Figure 6-5a show the electrochemical oxidation mechanism of EP (explained in Section 6.1.3.1) and AA. The oxidation mechanism of AA is characterized by the transfer of two electrons and two protons in which dehydroascorbic acid (DHA) is electrogenerated, followed by an irreversible chemical reaction, hydrolysis of DHA, yielding 2,3-diketogulonic acid, electrochemically inactive.²⁸⁰ This mechanism can be observed in the AA CV (green line, Figure 6-5a), which only presents the anodic peak at +0.15 V. It should be highlighted that the oxidation of AA starts at 0 V, before the oxidation of EP, that begins at +0.15 V.

The CV corresponding to the mixture of EP and AA shows two overlapped anodic peaks, the first one related to the oxidation of AA and the second one to the oxidation of EP (orange line, Figure 6-5a). Comparing the CVs of EP and the mixture of EP and AA, and considering that both solutions have the same concentration of EP, several differences can be observed. On the one hand, the anodic peak of EP is 20 % more intense in the mixture of compounds than in the solution containing only EP. On the other hand, the anodic peak

potential is slightly higher in the mixed solution than in the EP solution. It can be concluded that AA interferes with the electrochemical detection of EP.

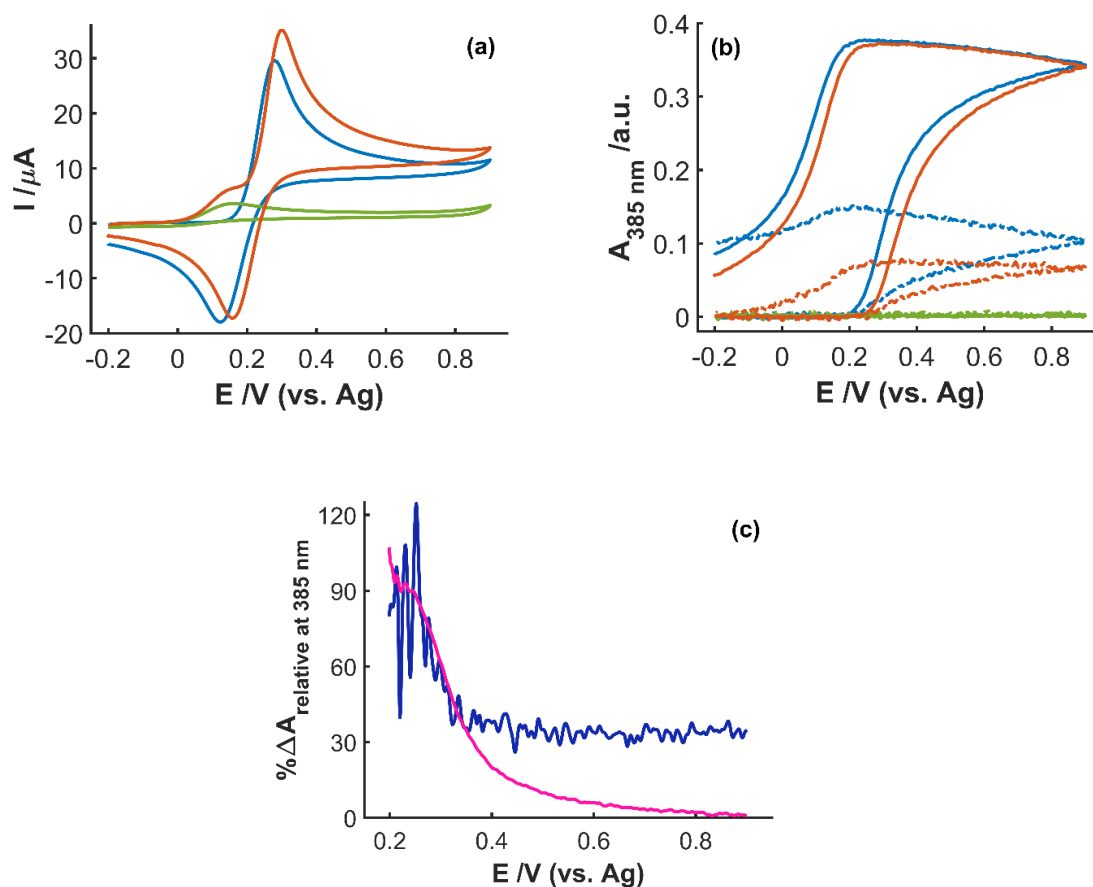


Figure 6-5. Comparison between (a) CVs and (b) the corresponding CVAs at 385 nm for the BSEC experiments of $2 \cdot 10^{-3}$ M EP (blue lines), $3 \cdot 10^{-4}$ M AA (green lines), and a solution mixture of $2 \cdot 10^{-3}$ M EP and $5 \cdot 10^{-4}$ M AA (orange lines), all in 0.1 M HClO_4 , between -0.20 V and +0.90 V at $0.01 \text{ V} \cdot \text{s}^{-1}$. The absorbance in normal configuration is denoted by dotted lines and the absorbance in parallel configuration by solid lines. (c) Relative variation of absorbance in normal (blue line) and parallel configuration (pink line) between the solution with EP and the one containing EP and AA in the forward scan.

To compare the spectroscopic responses, the CVAs at 385 nm of EP, AA, and EP + AA in normal and parallel configurations are plotted in Figure 6-5b. This wavelength was selected because it is related to the oxidation of EP to EPQ. For this reason, no change in absorbance values is observed in the CVAs recorded during the oxidation of the ascorbic acid solution (green lines, Figure 6-5b). However, several differences can be observed between both arrangements in the signals related to EP and EP + AA solutions. In both cases, the absorbance achieved in normal configuration is lower than in parallel

arrangement, which is due to the shorter optical pathway, as was explained in Section 6.1.3.1 for EP signals. Both, in normal and parallel configuration, it is observed that the oxidation of EP starts at +0.20 V. From this potential onwards, until the vertex potential, the absorbance increases due to the oxidation of EP to EPQ in a diffusion-controlled process. In the reduction scan, the absorbance decreases from +0.19 V due to the consumption of EPQ.

The absorbance value reached in the CVAs in both configurations due to the oxidation of EP in presence of AA is always lower than the absorbance value achieved in absence of this vitamin. This is due to the antioxidant effect of AA. When the potential applied is high enough, electrochemical oxidation of EP to EPQ occurs, which is then chemically reduced to EP by AA. This antioxidant effect is also responsible for the delay in detecting the increase in absorbance related to the generation of EPQ, which is noticeable from about +0.25 V. However, there are remarkable differences between normal and parallel configuration. Calculating the relative variation of absorbance at 385 nm in both configurations in the presence and absence of AA, according to Equation 6-2, in normal configuration absorbance in EP + AA solution is around a 35 % lower than in EP solution (blue line in Figure 6-5c) from +0.35 V and +0.90 V in the anodic scan. However, in parallel configuration this difference decreases along the anodic scan from a 35 % at +0.35 V to a 1 % at +0.90 V (pink line in Figure 6-5c).

$$\% \Delta A_{relative\ at\ 385\ nm}^X = \frac{A_{EP}^{X,385\ nm} - A_{EP+AA}^{X,385\ nm}}{A_{EP}^{X,385\ nm}} \times 100 \quad \text{Equation 6-2}$$

where absorbance values at 385 nm ($A^{385\ nm}$) correspond to the anodic scan between +0.20 V (where the oxidation of EP starts) and +0.90 V (where the anodic scan ends), and X denotes normal or parallel configuration.

The explanation for these differences in the optical signal is related to the different regions of the solution sampled with each arrangement. In parallel configuration, approximately, only the first 120 μm of the solution adjacent to the working electrode are sampled, considering the cladding of the optical fibers. At the end of the anodic scan in this arrangement practically all the AA is oxidized, and the antioxidant effect disappears along the experiment. Consequently, the more AA is oxidized, the more EPQ is present in the diffusion layer. Conversely, in normal configuration, both the diffusion layer and the bulk solution are sampled. In the solution near the electrode the AA is oxidized to DHA along

the anodic scan and its antioxidant action is less noticeable. But in the bulk solution AA still exists, which reacts with the electrogenerated EPQ that diffuses beyond 120 μm of the solution sampled by the optical fibers in parallel configuration, regenerating EP again. This causes that in normal configuration, the absorbance measured in the presence of AA is 35 % lower than that recorded in the absence of this antioxidant along the whole anodic scan.

Summarizing, as it is demonstrated, EPQ, which is the compound followed at 385 nm, only exists in the diffusion layer, corresponding to the first 120 μm of the solution adjacent to the working electrode where the amount of AA is negligible due to its electrochemical oxidation along the anodic scan and the chemical reaction of AA with EPQ. Consequently, as normal configuration provides information not only about the solution interrogated by the optical fibers, close to the electrode, but also about the bulk solution, where the presence of AA is practically constant, the differences between the absorbance values are remarkable and quite constant. In contrast, parallel configuration provides optical information only about the solution adjacent to the electrode surface, the first 120 μm , where the amount of AA greatly diminishes along the anodic scan. So, the EPQ can be successfully detected at the end of the oxidation scan in parallel configuration.

Finally, these results allow us to compare the differences between the normal and parallel configuration in a system with the presence of an antioxidant compound, understanding what signal can be used, in this case, to carry out the quantification of EP in the presence of a highly interfering species, and which ones cannot be used. In this way, as demonstrated below, the optical response in parallel configuration improves considerably the results of the EP detection using electrochemistry and the spectral response in normal arrangement, which is the most important fact to consider for carrying out the quantitative analysis.

6.1.3.3. Determination of EP in presence of AA using BSEC chronoamperometric detection

To quantify EP in presence of AA, chronoamperometric detection is selected due to its higher efficiency in the oxidation process by applying a sufficiently anodic fixed potential for a fixed time, which also shortens the length of the experiments. Since it is observed

in Figure 6-5a, EP is completely oxidized at +0.90 V and the process is diffusion-controlled.

BSEC chronoamperometry experiments were performed applying a potential of +0.90 V during 125 s. Figure 6-6 shows chronoabsorptograms (CABs) at 385 nm of EP, AA, and EP + AA, in normal and parallel configuration. When the potential of +0.90 V is applied, the absorbance increases because of the oxidation of EP generating EPQ (blue lines, Figure 6-6). As it has been explained above, the absorbance achieved in normal configuration (dotted lines, Figure 6-6) is always lower than in parallel configuration (solid lines, Figure 6-6), because of the shorter optical pathway. Around 125 s, the absorbance in parallel configuration reaches a constant and maximum value, because almost all the EP in the 100 μm sampled by the light beam is transformed in EPQ. As can be inferred, the interpretation of Figure 6-5 considering the different information obtained with normal and the parallel arrangements are also valid to chronoamperometric experiments depicted in Figure 6-6. As shown in the CVAs in Figure 6-5b, the CABs in normal configuration for EP + AA solution is around a 35 % lower than for EP solution because of the antioxidant effect of AA (orange lines, Figure 6-6). However, the antioxidant activity of AA in parallel configuration decreases significantly along the chronoamperometric experiment, reaching at 125 s almost the same absorbance value. In a similar way to CVs experiments, absorbance does not change during the whole experiment for solutions that only includes AA (green lines, Figure 6-6).

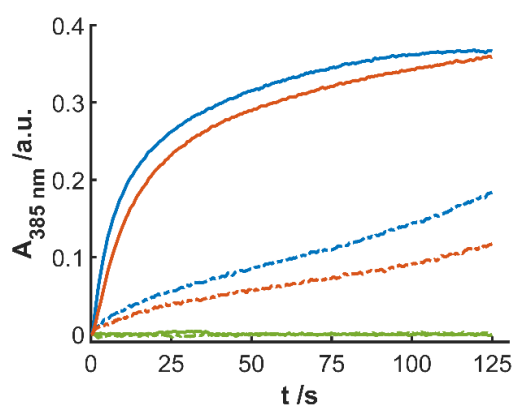


Figure 6-6. Comparison between the corresponding CABs at 385 nm for the BSEC experiments of $2 \cdot 10^{-3}$ M EP (blue lines), $3 \cdot 10^{-4}$ M AA (green lines), and a solution mixture of $2 \cdot 10^{-3}$ M EP and $5 \cdot 10^{-4}$ M AA (orange lines), all in 0.1 M HClO_4 , applying +0.90 V during 125 s. The absorbance in normal configuration is denoted by dotted lines and the absorbance in parallel configuration by solid lines.

From the absorbance values in normal and parallel configuration and considering Equation 6-1, the thickness of the diffusion layer is approximately 500 μm . Therefore, from the approximation $w = \sqrt{\pi Dt}$, an approximate diffusion coefficient of $6.37 \cdot 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ is estimated. Assuming a similar diffusion coefficient for AA, at 125 s, few AA is located close to the optical fibers and its interference in the oxidation of EP should be really low in parallel configuration, where only the 120 μm closest to the electrode surface are interrogated.

A set of 12 chronoamperometric BSEC experiments applying +0.90 V during 125 s were performed to construct a BSEC calibration model, with the aim of demonstrating that EP can be determined not only in the absence but also in the presence of different concentrations of AA when parallel arrangement is used. Hence, different calibration samples were prepared with concentrations of EP between 0 and $5 \cdot 10^{-3} \text{ M}$ in 0.1 M HClO_4 and with different concentrations of AA, between 0 and $5 \cdot 10^{-4} \text{ M}$ (Table 6-1).

Table 6-1. Set of samples prepared with different concentrations of EP and AA for BSEC calibration.

Sample	C_{EP} (M)	C_{AA} (M)
01	0	0
02	$2 \cdot 10^{-3}$	0
03	0	$3 \cdot 10^{-4}$
04	$1 \cdot 10^{-3}$	$4 \cdot 10^{-4}$
05	$3 \cdot 10^{-3}$	$4 \cdot 10^{-4}$
06	$5 \cdot 10^{-4}$	$3 \cdot 10^{-4}$
07	$2 \cdot 10^{-4}$	$5 \cdot 10^{-4}$
08	$1 \cdot 10^{-4}$	$1 \cdot 10^{-4}$
09	$2 \cdot 10^{-3}$	$5 \cdot 10^{-4}$
10	$5 \cdot 10^{-3}$	$3 \cdot 10^{-4}$
11	$4 \cdot 10^{-3}$	$5 \cdot 10^{-4}$
12	0	0

Each experiment provides three different signals: the chronoamperogram and two spectroscopic ones (the CAbs in normal and in parallel configuration), being able to

obtain three calibration curves (Figure 6-7). It should be indicated that current intensity and absorbance values in normal and parallel configuration have been obtained at 125 s after starting the experiment, that, according to Figure 6-6, is the best time to quantify EP in presence of AA, particularly for the parallel configuration. The figures of merit obtained with each calibration model are tabulated in Table 6-2.

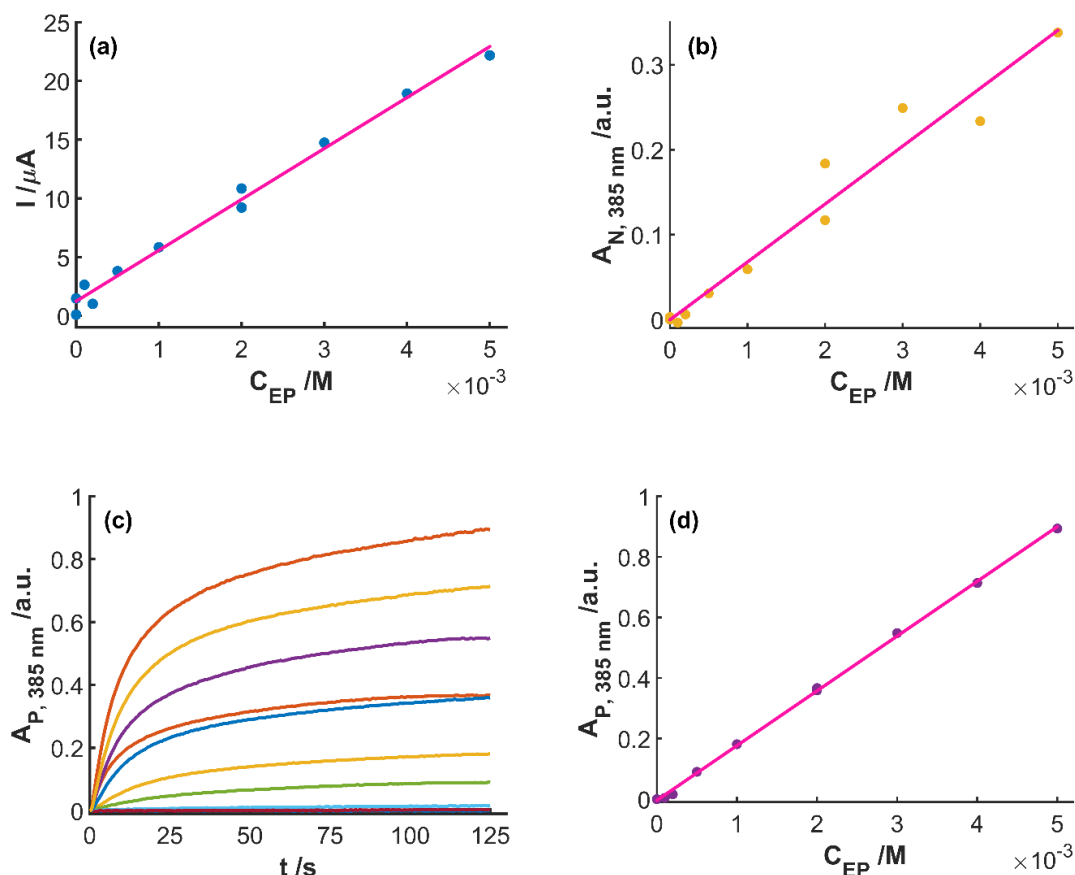


Figure 6-7. Calibration curves at 125 s, after starting to apply the potential of +0.90 V, for (a) the current intensity, (b) the normal absorbance at 385 nm and (d) the parallel absorbance at 385 nm, versus EP concentration. Twelve samples with different concentrations of EP and AA were measured. (c) CABS in parallel configuration for the 12 samples measured.

In the calibration curve obtained with the electrochemical data (Figure 6-7a), there are some aspects that must be remarked considering that current intensity includes the oxidation of both compounds, EP and AA. There is a great difference between the two calibration points with the same concentration of $2 \cdot 10^{-3}$ M EP. The point which is above the calibration curve has $5 \cdot 10^{-4}$ M AA (sample 09), while the other point does not have AA (sample 02). Therefore, the electrochemical calibration curve is very sensitive to the presence of the interfering compound because the electrochemical signal is due to the

oxidation not only of EP but also of AA. In addition, it should be highlighted that the calibration curve remarkably fails at the lowest concentrations of EP (see that the blank solution of EP in presence of AA, sample 03, gives a current intensity value very different to the expected value of zero, samples 01 and 12). Therefore, the experimental intercept is very different to the expected one if no interferent affects the quantification. Consequently, electrochemical data it is not suitable for the quantification of EP in presence of AA, even though the R^2 is close to 1.

Table 6-2. Figures of merit for the linear regression models obtained from current intensity and absorbance values at 385 nm, +0.90 V and 125 s, in normal and parallel configuration.

Calibration model	Slope	Intercept	R^2	S_{yx}
$I_{125\text{ s}}$ vs. C_{EP}	$4.3 \cdot 10^3 \text{ A} \cdot \text{M}^{-1}$	1.23 μA	0.9897	$8.1 \cdot 10^{-1}$
$A_{N,385\text{ nm}}$ vs. C_{EP}	$6.8 \cdot 10^1 \text{ M}^{-1}$	$3.0 \cdot 10^{-4}$ a.u.	0.9568	$2.7 \cdot 10^{-2}$
$A_{P,385\text{ nm}}$ vs. C_{EP}	$1.8 \cdot 10^2 \text{ M}^{-1}$	$-4.5 \cdot 10^{-3}$ a.u.	0.9993	$8.7 \cdot 10^{-3}$

R^2 : coefficient of determination; S_{yx} : standard deviation of residuals

Figure 6-6b and Figure 6-6d show the calibration curves obtained with the spectroscopic data, registered in normal and parallel configuration, respectively, where the absorbance at 385 nm and +0.90 V is represented versus EP concentration. It should be highlighted that also in normal configuration there is a great difference between the values with a concentration of EP of $2 \cdot 10^{-3}$ M. In this case, the point above the calibration curve has no AA (sample 02), while the point below the calibration curve has $5 \cdot 10^{-4}$ M AA (sample 09). As in the electrochemical calibration curve, the absorbance in normal arrangement is very sensitive to the presence of this interfering compound. Although this calibration curve is not adequate to predict the concentration of EP in presence of AA, there is an important advantage with respect to the electrochemical one. The intercept is very similar to the expected value of zero, because the AA does not absorb at 385 nm.

As expected from the previous Sections, the figures of merit obtained when the solution is sampled in a parallel configuration are clearly better. The slope is significantly higher because of the higher optical pathlength improving the sensitivity, the intercept is very close to zero noting that AA does not absorb at this wavelength and does not interfere in the absorbance values measured, the R^2 is almost 1 and the S_{yx} is really low stating that the regression predictions fit perfectly the data. Therefore, in parallel configuration, since

at 125 s there is practically no interfering effect of AA, quantification of EP in presence of AA in this concentration range can be suitably performed under these conditions (Figure 6-6c and Figure 6-6d).

Aiming to compare the capability of prediction of these calibration models, Table 6-3 shows the predicted concentrations of different measured samples of EP and AA and their relative errors. As can be observed, although none of the R^2 values are excessively far from 1 and S_{yx} values are rather low, the values obtained using the calibration model in parallel configuration are much better than those obtained for the other calibration curves. In addition, the relative errors obtained when the parallel calibration curve is used are lower than 5 %.

Table 6-3. Predicted concentrations with their relative errors for five different samples obtained using the calibration models in Figure 6-7 and Table 6-2.

Calibration model	C_{EP} , REAL (M)	C_{EP} , Predicted (M)	C_{AA} , REAL (M)	% ε_r
$I_{125\text{ s}}$ vs. C_{EP}	$5 \cdot 10^{-4}$	$5.91 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	18
	$1 \cdot 10^{-3}$	$1.06 \cdot 10^{-3}$	$4 \cdot 10^{-4}$	6
	$2 \cdot 10^{-3}$	$2.21 \cdot 10^{-3}$	0	11
	$2 \cdot 10^{-3}$	$1.83 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	-8
	$4 \cdot 10^{-3}$	$4.07 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	2
$A_{N,385\text{ nm}}$ vs. C_{EP}	$5 \cdot 10^{-4}$	$4.57 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	-9
	$1 \cdot 10^{-3}$	$8.73 \cdot 10^{-4}$	$4 \cdot 10^{-4}$	-13
	$2 \cdot 10^{-3}$	$2.69 \cdot 10^{-3}$	0	34
	$2 \cdot 10^{-3}$	$1.71 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	-14
	$4 \cdot 10^{-3}$	$3.43 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	-14
$A_{P,385\text{ nm}}$ vs. C_{EP}	$5 \cdot 10^{-4}$	$5.22 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	4
	$1 \cdot 10^{-3}$	$1.03 \cdot 10^{-3}$	$4 \cdot 10^{-4}$	3
	$2 \cdot 10^{-3}$	$2.05 \cdot 10^{-3}$	0	3
	$2 \cdot 10^{-3}$	$2.01 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	0.4
	$4 \cdot 10^{-3}$	$3.97 \cdot 10^{-3}$	$5 \cdot 10^{-4}$	0.8

ε_r : Relative error

Thus, with these results it has been demonstrated that, the electrochemical curve is not suitable for such determination because the current intensity is associated with the oxidation of both compounds (EP and AA). Similarly, spectroscopic in normal arrangement is not either adequate to such determination because it samples both the diffusion layer and the bulk solution. Only the spectral information in parallel configuration provides a valid calibration curve for the determination of the concentration of the analyte of interest (EP) present in a problem sample in presence of an antioxidant compound such as AA. The information provided in parallel arrangement is related to the first 120 μm adjacent to the electrode surface, where the presence of AA is practically negligible at a time greater than 125 s, thus removing the antioxidant effect at the AA concentrations studied in this work.

The results of this work demonstrate the capabilities of BSEC for analysis of EP in presence of AA, showing the negative influence of this interfering compound in the electrochemical signal and the optical signal in normal configuration, being the parallel configuration a good option to quantify EP in presence of AA. The method is robust for concentrations of AA lower than $5 \cdot 10^{-4} \text{ M}$. If AA concentrations higher than $5 \cdot 10^{-4} \text{ M}$ need to be analyzed, a strategy based on a thin-layer cell can be used. For example, for $5 \cdot 10^{-3} \text{ M}$ EP in presence of $5 \cdot 10^{-3} \text{ M}$ AA, a difference of the signal in presence and in absence of AA lower than a 3 % is observed at 125 s in parallel direction in an experiment in a thin-layer cell (Figure 6-8).

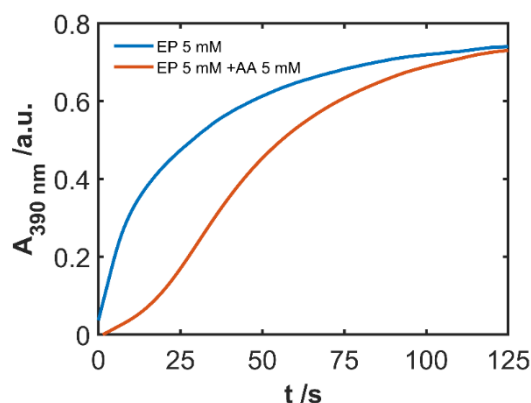


Figure 6-8. Comparison between the corresponding CAbs at 385 nm in a thin layer cell for the BSEC experiments of a $5 \cdot 10^{-3} \text{ M}$ EP solution (blue line) and a mixture of $5 \cdot 10^{-3} \text{ M}$ AA and $5 \cdot 10^{-3} \text{ M}$ EP (orange line), all in 0.1 M HClO_4 , applying +0.90 V during 125 s.

Moreover, the methodology presented in this work could be used to resolve more complex samples, including for example similar analytes (dopamine, L-DOPA or interfering compounds), but multivariate analysis would be needed to deconvolve the optical signals.

6.1.4. Conclusions

This work shows, for the very first time, quantitative analysis using BSEC, which is a powerful tool that permits to assess which is the best arrangement to perform quantitative measurements of an analyte in presence of a direct interfering and antioxidant compound quickly and easily. With this objective, the determination of EP in presence of AA in acidic media was selected.

It has been demonstrated that the electrochemical signal includes information of the oxidation process that occurs on the electrode surface, the oxidation of EP to EPQ and the oxidation of AA to DHA. On the other side, measurements of absorbance in normal configuration, where both the diffusion layer and the bulk solution are sampled, include information related to the distance-integrated concentration of reactants and products across the diffusion layer as a result of the oxidation reaction at the electrode surface. In the problem tackled in this work, the presence of AA significantly affects the detection of EP, being neither possible to quantify it correctly.

Conversely, absorbance in parallel arrangement allows the determination of EP despite the presence of AA. For this purpose, a sufficiently high potential has been chosen to effectively oxidize both, AA and EP, in order to practically eliminate the antioxidant effect of AA. In this case, sampling approximately the 120 μm of the solution adjacent to the working electrode in parallel configuration, where only the diffusion layer is observed, the amount of AA at long enough times can be negligible due to its electrochemical oxidation and the chemical reaction with EPQ. Actually, the process is more complicated because the formal potential of the two redox couples (AA/DHA and EP/EPQ) are very close, as is observed in Figure 6-5. Therefore, the methodology shown in this work is effective for AA concentrations lower than $5 \cdot 10^{-4}$ M, since the diffusion of AA from the bulk solution in more concentrated solutions can interfere in the optical response. For higher concentrations, working in a thin-layer set-up would be mandatory in order to avoid the diffusion of the interfering compound from the bulk solution. In summary, while the electrochemical and normal absorbance predicted values are very sensitive to the presence of the antioxidant species in the sample, the parallel absorbance provides

excellent results to quantify EP in presence of AA, which should be extrapolated to equivalent systems. Always, spectroelectrochemistry users must take care to select appropriate experimental conditions to electrochemically remove the interfering compounds.

This paper increases the advantages of spectroelectrochemistry in terms of selectivity, as well as demonstrates the need to select properly the optical mode to carry out the analysis of the compounds, being very important to know which configuration is more convenient to select in each case. This strategy lays the groundwork for spectroelectroanalysis for cases in which antioxidant compounds are present in the solution.

6.2. UV/Vis absorption spectroelectrochemistry of folic acid

The utility and versatility of UV/Vis absorption SEC as an analytical tool in the quantitative field is demonstrated in this Section, where the quantification of folic acid in a pharmaceutical tablet by UV/Vis SEC in the parallel configuration is carried out. For this purpose, first of all, the oxidation process of this target molecule in alkaline medium will be studied. The consumed folic acid and the oxidation products formed have been detected by this hyphenated technique. Afterwards, the development of an analytical protocol has been optimized, demonstrating that high repeatability and low detection limits are obtained working in a basic medium ($\text{pH} = 12.9$), as well as good linear regressions between analyte concentration and optical signal. Although only the spectroscopic signal can be used in this study when quantifying the analyte, SEC is highly essential, since the optical signal represents the changes that occur during the electrochemical oxidation of the FA. This study has been published in *Journal of Solid State Electrochemistry*, 2021, 26, 29–37.²⁸¹

6.2.1. Introduction

Instrumental analytical techniques commonly used to identify and quantify a specific molecule are typically based on the use of a single signal characteristic of the compounds under study. However, it is well known that the use of two or more techniques can be much more helpful in the determination of a molecule. Techniques such as spectroelectrochemistry (SEC) are extremely useful analytical tools in the quantification of molecules and have proven to be very useful when dealing with complex samples or when there is a large matrix effect.^{42,123,124,282,283} SEC encompasses a group of analytical techniques that are characterized by the simultaneous recording of electrochemical and spectroscopic signals.^{8,284–288} Among all the SEC techniques, one of the most widely used is UV/Vis absorption SEC (UV/Vis SEC), in which during the electrochemical oxidation or reduction of a molecule, changes in the molecular absorption spectrum of the studied analytes are observed due to the electrogeneration of the reaction products and/or the consumption of the native molecule.^{7,8,285,286,289}

SEC in general, and UV/Vis SEC in particular, is a group of instrumental techniques commonly used in the characterization of new materials and/or molecules, in the study of electron transfer processes, or in the study of reaction mechanisms.^{30,54,251,284,290–295} However, the great potential of these multi-response techniques for quantitative analysis

has not been enough explored.^{7,40,42,71,123,124,283,296,297} Among all SEC techniques, the coupling of electrochemistry and UV/Vis absorption spectroscopy is the most successful one in the field of electroanalysis. Significant improvements in instrumentation and SEC devices, such as SEC cells, have significantly contributed to the fruitful use of these operando and multi-response techniques for analytical purposes.^{6,8,30,42,52,265,287} From a quantitative point of view, it should be remarked that UV/Vis SEC can provide a dual determination of the analyte studied, using both the electrochemical and the spectroscopic signals, providing much more reliable and fully validated results. In some cases, the electrochemical signal is not good enough for quantitative purposes, but a good optical signal is obtained during the electrochemical process. Furthermore, UV/Vis SEC results are characterized by a high reproducibility and very low relative standard deviation values (RSD), parameters required by all analytical techniques used for quantitative purposes. Lastly, it should be pointed out that the information gathered in this type of experiments, concerning the reaction mechanism involved in the electron transfer process studied, helps significantly not only to detect outliers but also to obtain an explanation for why that point is an outlier.⁶

Vitamin B9 belongs to the group of water-soluble vitamins and its structure is derived from folic acid (FA). FA is a cofactor in methylation reactions, in biochemical reactions related to the synthesis of some nitrogen bases, such as adenine, guanine, or thymine, and in amino acid metabolism. Therefore, deficiency of this vitamin is directly related to improper DNA replication and, consequently, to improper cell division. Increased metabolic demand for folate is common in pregnancy due to megaloblastic anemia. A deficiency of this vitamin in pregnancy can lead to defects in the embryo, especially in the neural tube. Deficiency of vitamins B9 and B12 is also associated with some diseases related to the central nervous system such as dementia, depression, or Alzheimer's disease and with vascular diseases such as thrombosis.^{298–301} To avoid FA deficits, since the human body is not able to produce FA, adequate levels of vitamin B9 must be achieved through diet or food supplements.

FA derives from three chemical structures: a 6-methylpterin, *p*-aminobenzoic acid and glutamic acid.²⁹⁸ There are many different folates depending on the glutamate residues included in the chemical structure.³⁰⁰ On the other hand, folate exists in different oxidation states. The natural folates have the pterin ring in its reduced state such as 7,8-

dihydrofolate or 5,6,7,8-tetrahydrofolate, not being a natural physiological form of this vitamin the pterin ring in its high oxidized state.^{298,300,302}

The solubility of FA in water is low in neutral medium (1.6 mg/L at 25 °C) and depends strongly on the pH of the solution.³⁰³ Under high acidic conditions the solubility of FA increases significantly, but its stability decreases at pH values below 5. On the other hand, solubility increases significantly with increasing pH (50 mg/mL at 1 M NaOH), being stable solutions when protected from light.²⁹⁸

In alkaline medium, it presents three well-defined absorption bands in the UV/Vis region, at 256, 283 and 365 nm assigned to the $\pi \rightarrow \pi^*$ electronic transitions due to the pterin ring and *p*-aminobenzoic acid moieties of FA,^{206,304,305} and its electrochemical properties, both in oxidation and reduction processes, are related to the conjugated pteridine ring.^{200,306}

FA has been studied and quantified with different analytical techniques such as photoluminescence,^{307–309} UV/Vis absorption spectroscopy,^{209,310} electrochemistry,^{200,221,311–313} HPLC^{314,315} or HPLC-MS.^{215,316} Some of these techniques require complex and long sample pretreatments, others imply the modification of the surface used to determine this molecule such as in electrochemistry. Since FA has a characteristic UV/Vis absorption spectrum and its oxidation process is previously described, UV/Vis SEC can be a suitable instrumental technique to study the electron transfer reaction of vitamin B9.

In this work, a UV/Vis SEC based methodology is proposed to quantify FA in a pharmaceutical drug. Specifically, in this work, FA is electrochemically oxidized while changes in the UV/Vis absorption spectra, which are used to identify and quantify FA, are simultaneously recorded. The determination protocol developed is simple, not time-consuming, and provides optimal results in the identification and quantification of FA. By adapting this protocol to other substances and other matrices, this approach can be used for the analysis of other drugs in real samples.

6.2.2. Experimental

6.2.2.1. Chemicals and Materials.

FA (97 %, Sigma-Aldrich), KCl (99+ %, Acros Organics), NaOH (Acros Organics), glacial acetic acid (VWR) and sodium acetate (>99 %, VWR) were of analytical grade

and were used as delivered without further purification. Yodocefol® (ItalFarmaco). The protocols used in all the experiments performed in this work ensure safe and careful handling of all reagents.

All solutions were freshly prepared using ultrapure deionized water (18.2 MΩ cm resistivity at 25 °C, Milli-Q Direct 8, Millipore).

6.2.2.2. *Instrumentation*

UV/Vis SEC measurements were performed using a customized UV/VIS SPELEC instrument (Metrohm-DropSens), employing the experimental set-up described previously.^{42,283} A deuterium lamp was used because the reagents and products of this electrochemical process absorb electromagnetic radiation in the UV region. Carbon DRP-110 screen-printed electrodes (SPE, Metrohm-DropSens) were used for UV/Vis SEC measurements. These C-SPEs consist of a carbon working electrode (C-WE) of 4 mm diameter, a silver pseudo-reference electrode and a carbon counter electrode.

UV/Vis SEC experiments in normal configuration were carried out using the Teflon® SEC cell for reflection experiments (DRP-REFLECELL, Metrohm-DropSens) and a reflection probe (DRP-RPROBE, Metrohm-DropSens).

UV/Vis SEC experiments in parallel configuration were carried out by facing and attaching two 100-μm-bare optical fibers (Avantes) to the surface of the C-WE of the SPEs, that were placed at the boxed connector for SPEs (DSC, Metrohm-DropSens). The optical pathway was measured for each C-SPE used, which is in all cases between 2 and 3 mm in length. A 50-μL aliquot of the solution studied is used in each experiment.

6.2.2.3. *UV/Vis SEC study of the effect of pH on FA oxidation*

0.1 M acetic/acetate buffer solutions were prepared, being their pH = 5. 0.1 M KCl solutions were prepared, being their pH = 7. Also, 0.1 M NaOH solutions were prepared, being their pH = 12.9.

UV/Vis SEC experiments in normal configuration were performed to test the influence of the pH on the process of electrochemical oxidation of FA. Solutions of 100 μM FA in the specific medium tested (buffer acetic/acetate, KCl or NaOH) were prepared and cyclic voltammetry experiments were carried out. The potential was scanned in the anodic direction at 0.01 V·s⁻¹ between 0.00 and +0.90 V (0.1 M NaOH) or between +0.70 and

+1.30 V (0.1 M acetic/acetate buffer and 0.1 M KCl) and simultaneously the evolution of the absorbance between 240 and 900 nm is recorded along the whole experiments. The spectrum of the sample at the starting potential was taken as reference and changes of absorbance respect to this reference spectrum (ΔA) were recorded. The integration time for absorptometric data in all experiments was 400 ms.

6.2.2.4. UV/Vis SEC quantification Protocol

UV/Vis SEC measurements were performed in 0.1 M NaOH (pH = 12.9), changing the concentration of FA between 5 and 100 μM . SEC experiments were performed collecting the evolution of absorption spectra between 240 and 900 nm during a cyclic voltammetry experiment. The potential was scanned between 0.00 and +0.90 V at $0.02 \text{ V}\cdot\text{s}^{-1}$, starting all the experiments at 0.00 V in the anodic direction. In all UV/Vis SEC experiments, the first spectrum of the sample at 0.00 V was taken as reference and, therefore, the change of absorbance respect to the reference spectrum was recorded, expressed as ΔA in all figures. Time-resolved UV/Vis SEC measurements were performed using an integration time of 400 ms.

The calibration models were constructed by randomly measuring the different FA solutions, varying its concentration between 5 and 100 μM . Each point in the calibration models was replicated three times. Before performing the spectroelectrochemical measurements, the SPEs were pretreated by placing 50 μL of a 0.1 M NaOH solution and performing 10 potential scans between 0.00 and +0.90 V at $0.02 \text{ V}\cdot\text{s}^{-1}$. The same C-SPE was used for each calibration test. The blank solution containing 0.1 M NaOH was measured before and after sampling all the calibration samples to check that the WE does not undergo any modification.

6.2.2.5. Test sample preparation

Yodocefol® tablets were used as test sample. Each tablet of this pharmaceutical drug contains 400 μg of FA, 262 μg of potassium iodide, 2 μg of vitamin B12, and different excipients (110 mesh lactose monohydrate, microcrystalline cellulose, sodium potato starch glycolate, calcium stearate, trisodium citrate, citric acid and maltodextrins). To prepare test samples of this drug, first a dispersion of the tablet is prepared in 25 mL of 0.1 M NaOH. After decantation of the non-water-soluble excipients, 1 mL-aliquots of the supernatant were centrifuged at 4000 rpm for 28 minutes in Eppendorf vials. The

supernatant obtained was directly used as drug sample to be quantified, that considering the starting dilution, the FA concentration in this test sample was 36 μM .

6.2.2.6. *Data acquisition and analysis Software*

SPELEC instrument was controlled by DropView SPELEC software (Metrohm-DropSens), performing operando, time-resolved SEC experiments with fully synchronized data acquisition.

Matlab 2018 and R 3.6.1 were used to analyze all UV/Vis SEC experiments, to generate the calibration models and to predict the FA concentration of test samples.

6.2.3. Results and discussion

6.2.3.1. *FA oxidation with UV/Vis absorption spectroelectrochemistry*

UV/Vis SEC can be performed using a normal or parallel arrangement. When soluble products of reaction are formed during the electrochemical process, UV/Vis in parallel arrangement is more suitable for quantitative purpose. Nevertheless, UV/Vis SEC in normal arrangement is very suitable for performing a rapid screening of the best experimental conditions used for quantification. Thus, UV/Vis SEC in normal arrangement was used to look for the best electrolytic medium to obtain a sensitive and reproducible optical response.

Three different electrolytic media were explored to determine FA using UV/Vis SEC in normal configuration: (a) 0.1 M NaOH (pH = 12.9), (b) 0.1 M KCl (pH = 7) and (c) 0.1 M acetic/acetate buffer (pH = 5). Figure 6-9 shows the contour plots for the three different electrolytic media tested, where evolution of absorption spectra during a UV/Vis SEC experiment were shown. As can be observed, only in alkaline media, well-defined and sufficiently intense UV/Vis absorption signals with suitable spectral resolution were recorded.

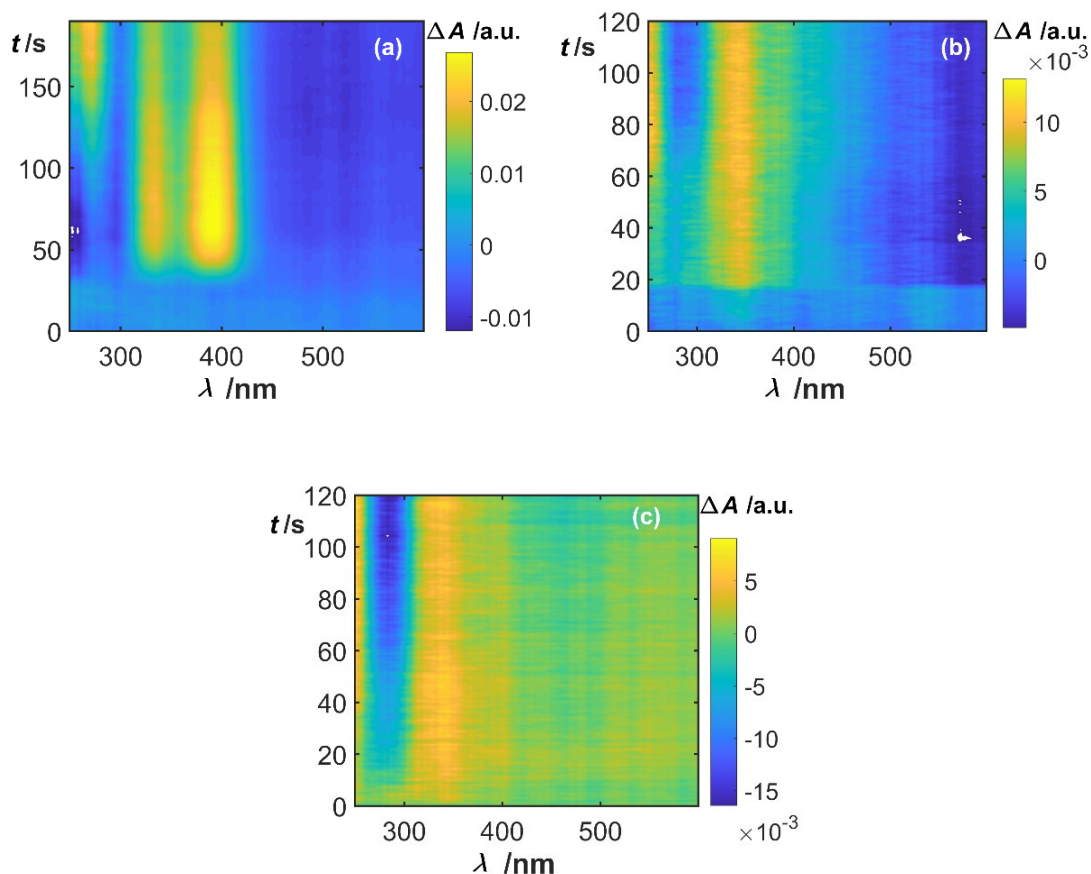


Figure 6-9. Contour plots of the absorbance with time/potential during SEC experiments in a 100 μM FA solutions in (a) 0.1 M NaOH (pH = 12.9), (b) 0.1 M KCl (pH = 7) and (c) 0.1 M acetic/acetate buffer (pH = 5). Potential was scanned between 0.00 V and +0.90 V at $0.01 \text{ V}\cdot\text{s}^{-1}$ in (a), between +0.70 V and +1.30 V at $0.01 \text{ V}\cdot\text{s}^{-1}$ in (b) and between +0.70 V and +1.30 V at $0.01 \text{ V}\cdot\text{s}^{-1}$ in (c). ΔA values in normal configuration were measured taking the starting FA solution as reference spectrum. Integration time was 400 ms.

Figure 6-10 shows the SEC responses obtained in parallel configuration with a 100 μM FA and 0.1 M NaOH solution (pH = 12.9). As can be seen, a small and ill-defined anodic peak can be observed in the cyclic voltammogram between +0.50 and +0.60 V (CV, Figure 6-10a). This ill-defined anodic peak must be related to the oxidation of FA since it is the main difference with respect to the CV of the blank solution. However, a clear evolution of the UV/Vis absorption spectra is appreciated during the CV (Figure 6-10b). These absorbance changes are related to the oxidation of FA as can be clearly observed in the cyclic voltabsorptograms (CVA, Figure 6-10c).

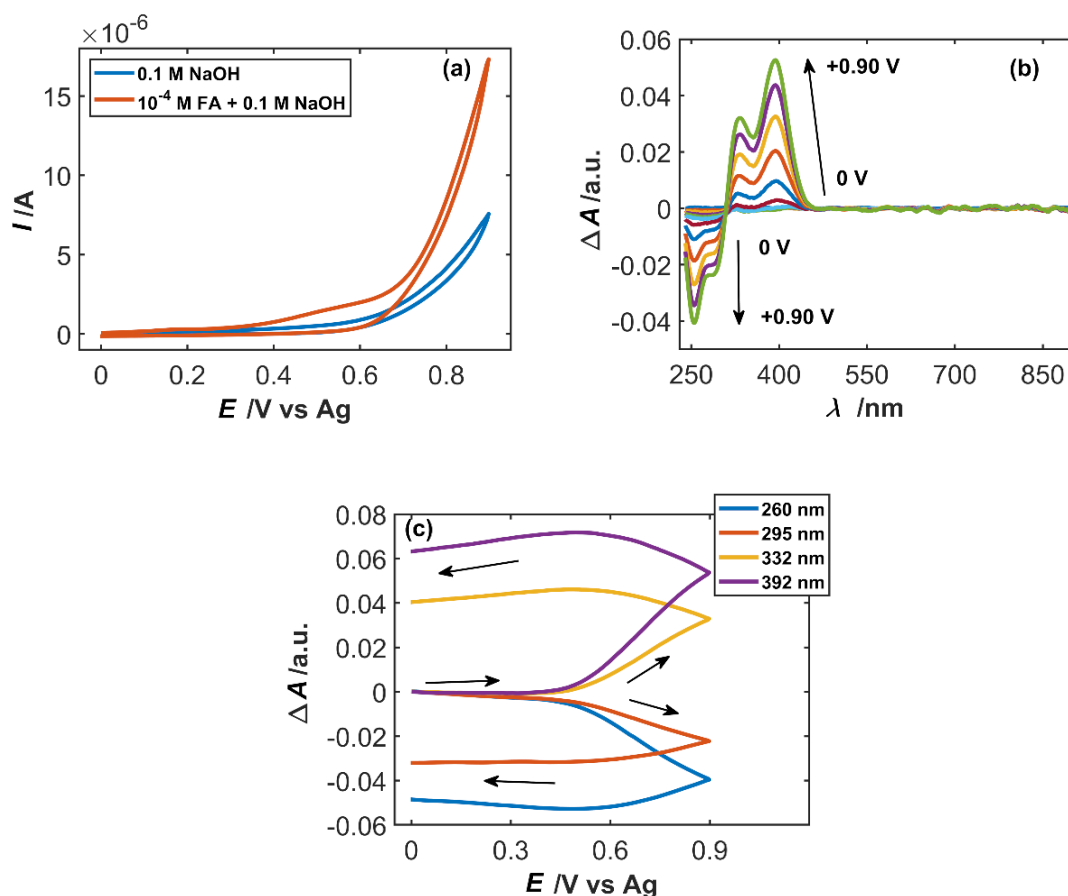


Figure 6-10. (a) CV of the blank solution (0.1 M NaOH, blue line) and of the FA solution (orange line). (b) Absorption spectra recorded at different potentials during the anodic scan, and (c) CVAs at 260 (blue line), 295 (orange line), 332 (yellow line) and 392 (violet line) nm in a 100 μ M FA and 0.1 M NaOH solution. Potential was scanned between 0.00 V and +0.90 V at 0.02 V/s. ΔA values in parallel configuration were measured taking the starting FA solution at 0.00 V as reference spectrum. Integration time for absorbance measurements was 400 ms. Optical path-length = 2.3 mm.

In the evolution of the spectra during the anodic scan (Figure 6-10b), four absorption bands were observed, two overlapped positive ones at 332 and 392 nm and two small overlapped negative ones at 260 and 295 nm, showing a single isosbestic point at 312 nm. This isosbestic point indicates that there is an interconversion of the reactant to a single product that absorbs electromagnetic radiation in this spectral range. Negative absorbance values are observed because the reference spectrum is taken in the FA initial solution. At alkaline pH values, FA is found as folate anion,³¹⁷ showing three bands around 256, 285 and 365 nm according to literature.^{209,304,305,318,319} According to the literature,²⁰⁶ the absorption band decreasing at 260 nm is related to the $\pi \rightarrow \pi^*$ transition of the pterin moiety, while the absorption band at 295 nm is related to the $\pi \rightarrow \pi^*$ transition of the amino-benzoyl ring. Thus, the two absorption bands with positive values can be ascribed

to the formation of a new compound during the oxidation of FA, while the two absorption bands with negative values correspond to the FA that is consumed from the solution adjacent to the C-WE during the oxidation process. CVA at these four absorption bands (Figure 6-10c) shown how the oxidation of FA begins at +0.55 V, since the absorbance increases in the bands associated with the oxidation product of FA and decreases in the bands associated with the consumption of FA. Once the vertex potential (+0.90 V) has been reached, absorbance continues increasing in the cathodic scan until it reaches a maximum value around +0.60 V. From this potential downwards, the absorbance only decreases slightly during the cathodic scan because of the diffusion of the products of the electrochemical process.

As was described in bibliography, FA oxidation reaction is irreversible, since FA is oxidized to an enamine that subsequently suffers a hydrolysis process, breaking this enamine into the pterin moiety as 6-formylpterin and the *p*-aminobenzoyl-L-glutamic acid (PGA).^{305,311} After oxidation, absorption spectral changes (ΔA) at wavelengths longer than 300 nm should correspond to the 6-formylpterin fragment. This hydrolysis of FA in alkaline medium has been previously reported³⁰⁶ and, according to the literature, the ΔA spectra of derivatives of the 6-formylpterin fragment shown absorption bands between 300 and 400 nm.^{318,320,321} In our case, we observe absorption bands at 332 and 392 nm, which confirms this hydrolysis process.

From the electrochemical signal is not easy to follow the oxidation of FA due to the ill-defined anodic peak recorded, however the optical signal clearly confirms that the molecule is oxidized in this alkaline electrolytic medium. This is one of the main advantages of UV/Vis SEC because the electrochemical oxidation process of molecules can be studied without a clear evidence of the process in the electrical signal.

6.2.3.2. *FA determination with UV/Vis absorption spectroelectrochemistry*

Once the optimal conditions for the study of FA were determined, the calibration of FA using the optical signal was performed. In this case, the electrochemical signal cannot be used as no oxidation peak is observed. Different tests and preliminary experiments were carried out to determine the linear range. At concentrations greater than 100 μM , no linear relationship between concentration and absorbance was found. On the other side, concentration values below 5 μM cannot be easily differentiated from the signal noise. Thus, a calibration set of known FA concentration samples between 5-100 μM was

prepared, with standards of 5, 8, 10, 20, 40, 60, 80 and 100 μM of FA, always in 0.1 M NaOH to ensure that the desired pH is the same in all solutions ($\text{pH} = 12.9 \pm 0.1$). A parallel configuration UV/Vis SEC cell was selected for the measurements because it is more sensitive than normal arrangement for soluble products of the electrochemical reaction due to its longer optical path-length.

Figure 6-11 shows the optical responses obtained during the calibration procedure for some of the tested samples: 0, 8, 40, 80 and 100 μM . Figure 6-11a shows the UV/Vis absorption spectra of these FA samples recorded at +0.55 V in the reverse scan, in the spectral range of 200-500 nm where absorbance changes are detected. As was expected, the higher the FA concentration, the higher the recorded absorbance related to the oxidation/hydrolysis products. A good reproducibility between measurements was obtained as can be deduced from the two samples shown at 40 μM , which practically are overlapped. The CVAs at 392 nm for the solutions of concentration equal to those shown in Figure 6-11a are shown in Figure 6-11b. This wavelength is selected because it corresponds to the most sensitive absorption band. Oxidation starts from a potential of +0.55 V onwards, observing an increase of absorbance, reaching a maximum during the reverse scan at +0.55 V.

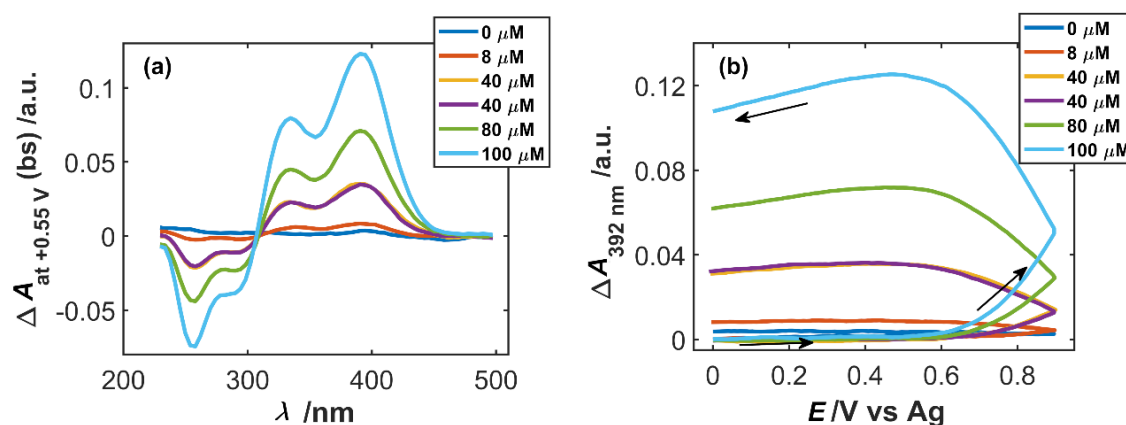


Figure 6-11. (a) Absorption spectra at +0.55 V obtained during the reverse scan recorded at different concentrations (b) CVAs at 392 nm obtained at different concentrations. Experimental conditions in the Figure 6-10 caption.

Figure 6-12 shows the calibration curves obtained for the concentration range of 5 to 100 μM using the bands at 332 nm and 392 nm, the two positive absorption bands shown in Figure 6-10b and Figure 6-11a. Figures of merit of the two calibrations are summarized in Table 6-4.

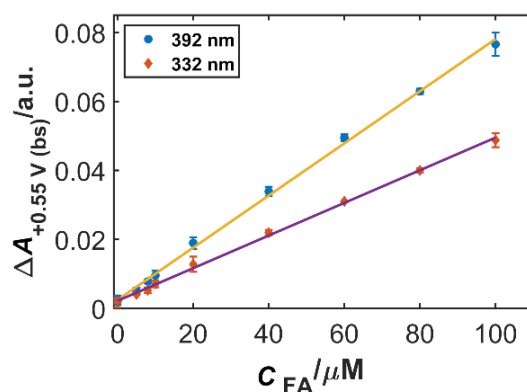


Figure 6-12. Calibration curves of FA obtained from the CVAs at +0.55 V in the reverse scan at 332 (violet line) and 392 nm (yellow line). Experimental conditions in the caption of Figure 6-10. Each point was replicated three times. Optical path-length = 2.3 mm.

The calibration model at 392 nm is more sensitive than the one at 332 nm as deduced from the slope. Very good and similar coefficients of determination (R^2) were obtained for the two calibration models, which provide similar limits of detection in the two cases. The low values of the standard deviation of residuals (S_{yx}) indicate the low dispersion of data, as can be observed in Figure 6-12 for the three replicates of each point, where the error bars are very small. The limits of detection (LOD) are very similar with the two wavelengths.

Table 6-4. Figures of merit for the linear regression models obtained from the absorbance values at 332 and 392 nm, at +0.55 V in the reverse scan.

Wavelength (nm)	Slope (μM^{-1})	Intercept (a.u.)	R^2	S_{yx}	LOD (μM^{-1})
332	$4.7 \cdot 10^{-4}$	$2.2 \cdot 10^{-3}$	0.998	$7.4 \cdot 10^{-4}$	4.7
392	$7.5 \cdot 10^{-4}$	$2.5 \cdot 10^{-3}$	0.998	$1.2 \cdot 10^{-3}$	4.8

R^2 : coefficient of determination; S_{yx} : standard deviation of residuals; LOD: limit of detection

Repeatability of the UV/Vis SEC method was evaluated by constructing three regression models for each wavelength (332 and 392 nm) under the same experimental conditions described above and evaluating the repeatability of the slopes. For the calibrations at 332 and 392 nm, a %RSD of 4.36 and 2.20 were obtained, respectively. From these values, we can conclude that the UV/Vis SEC method has a very good repeatability in the quantification of FA in alkaline medium.

6.2.3.3. *FA determination in a pharmaceutical tablet*

Once demonstrated that this UV/Vis SEC method is reliable, repeatable, and sensitive, the determination of FA in a drug (Yodocefol®) was performed, probing the capability of the method for real samples. Yodocefol® tablet contains 262 µg of KI (equivalent to 200 µg of I₂), 400 µg of FA and 2 µg of vitamin B12 and different excipients. Therefore, the analyte is accompanied with potential interfering compounds. The drug sample was prepared as described in the experimental Section.

A new calibration procedure was performed to carry out the determination of a tablet of Yodocefol® using the UV/Vis SEC method described above. Since the tablets are dissolved in 25 mL of 0.1 M NaOH solution, and they contain 400 µg of FA, the real concentration of the test sample was 36 µM of FA.

Figure 6-13a shows the UV/Vis absorption spectra registered during the oxidation of the Yodocefol® sample prepared in alkaline medium. Only the four bands that had been observed for the FA standard solutions are detected, although they are slightly noisy due to scattering of light, possibly caused by the presence of small particles of the excipient that have not been completely removed during the centrifugation process. It is noteworthy the robustness of the method, allowing us to perform the determination of the analyte despite the dispersion produced by this complex matrix. Figures of merit for the new calibration models at 332 and 392 nm are very similar to the ones obtained in the calibration shown in Table 6-4. In this case, the slope is higher ($8.1 \cdot 10^{-4} \mu\text{M}^{-1}$ at 332 nm and $13.4 \cdot 10^{-4} \mu\text{M}^{-1}$ at 392 nm) because the optical pathway is longer (2.90 mm). Two replicated experiments are shown in Figure 6-13. As can be seen, the two signals (spectra, inset Figure 6-13a, and CVAs at 392 nm, Figure 6-13b) are very similar indicating the good reproducibility of the method, even in this complex matrix. The same absorption spectrum at +0.55 V in the reverse scan is obtained for the Yodocefol® sample and for a standard solution, as well as the same CVAs at 392 nm independently on the sample matrix. These results led us to conclude unequivocally that the only molecule that is oxidized is the FA, which is present in the Yodocefol® sample. The oxidation begins at a potential of +0.55 V, as in the previous cases, increasing the absorbance, until it reaches a maximum absorbance value at +0.55 V in the reverse scan, the potential selected for quantification (Figure 6-13b).

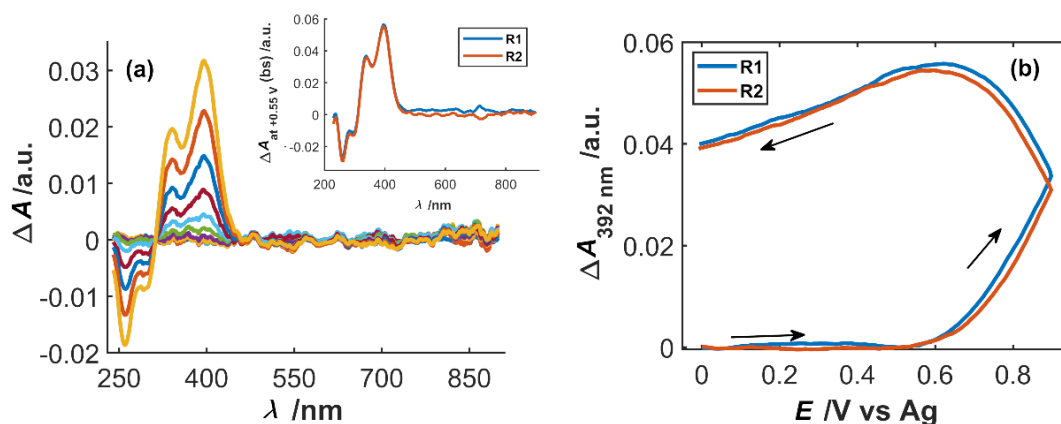


Figure 6-13. (a) Absorption spectra recorded at different potentials during the oxidation of the Yodocefal® sample. Inset shows the spectra of two Yodocefal® samples at +0.55 V in the reverse scan. (b) CVAs at 392 nm for two Yodocefal® samples. Experimental conditions in the Figure 6-10 caption. Optical path-length = 2.9 mm.

A concentration of $37 \pm 4 \mu\text{M}$ for the linear regression model obtained with ΔA values at +0.55 V in reverse scan at 332 nm and $38 \pm 5 \mu\text{M}$ for the corresponding calibration at 392 nm were obtained, with the real concentration being $36 \mu\text{M}$. Therefore, a good prediction of the test sample is obtained. Moreover, a low %RSD, 4.8 %, was assessed for the regression models at the two characteristic wavelengths, which indicates a high degree of coincidence between repeated determinations.

From these results, we can conclude that the new analytical method based on UV/Vis SEC allows us the determination of FA in complex matrices with very good figures of merit.

6.2.4. Conclusions

UV/Vis SEC has been demonstrated to provide very good analytical figures of merit for the determination of FA. It should be noted that electrochemistry is used to generate the oxidation product, that combined with spectroscopy, allows us to obtain a determination of this molecule. In many cases, a double determination of the sample is possible, but sometimes, as in the system shown in this work, the electrochemical signal cannot be used for quantitative purposes, and the determination is made only with the spectroscopic data generated during the oxidation process of the FA. Therefore, the marriage of optical techniques with electrochemistry facilitates the development of analytical methods. It is noteworthy that, in the determination of FA, UV/Vis spectroscopy is used to detect the

products of a reaction that are not observed in the electrochemical signal. FA contained in Yodocefol® has been successfully determined. The interfering compounds in this commercial drug do not affect the quantification process, demonstrating the capability of these techniques in the analysis of complex samples. UV/Vis SEC shows a very good reproducibility, which is remarkable considering the simple pretreatment of the sample prior to its quantification. Moreover, some non-soluble excipient compounds were observed in the test sample, but despite that, high quality analytical figures of merit were obtained. A simple univariate calibration has been used for the determination of test samples, but as has been demonstrated, different absorption bands can be used to carry out the quantification of the sample. Therefore, analytical solutions based on multivariate analysis could be easily implemented to extract the analytical information in case of strong interferences. Additionally, UV/Vis SEC provides information on the electrochemical oxidation process, allowing to obtain spectra related to the products of the reaction.

6.3. Determination of ofloxacin in urine using UV/Vis absorption spectroelectrochemistry

This sub-Section demonstrates once again the effectiveness of parallel UV/Vis SEC as an analytical tool in the quantitative field. On this occasion, a relevant fluoroquinolone (ofloxacin) is quantified in a urine sample, without any sample pretreatment. First of all, it is shown that the electrode surface is modified during the oxidation of this molecule, which makes it necessary to change the electrode between measurements. Thus, the requirement arises to develop a SEC cell that allows changing the electrode while guaranteeing reproducibility, i.e. keeping the distance between the optical fibers. With this aspect in mind, this new cell has been used and an analytical protocol has been optimized to determine ofloxacin in this complex urine matrix. In addition, due to the presence of interferents that can be found in the urine sample and affect the quantification of the target molecule, the use of PARAFAC is required. This study has been published in [*Microchemical Journal*, 2024, 198, 110186.](#)³²²

6.3.1. Introduction

Quinolones and, in particular, fluoroquinolones (FQs) are a family of synthetic antibiotics with a high antibacterial activity and relevant pharmacokinetic properties.^{134,323} The basic FQ structure consists of a bicyclic core with a N in carbon 1, a carboxyl group at carbon 3, a carbonyl group at carbon 4, and a fluorine atom at carbon 6 of the quinolone scaffold.¹³⁰ FQs are commonly prescribed to treat bacterial infections caused by both Gram-positive and Gram-negative bacteria, including, among others, urinary tract infections, sexually transmitted diseases, tuberculosis, pneumonia, and skin and soft tissue infections. These antibiotics inhibit bacterial RNA and DNA synthesis through the formation of a quinolone-enzyme-DNA complex that blocks the catalytic activity of DNA gyrase and topoisomerase IV, causing cell and bacterial death.^{324–326}

Ofloxacin (OFL) is considered a second generation FQ with a N-methylpiperazine group attached to carbon 7, widely used in the treatment of respiratory, digestive and urinary tract infections against Gram-negative bacteria in both humans and animals.^{134,137,140,323,327} However, despite its great potential to address various bacterial infections, OFL is under surveillance. The World Health Organization and National Academy of Medicine in the United States of America are currently concerned about the

prevalence of trace amounts of FQs in the environment;¹³⁴ in particular, a large concentration of OFL has been detected in many aquatic environments. It is estimated that 70-80 % of ingested OFL is excreted unmetabolized via urine to domestic wastewater and sewage treatment plants.³²⁸ Overexposure to OFL-contaminated waters leads to adverse reactions in the human body and results in multiple ecosystem and environmental problems, such as microbial resistance.^{132,329,330}

To date, liquid chromatography, mass spectrophotometry, UV/Vis spectrometry and fluorescence represent the most relevant techniques to study OFL.^{137,143,145,146,327} Nevertheless, the application of these techniques usually requires the use of expensive instrumentation and complex working protocols. Overcoming the main problems of these techniques, the development of electrochemical sensors has led to simpler methods that allow the study and detection of this type of molecules. Despite the great advantages of electrochemical methods such as simplicity, quickness, sensitivity and reproducibility, it is often necessary to carry out prior optimization work or even the development of specific modified electrodes to improve the selectivity and/or sensitivity.^{137,140,143–147,160,161,327}

In this work, spectroelectrochemistry (SEC) is proposed to develop a working protocol that allows the quantification of OFL in a complex media in a simple and reproducible way. The specific photophysical properties of OFL, such as its characteristic absorption spectra, its acid-base behavior and the possibility of electrochemical oxidation, make it a perfect candidate to be studied by SEC techniques, as in this case UV/Vis absorption SEC (UV/Vis SEC). This technique, which provides two signals of different nature but fully correlated, one electrochemical and other spectroscopic, is increasingly used in chemical analysis, characterization of new materials or in the study of electrochemical processes.⁵² Considering that a light beam can interrogate the diffusion layer in different ways, a distinction is made between two optical arrangements known as normal configuration and parallel configuration.⁷ In this work, since OFL can be detected in the solution adjacent to the electrode surface, a parallel configuration respect to the electrode surface is chosen, aligning two optical fibers on the working electrode (WE) surface to record all the changes taking place during the OFL oxidation process. Usually, the SEC cells described in literature based on optical fibers, fix them directly on the surface of the working electrode.⁴² However, an experimental set-up based on fixed optical fibers on the WE entails certain problems which must be overcome. For example, when the generated

product is adsorbed on the WE surface the set-up is not useful because a different electrode is required for each measurement.⁴² In that case, the optical fibers must be fixed to a new electrode after each measurement and, consequently, the distance between the fibers may change, affecting the optical path length and losing the necessary reproducibility within the set of experiments.⁴²

Some alternatives have been proposed to overcome this drawback such as aligning and fixing the optical fibers on a quartz plate subsequently placed on the working electrode, which allows the electrode change while maintaining the optical path.⁵² This set-up involves working in a thin-layer regime, since the solution is confined in a small space, which can be very useful in the quantification field as total electrolysis of the analyte occurs.⁵² However, for other processes, such as the study of reaction mechanisms, it may be more convenient to work under semi-infinite diffusion conditions. In this work, a new SEC cell has been developed, which allows changing the electrode without modifying the optical pathway, obtaining very reproducible experiments in the semi-infinite diffusion regime. The new SEC cell allows us to follow and study processes that modify the electrode surface and the electrode can be changed easily in a very reproducible way, without modifying the optical path-length, which is mandatory for analytical purposes.

Finally, time-resolved SEC experiments provide a large amount of data which can be used to resolve complex problems, especially when the determination of an analyte is carried out in a complex matrix. The trilinear character of SEC data allows us to use powerful chemometric tools for quantification of FQs, particularly OFL, in real matrices such as urine samples. The presence of potential interfering compounds in the determination of OFL is a significant problem, because it can undoubtedly affect the quantification of this antibiotic, compromising the validity of the obtained results.

Therefore, the main objective of this work is to quantify OFL in a real urine sample using a new UV/Vis SEC cell working in parallel configuration and using PARAFAC as chemometric tool to extract the quantitative information of the SEC data set. The development of methodologies for the quantification of these drugs in urine can help to determine the efficiency of medical treatments and to optimize the appropriate dose to be administered to the patient.

6.3.2. Experimental Section

6.3.2.1. *Reagents and Materials*

Ofloxacin (OFL, Sigma-Aldrich) and uric acid (UA, ACS reagent, Acros Organics), were used as received without further purification. All solutions were prepared in 0.1 M KCl (Acros Organics) and in 0.1 M Britton-Robinson (BR) as the supporting electrolyte, being the final pH of this solution 4.02. This buffer solution contains boric acid (H_3BO_3 , Panreac), phosphoric acid (H_3PO_4 , 85 %, Panreac), acetic acid (HAc, VWR Chemicals) and sodium hydroxide (NaOH, Acros Organics).

All these solutions were prepared using high-quality ultrapure deionized water (18.2 M Ω cm resistivity at 25 °C, Milli-Q Direct 8, Millipore) and the final pH of the solution was measured with a pH electrode (sensION). All experiments were performed at room temperature.

6.3.2.2. *Instrumentation and Software*

UV/Vis SEC experiments were performed using a customized SPELEC instrument (Metrohm-DropSens) controlled by DropView SPELEC software (Metrohm-DropSens). This instrument includes a potentiostat/galvanostat, a spectrometer, and a halogen-deuterium light source.

In addition, a SEC cell was designed and fabricated to carry out the experimental work. A detailed description of the SEC cell is given in Section 6.3.2.3. Moreover, a new carbon screen printed electrode (SPE, DRP-110, Metrohm-DropSens) was used in each SEC measurement. These SPEs consist of a 4 mm diameter disc screen printed carbon working electrode (WE), a carbon counter electrode (CE) and a silver pseudo-reference electrode (RE). Two bare optical fibers (100 μm , Ocean Optics) were used to illuminate the sample and to collect the light beam, which is conducted to the spectrometer.

6.3.2.3. *Design and fabrication of the SEC cell*

As previously explained, adsorption of the reaction product on the WE surface causes the modification of the electrode, leading to changes in both the electrochemical and the spectroscopic responses. Therefore, the use of a new electrode after each experiment is essential. In addition, in parallel UV/Vis SEC, working with a fixed and known optical-pathway length during a set of experiments is mandatory. With all these factors in mind,

a new parallel UV/Vis SEC cell that works in semi-infinite regime has been developed, allowing the replacement of the SPE without modifying the optical-pathway length in a very easy and reproducible way. For this purpose, the optical fibers were fixed and aligned directly on the upper body of the cell, instead of on the WE. In this way, the quantification of OFL is simplified.

Figure 6-14a shows the SEC cell, which consists of two different bodies with four 5 mm holes positioned at each corner where magnets are placed, which allow a reproducible magnetic fixation. The lower body has been designed and created with a 3D printer (Photon Mono SE, ANYCUBIC) using UV sensitive 405 nm resin (ANYCUBIC), while the upper body was fabricated in poly(methyl methacrylate) (PMMA) using a high-precision CO₂ laser cutting machine. The lower body measures 65.0 x 40.0 x 8.0 mm and features a rectangular space (29 x 10 mm) to insert the SPE, as well as a small opening to connect the SPE to the potentiostat. The upper body measures 47.5 x 40.0 x 9.8 mm and features a circular cavity of 16 mm in diameter with two small 2.6 mm diameter pillars to support the optical fibers. These pillars facilitate the alignment of the optical fibers at different optical path lengths, as can be observed in the Figure 6-14b. The circular cavity allows working in semi-infinite regime during the SEC experiments. In this way, the light beam passes close to the WE surface in parallel configuration and is conducted to the spectrometer, which collects the absorbance changes occurring in the solution adjacent to the WE.

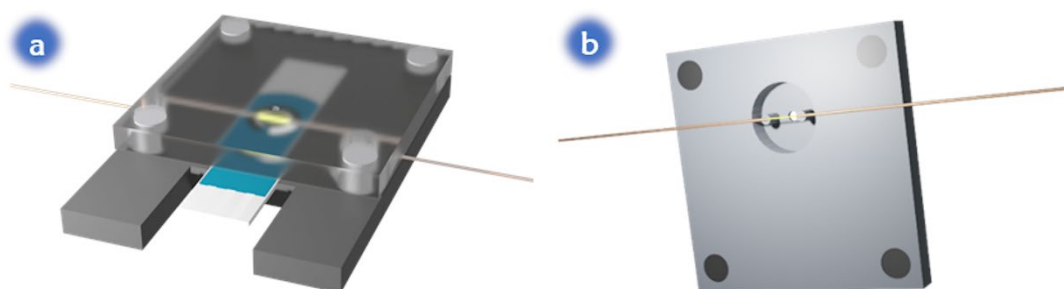


Figure 6-14. (a) Schematic image of the parallel UV/Vis SEC cell to work in semi-infinite diffusion regime, and (b) detailed schematic view of the upper body of the cell.

In summary, the home-made UV/Vis SEC cell described above has been optimized to perform experiments in parallel configuration and in a semi-infinite diffusion regime in a simple and reproducible way. Additionally, the replacement of the SPE between experiments without altering the optical path length is facilitated.

6.3.2.4. *Experimental set-up for SEC measurements*

In all the experiments carried out in this work, the light beam interrogates the solution in a parallel direction to the WE surface. For this purpose, two 100 μm bare optical fibers were aligned and fixed with nail polish in the upper piece on the two pillars described in Figure 6-14. One of these fibers is coupled to the light source while the other one is connected to the spectrometer. The optical pathway is the distance between the two optical fibers, which is measured using ImageJ,³³¹ obtaining in this set of experiments a value of 2.07 mm.

To perform the parallel UV/Vis SEC experiments, the SPE is first placed in the lower body and 50 μL of the test solution is added, making sure that the drop completely covers the three electrodes of the SPE. Then, the upper body with the optical fibers is perfectly attached to the lower part, thanks to the four magnets present in the corners of both pieces. Finally, the SPE is connected to the potentiostat.

6.3.3. Results and discussion

The main objective of this work is to measure OFL levels in real urine samples. It should be noted that urine is a complex matrix containing potentially interfering compounds. One of the most common compounds found in urine is uric acid (UA), which can be also oxidized. For this reason, before starting a complex analysis, the individual study of the UV/Vis SEC behavior of both OFL and UA has been carried out.

6.3.3.1. *OFL study by UV/Vis SEC*

First, a 100 μM OFL solution in 0.1 M KCl and BR buffer ($\text{pH} = 4.02$) was measured by performing cyclic voltammetry between +0.70 V and +1.10 V at $0.01 \text{ V}\cdot\text{s}^{-1}$. Figure 6-15a shows the cyclic voltammogram (CV), where an anodic peak related to the oxidation of OFL evolves around +0.99 V. During the backward scan, no reduction peak is observed, indicating that the oxidation of OFL is an irreversible process. Concomitantly, the evolution in the UV/Vis absorption spectra was recorded every 10 ms. The corresponding contour map of the spectra during the experiment is shown in Figure 6-15b. It should be noted that the UV/Vis absorption spectrum of OFL at OCP was taken as reference, so the spectral changes occurred during the cyclic voltammetry experiment were recorded. The oxidation of OFL leads to the evolution of three absorption bands. One increases with the applied potential at 245 nm being related to the generation of the oxidized form of OFL.

The strong absorption band that decreased at 294 nm corresponds to a $\pi \rightarrow \pi^*$ transition of the chromophore involving from the N-1 position to the carboxylic acid group; on the other hand the weak absorption band at 340 nm is linked to the $n \rightarrow \pi^*$ transition of the chromophore involving the nitrogen of the piperazinyl group to the carbonyl group.^{332,333} Both absorption bands with a negative behavior are associated to the consumption of OFL during the oxidation process. The cyclic voltabsorptograms (CVAs, Figure 6-15c) show the evolution of the absorbance at 294 nm and 245 nm with the applied potential. From +0.90 V onwards in the anodic scan, an abrupt change in absorbance occurs, which corresponds to the oxidation process of OFL shown in Figure 6-15a. The derivative voltabsorptogram (DVA) at 294 nm, Figure 6-15d, shows the correlation between the electrochemical and spectroscopic signals, demonstrating that both responses are only related to the oxidation of OFL.

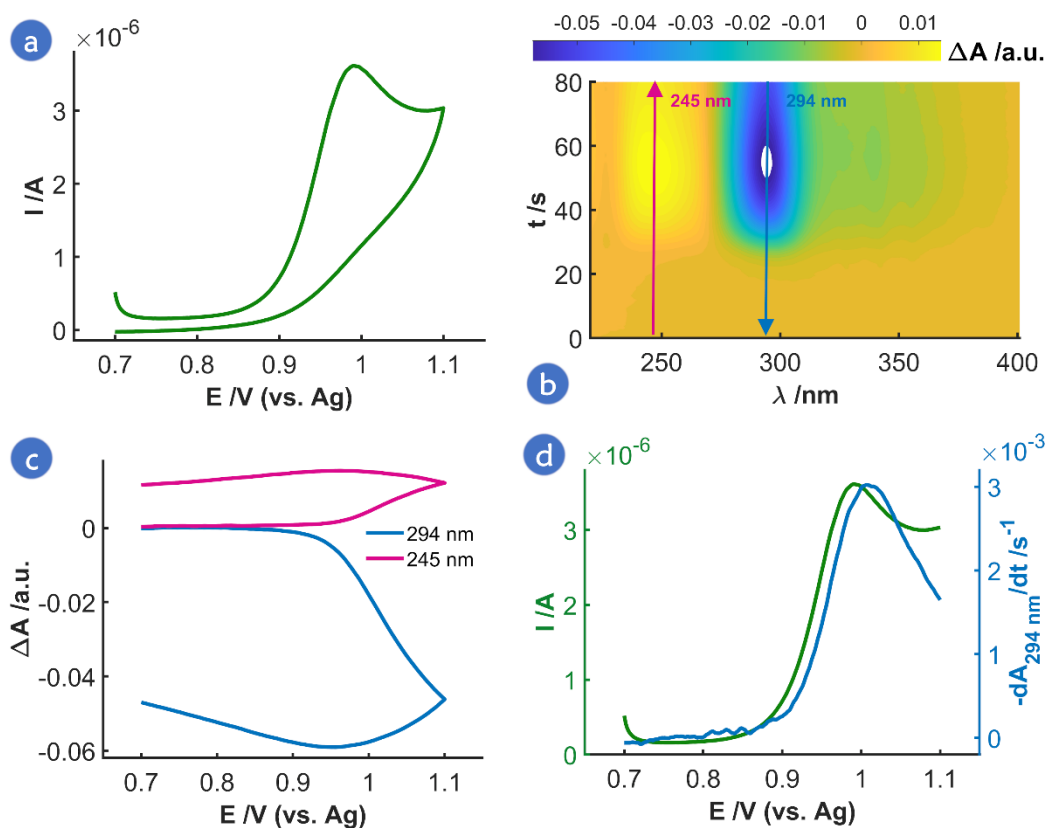


Figure 6-15. SEC experiment of 100 μ M OFL in BR buffer (pH = 4.02) and 0.1 M KCl between +0.70 V and +1.10 V at 0.01 $V \cdot s^{-1}$. (a) CV, (b) contour plot with spectra evolution during the potential scan (pink arrow: band at 245 nm, blue arrow: band at 294 nm), (c) CVA at 245 nm (pink line) and 294 nm (blue line), (d) comparison of CV (green line) and DVA at 294 nm (blue line).

6.3.3.2. UA study by UV/Vis SEC

Similarly, a comparable analysis was carried out in a 100 μM UA in 0.1 M KCl and BR buffer (pH = 4.02) solution. In this case, the CV recorded between 0.00 V and +0.70 V at 0.01 $\text{V}\cdot\text{s}^{-1}$ is also characterized by a single anodic peak around +0.45 V (Figure 6-16a) assigned to the irreversible oxidation process of UA.

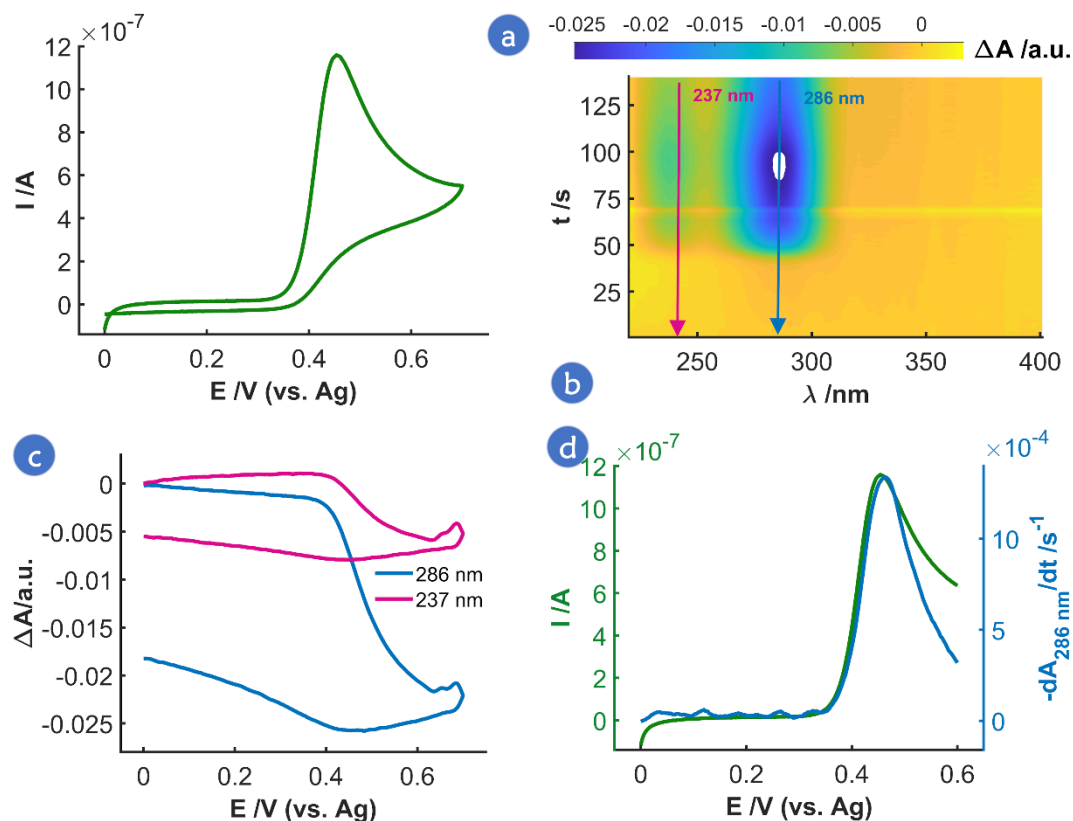


Figure 6-16. SEC experiment of 100 μM UA in BR buffer (pH = 4.02) and 0.1 M KCl between 0.00 V and +0.70 V at 0.01 $\text{V}\cdot\text{s}^{-1}$. (a) CV, (b) contour plot with spectra evolution during the potential scan (pink arrow: band at 237 nm, blue arrow: band at 286 nm), (c) CVA at 237 nm (pink line) and 286 nm (blue line), and (d) comparison of CV (green line) and DVA at 286 nm (blue line).

The evolution of the UV/Vis absorption spectra recorded simultaneously every 10 ms (Figure 6-16b), taking the initial UA solution as reference spectrum, displays two absorption bands that decrease during the anodic scan. These spectra are defined by a weak negative band at 237 nm and a more intense band at 286 nm, both related to the depletion of UA in solution.^{124,235} The CVAs at 237 and 286 nm, represented in Figure 6-16c, show that from +0.40 V onwards the absorbance decreases, which clearly coincides with the onset potential of the anodic peak recorded in the CV (Figure 6-16a). The absorbance continues to decrease until reaching again this potential of +0.40 V in the

backward scan. The relationship of the 286 nm absorption band with UA oxidation can be confirmed with the similarity between the evolution of DVA at 286 nm and the CV represented in Figure 6-16d, where only the anodic scan is plotted.

6.3.3.3. Study of mixtures of OFL and UA using UV/Vis SEC

Once the behavior of each components is identified, the UV/Vis spectroelectrochemical study of a synthetic sample containing the two molecules, OFL and UA, was performed. For this purpose, a solution of 75 μM OFL and 200 μM UA in 0.1 M KCl and BR buffer (pH = 4.02) was prepared. In this case, Figure 6-17a shows the CV performed between 0.00 V and +1.10 V at $0.01 \text{ V}\cdot\text{s}^{-1}$. Two anodic peaks are observed at +0.45 V and +0.96 V related to the oxidation of UA and OFL, respectively.

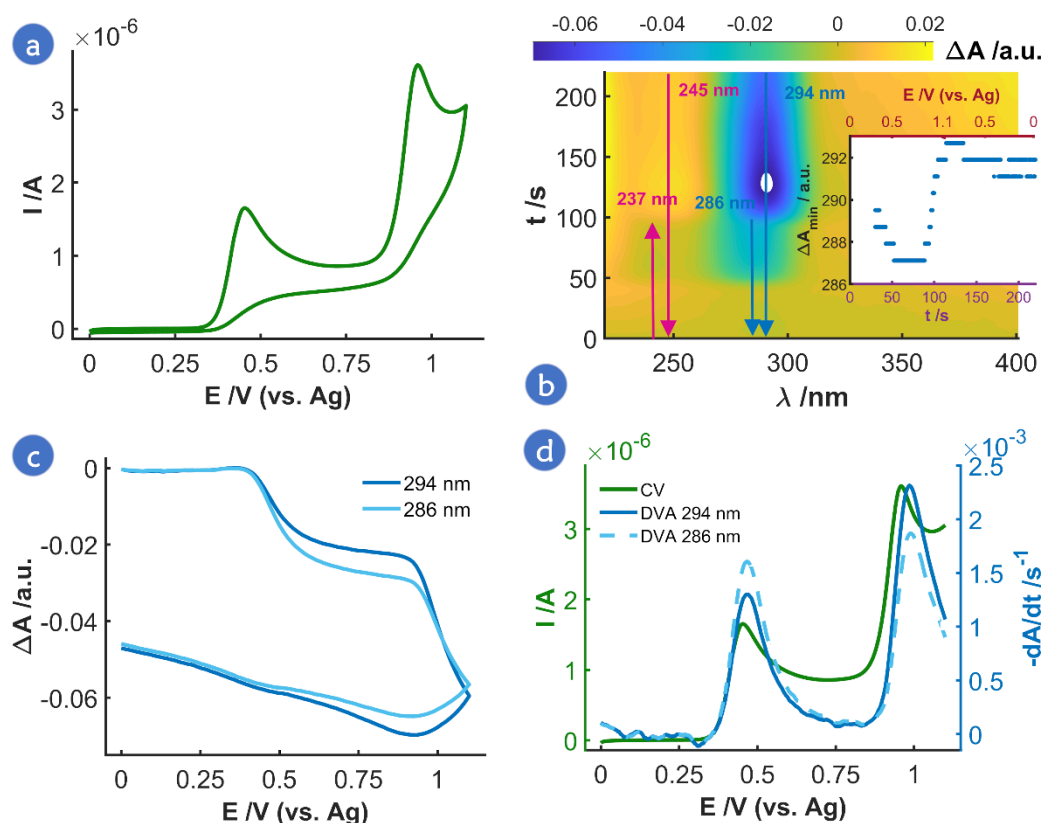


Figure 6-17. SEC experiment of 75 μM OFL and 200 μM UA in BR buffer (pH = 4.02) and 0.1 M KCl between 0.00 V and +1.10 V at $0.01 \text{ V}\cdot\text{s}^{-1}$. (a) CV, (b) contour plot with spectra evolution during the potential scan; inset: wavelength at which the minimum of absorbance is reached during the experiment, (c) CVA at 286 and 294 nm, and (d) comparison of CV and DVA at 286 and 294 nm.

The evolution of the spectra with the potential during the anodic scan is shown in Figure 6-17b, taking the spectrum of the initial solution of UA and OFL as reference. As can be

observed, initially a band appears at 287 which shifts up to 294 nm (inset Figure 6-17b), indicating that from 0.00 to +0.85 V approximately, only the oxidation of UA is occurring, and from +0.85 V onwards the oxidation of OFL is taking place. Selecting 286 and 294 nm as representative wavelengths for each one of the molecules, it can be seen that the CVAs clearly show changes related to the initial oxidation of UA and the subsequent oxidation of OFL from +0.85 V onwards (Figure 6-17c). Both absorption bands are fully overlapped, being difficult to discriminate the absorbance related only to OFL due to the initial presence of UA which is easily oxidized. The same behavior is observed in the DVAs at these two wavelengths that are fully correlated with the electrochemical signal (Figure 6-17d). The sample was measured in triplicate.

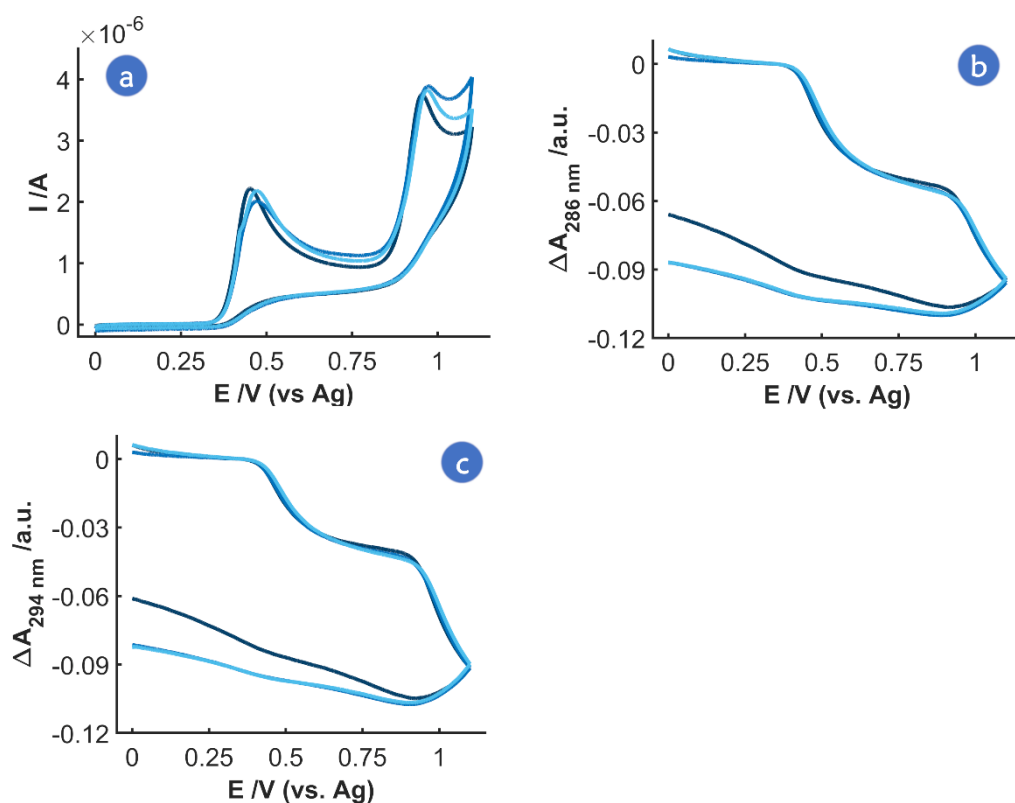


Figure 6-18. UV/Vis SEC experiments of a solution containing 75 μM OFL and 200 μM UA in BR buffer (pH = 4.02) and 0.1 M KCl showing the three replicates performed. (a) CVs, (b) CVAs at 286 nm and (c) CVAs at 294 nm.

Figure 6-18 shows the corresponding CVs and CVAs at 286 and 294 nm. The overlapping between the three measurements demonstrates the good repeatability of the spectroelectrochemical measurements carried out with the new UV/Vis SEC cell, which is also corroborated by the estimation of the %RSD at the vertex potential (+1.10 V),

which yields a value around 1 % for the spectroscopic signals and 11 % for the electrochemical response (Table 6-5).

Table 6-5. Repeatability data obtained with 3 replicates of UV/Vis SEC measurements carried out with a sample of 75 μM OFL and 200 μM UA in BR buffer (pH = 4.02) and 0.1 M KCl.

Signal	S_{yx}	%RSD
$I_{+1.10\text{ V}}$	$4.12 \cdot 10^{-1}$	11.45
$A_{286\text{ nm}, +1.10\text{ V}}$	$8.87 \cdot 10^{-4}$	-0.94
$A_{294\text{ nm}, +1.10\text{ V}}$	$9.83 \cdot 10^{-4}$	-1.08

S_{yx} : residual standard deviation

6.3.3.4. Study of real urine samples using UV/Vis SEC

Considering that the main objective of this work is to determine OFL in urine samples, the subsequent experiments involved the comparison of the signals acquired from synthetic and urine samples, spiked and non-spiked with OFL and UA. Different test samples, T1, T2, T3 and T4, were prepared as follows. The test urine sample without OFL (T1) contains 0.25 mL of urine in a final volume of 5 mL of BR buffer (pH = 4.02) in 0.1 M KCl. The OFL-enriched urine test sample (T2) was prepared by dissolving 0.00300 g of OFL in 10 mL of urine to simulate a real problem where the concentration of OFL in urine would be 830.18 μM . This test sample was prepared taking into account that the concentration of OFL excreted in urine by a patient taking this drug ranges from 500 to 1250 μM .¹³⁰ Next 0.25 mL of this test sample was diluted in 5 mL of BR buffer (pH = 4.02) in 0.1 M KCl, with a final OFL concentration of 41.51 μM . No other pretreatment of the urine sample was carried out. The UA-enriched urine sample (T3) contains 0.25 mL of urine and 73.57 μM UA in a final volume of 5 mL of BR buffer (pH = 4.02) in 0.1 M KCl. The UA-OFL-enriched urine sample (T4) contains a final concentration of 73.57 μM UA, 41.51 μM OFL, 0.25 mL of urine and 0.1 M KCl, all in a final volume of 5 mL of BR.

The CVs of the four samples (Figure 6-19a) show a first anodic peak at +0.45 V related to the UA which is present in urine. The same anodic peak is shown in Figure 6-16a and Figure 6-17a. A second slight oxidation peak overlapped with the one of UA can be observed, which means that other products in the urine besides UA are oxidized around +0.52 V. As can be seen, the UA oxidation peak of the urine samples is broader than in

synthetic samples, and its slope is less pronounced than the observed in the oxidation peak of the sample containing only UA and OFL, Figure 6-17a. Next, a third anodic peak at +0.84 V is observed in the urine samples without OFL (T1 and T3, purple and blue lines, Figure 6-19a) which could interfere and difficult the electrochemical OFL quantification in this these medium. On the other hand, the CVs of the urine samples spiked with OFL (T2 and T4, green and orange lines, Figure 6-19a) displays the fourth anodic peak at +0.96 V due to the oxidation of OFL added, as explained in Figure 6-15a and Figure 6-17a. Furthermore, this experiment confirms that the first oxidation peak in urine, observable in T1, corresponds to UA, since in T3, where UA is spiked to the sample, the first oxidation peak increases in intensity.

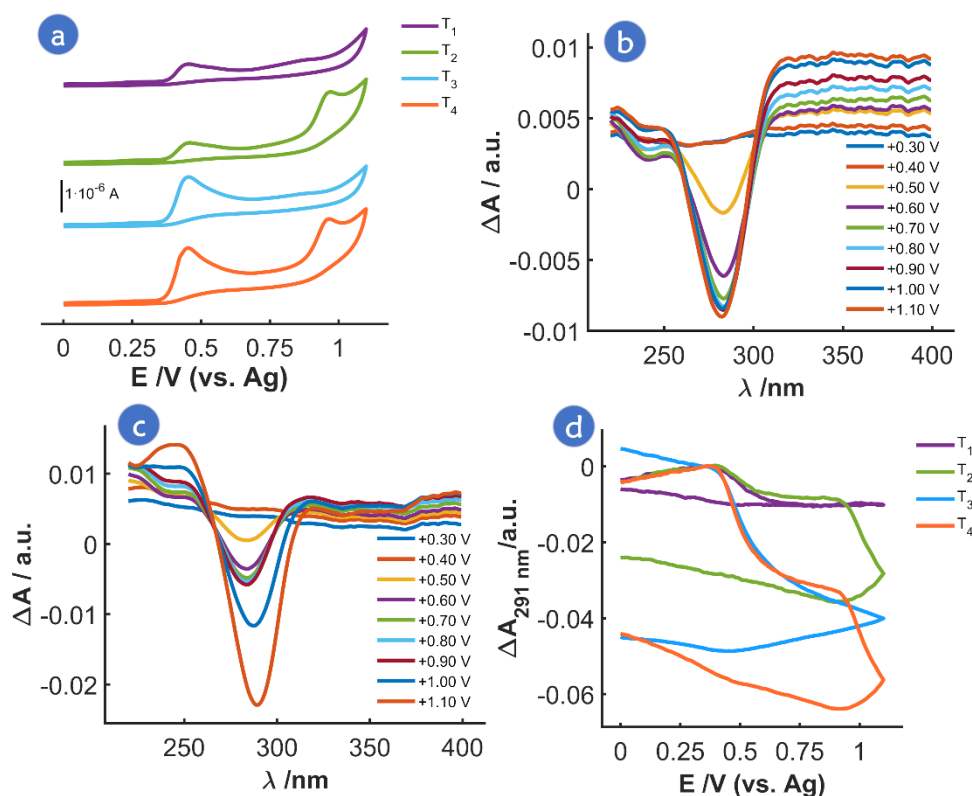


Figure 6-19. SEC experiment of T1 (urine), T2 (urine spiked with OFL), T3 (urine spiked with UA) and T4 (urine spiked with OFL and UA) test samples in 0.1 M KCl and in BR buffer (pH = 4.02) between 0.00 V and +1.10 V at 0.01 V·s⁻¹. (a) CVs, (b) spectra evolution during the potential scan of T1, (c) spectra evolution during the potential scan of T2, (d) CVAs at 291 nm.

The evolution of the UV/Vis absorption spectra during the oxidation of the molecules present in the urine samples T1 and T2 is shown in Figure 6-19b and Figure 6-19c. In the sample in which only urine is present (T1, Figure 6-19b), the decrease of an absorption band peaking at 286 nm is observed, related to the oxidation of UA, coinciding with the

anodic peak at +0.45 V in the CV (T1, purple line, Figure 6-19a). This specific oxidation process is clearly observed in the CVA at 291 nm shown in Figure 6-19d (purple line), where only the decrease of absorbance is appreciated around +0.45 V. However, in the urine sample spiked with OFL (T2, Figure 6-19c) is observed a shifting of the absorption band related to OFL from 286 nm to 291 nm at the end of the potential scan. This behavior is comparable to the spectral evolution illustrated in Figure 6-17b for the UA and OFL mixture, demonstrating that the spectral changes observed for solutions T2 and the mixture of 75 μ M OFL and 200 μ M UA are related to identical processes. UA initially undergoes an oxidation (evidenced by the absorption band at 286 nm), followed by OFL oxidation (absorption band at 291, slightly shifted to shorter wavelengths in the T2 solution). The CVA at 291 nm of T2 solution (green line, Figure 6-19d) shows the two anodic processes occurring at +0.45 V and +0.96 V as mentioned for the oxidation of UA and OFL, respectively. T3 sample shows a similar behavior to T1 sample where only the oxidation of UA is observed both in the CV and in the CVA at 291 represented in Figure 6-19d (line blue). Lastly, the CVA at 291 of sample T4 (line orange, Figure 6-19d.) shows a similar behavior to T2, where both the oxidation of UA and OFL is observed.

Samples T1, T2, T3 and T4 were measured three times with the cell developed to perform UV/Vis SEC in order to evaluate de reproducibility. Figure 6-20 shows the corresponding CVs, and CVAs at 286 and 291 nm. In addition, %RSD values at +1.10 V were estimated and summarized in Table 6-6. These results show the good repeatability of the signals, since in the most of the cases values of %RSD lower than 5 % were obtained.

Two different OFL and urine concentrations are plotted in Figure 6-21, demonstrating the well-defined spectroelectrochemical responses obtained in our measurements in linear sweep voltammetry experiments in spite of the presence of interfering compounds in this complex matrix. As can be seen, SEC responses using voltammetry has been demonstrated to be very useful for the comprehension of these complex responses.

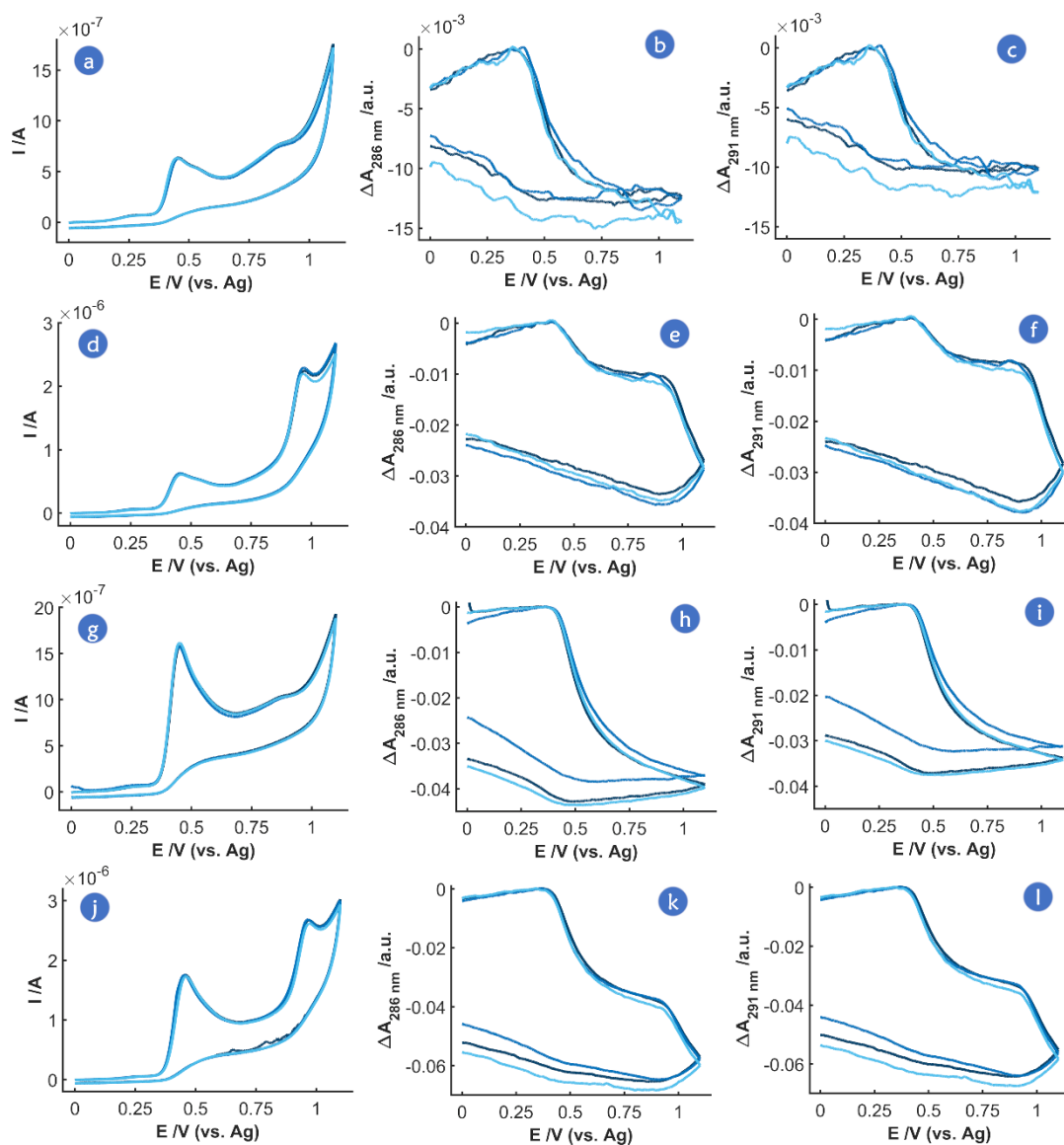


Figure 6-20. UV/Vis SEC experiments of urine samples in BR buffer (pH = 4.00) and 0.1 M KCl showing the three replicates performed. (a) CVs of T1, (b) CVAs at 286 nm of T1, (c) CVAs at 291 nm of T1, (d) CVs of T2, (e) CVAs at 286 nm of T2, (f) CVAs at 291 nm of T2, (g) CVs of T3, (h) CVAs at 286 nm of T3, (i) CVAs at 291 nm of T3, (j) CVs of T4, (k) CVAs at 286 nm of T4, (l) CVAs at 291 nm of T4.

Table 6-6. Repeatability data obtained with 3 replicates of UV/Vis SEC measurements carried out with the urine test samples in BR buffer (pH = 4.02) and 0.1 M KCl.

Sample	Signal	S_{yx}	%RSD
T1 (urine)	$I_{+1.10\text{ V}}$	$4.93 \cdot 10^{-2}$	2.88
	$A_{286\text{ nm}, +1.10\text{ V}}$	$1.20 \cdot 10^{-3}$	-8.85
	$A_{294\text{ nm}, +1.10\text{ V}}$	$1.10 \cdot 10^{-3}$	-10.51
T2 (OFL spiked urine)	$I_{+1.10\text{ V}}$	$9.09 \cdot 10^{-2}$	3.47
	$A_{286\text{ nm}, +1.10\text{ V}}$	$8.68 \cdot 10^{-4}$	-3.11
	$A_{294\text{ nm}, +1.10\text{ V}}$	$9.87 \cdot 10^{-4}$	-3.36
T3 (UA spiked urine)	$I_{+1.10\text{ V}}$	$2.67 \cdot 10^{-2}$	1.40
	$A_{286\text{ nm}, +1.10\text{ V}}$	$1.40 \cdot 10^{-3}$	-3.63
	$A_{294\text{ nm}, +1.10\text{ V}}$	$1.60 \cdot 10^{-3}$	-4.77
T4 (OFL and UA urine)	$I_{+1.10\text{ V}}$	$4.29 \cdot 10^{-2}$	1.43
	$A_{286\text{ nm}, +1.10\text{ V}}$	$1.40 \cdot 10^{-3}$	-2.46
	$A_{294\text{ nm}, +1.10\text{ V}}$	$1.60 \cdot 10^{-3}$	-2.79

S_{yx} : residual standard deviation

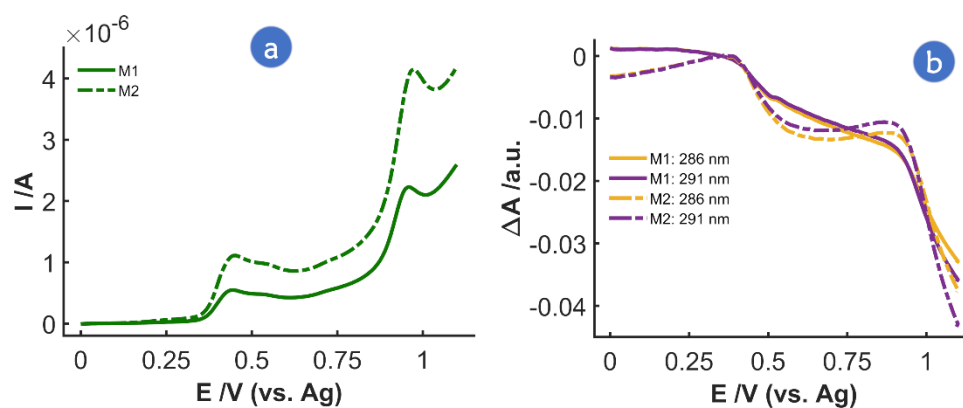


Figure 6-21. UV/Vis SEC experiments of two urine samples with different amounts of OFL and UA (a) CVs and (b) CVAs. Solid line: 0.25 mL of urine and 43 μM OFL in BR buffer (pH = 4.02). Dashed line: 0.50 mL of urine and 85 μM OFL in BR buffer (pH = 4.02).

6.3.3.5. *Calibration model to determine OFL in urine matrix samples*

The analysis of the spectroelectrochemical signals presented above demonstrates that various oxidation processes could interfere the determination of OFL in urine. In order to control the oxidation process taking place in the urine sample, multipulsed amperometric detection (MAD) was selected as electrochemical technique. For this purpose, a first potential pulse of +0.75 V was applied for 90 s to oxidize and remove from the closest solution to the electrode any interfering component, such as UA. This potential is high enough to oxidize UA but low enough to avoid the oxidation of the OFL. In a second step, a potential pulse of +1.10 V was applied during 90 s to specifically oxidize the target molecule, OFL.

Considering these conditions, calibration samples containing different concentrations of both UA and OFL were prepared to build a calibration model capable of predicting the concentration of OFL in a test urine sample. All these solutions were prepared in BR buffer and 0.1 M KCl (pH = 4.02), randomly varying their concentrations in the range of 10-200 μ M, as is shown in Table 6-7. Additionally, a solution containing only OFL and another containing only UA were also prepared and measured. Finally, the solution with 100 μ M OFL and 75 μ M UA was selected as test sample and was measured four times to determine the repeatability of the calibration model.

From each experiment, the corresponding multipulse chronoamperogram and the spectra evolution along the two applied potentials were recorded. Figure 6-22 shows the signals recorded from experiment E08 (Table 6-7): the chronoamperogram (CA, Figure 6-22a) and the spectra evolution during the two potential steps (Fig. Figure 6-22b). The obtained spectra after applying a potential of +0.75 V for 90 s displays a weak negative absorption band at 237 nm and a more intense band at 286 nm (Figure 6-22b, green lines), which coincides with the spectra previously shown in the UA oxidation during the CV (Fig. S1b). On the other hand, the spectrum obtained after applying a second potential step of +1.10 V for 90 s shows a positive absorption band at 250 nm and a negative band at 292 nm related to OFL consumption in the solution/electrode interface (Figure 6-22b, red lines).

Table 6-7. Set of calibration samples prepared with different concentrations of OFL and UA for UV/Vis SEC calibration.

Experiment	$C_{\text{OFL}} (\mu\text{M})$	$C_{\text{UA}} (\mu\text{M})$
E01	0	0
E02	100	0
E03	0	100
E04	10	25
E05	25	50
E06	50	150
E07	75	200
E08	100	75
E09	150	50
E10	200	100
E11	25	50
E12	100	75
E13	100	75
E14	100	75

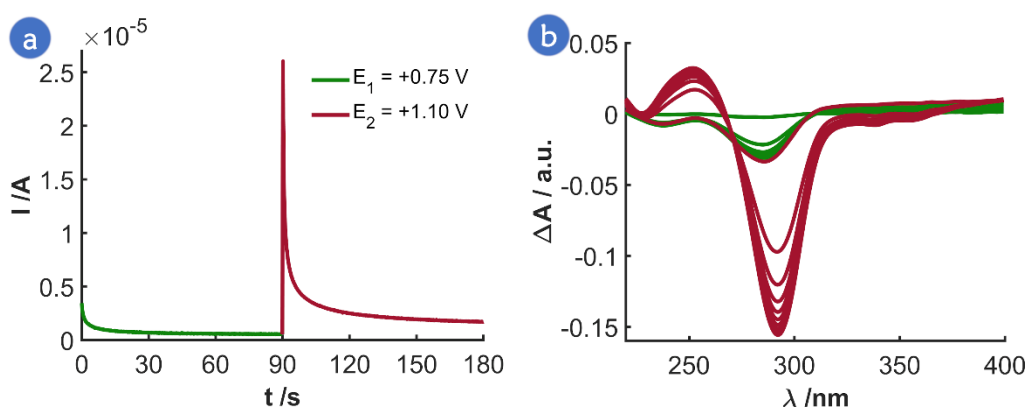


Figure 6-22. MAD-SEC experiment of a solution containing 100 μM OFL and 75 μM UA in BR buffer and 0.1 M KCl (pH = 4.02). (a) CA and (b) spectra evolution during the first potential step (+0.75 V, 90 s, green lines) and the second potential step (+1.10 V, 90 s, red lines).

Linear calibration models can be assessed with both the electrochemical and the spectroscopic signals for the two analytes, UA and OFL, using the samples indicated in Table 6-7. The regression parameters obtained with the four calibration models are summarized in Table 6-8.

Table 6-8. Regression parameters obtained from the 4 linear regression models built with the electrochemical and spectroscopic signals registered with the mixtures of OFL and UA in Table 6-7.

Molecule	Calibration model	R ²	Sensitivity	S _{yx}
UA (E _{ap} = +0.75 V)	CA: I _{10 s} vs. C _{UA}	0.9553	9.34·10 ⁻⁹ A·μM ⁻¹	1.14·10 ⁻⁷
	Spectra: A _{286 nm, 79 s} vs. C _{UA}	0.5909	2.82·10 ⁻⁴ μM ⁻¹	1.34·10 ⁻²
OFL (E _{ap} = +1.10 V)	CA: I _{100 s} vs. C _{OFL}	0.9773	2.80·10 ⁻⁸ A·μM ⁻¹	2.63·10 ⁻⁷
	Spectra: A _{292 nm, 179 s} vs. C _{OFL}	0.9438	1.28·10 ⁻³ μM ⁻¹	1.92·10 ⁻²

R²: Determination coefficient. S_{yx}: residual standard deviation.

The capability of prediction of the OFL calibration models evaluated by measuring four replicates of two OFL-enriched urine samples was very low, with relative errors between 22 % and 67 % for the CA calibration model and between 12 % and 58 % for the spectroscopic calibration model. The overlapping of UA and OFL absorption bands makes difficult the determination of OFL by simple linear regression models. This problem is also evident within the electrochemical calibration model. Although the coefficient of determination is not as low as in the other cases (R² = 0.9773), the model does not provide enough accuracy to determine OFL in this complex sample.

Parallel Factor Analysis (PARAFAC)²⁴⁸ was used to measure OFL levels in urine samples. Given the trilinear character of the time-resolved SEC data, PARAFAC can be used to determine the analyte in complex matrices without any sample pretreatment. Absorbance data at different wavelengths and at different times during a double-pulse chronoamperometric experiments are recorded for mixtures of different OFL and UA concentrations (Table 6-7), providing a spectroelectrochemistry data cube.

To construct the calibration model with PARAFAC, the cube of data is defined with the values of absorbance from 10 to 175 s, obtained between 225 and 380 nm for all samples. The selected time window corresponds practically to the whole experiment, removing only the initial values of absorbance because of its low value. The wavelengths window

belongs to the UV/Vis spectral region where the most significant changes of absorbance occurred, i.e. the selected wavelengths provide all the spectral information about the electrode process. In this way, the data cube is defined by the absorbance values at 1741 times $\times$ 196 wavelengths $\times$ 14 concentrations. Although the calibration samples contain only two known components (OFL and UA), the PARAFAC model was calculated using three components. The third component could be related to other interfering compounds present in the urine sample. A corcondia value of 96.02 % was obtained for the fitted model. This value indicates that the model constructed is suitable and explains the absorbance changes occurred during the oxidation of the samples shown in Table 6-7, as the corcondia value is close to 100 %.²⁵⁵

Figure 6-24 illustrates the deconvolution of the spectroelectrochemical data using the PARAFAC model for the samples listed in Table 6-7. Figure 6-23a shows the loadings of the three components obtained with the PARAFAC model plotted versus the wavelength; the spectra of UA (sample E03) and OFL (sample E02) after the oxidation process have also been plotted for comparison. The sign of the absorbance is changed in the experimental spectra to facilitate the comparison. As can be seen, component 1 is fully correlated with UA with a maximum of absorbance at 286 nm, while component 3 is linked with OFL, showing a maximum of absorbance at 294 nm; the spectra are almost identical to the shape of the signals obtained from the loadings of these factors. The loading signal related to the second component is significantly lower than the one of the other two components, but it has been proven to be needed to include this component in order to get a PARAFAC model which explains and fits the spectroelectrochemical data obtained with the set of samples listed in Table 6-7.

Figure 6-23 shows the evolution of the loadings with time during the two applied potential in the experiment; the two chronoabsorptograms at 286 nm for UA (sample E03) and at 294 nm for OFL (sample E02) are overlapped as dotted lines for comparison. Again, the sign of the absorbance has been changed for an easier comparison. As can be observed, the 3rd component assigned to OFL starts to increase when the second potential step is applied, with a value high enough (+1.10 V) to oxidize the OFL; this signal follows a behavior very similar to the chronoabsorptogram registered with the sample E02 prepared only with OFL. On the other hand, the 1st component, ascribed to UA, shows a very similar behavior to the chronoabsorptogram of UA registered for sample E03 which contains only this molecule. As can be observed, the 2nd component is oxidized at the

same time than UA, which could be related to any other molecule in this complex sample, being oxidized before the OFL oxidation process.

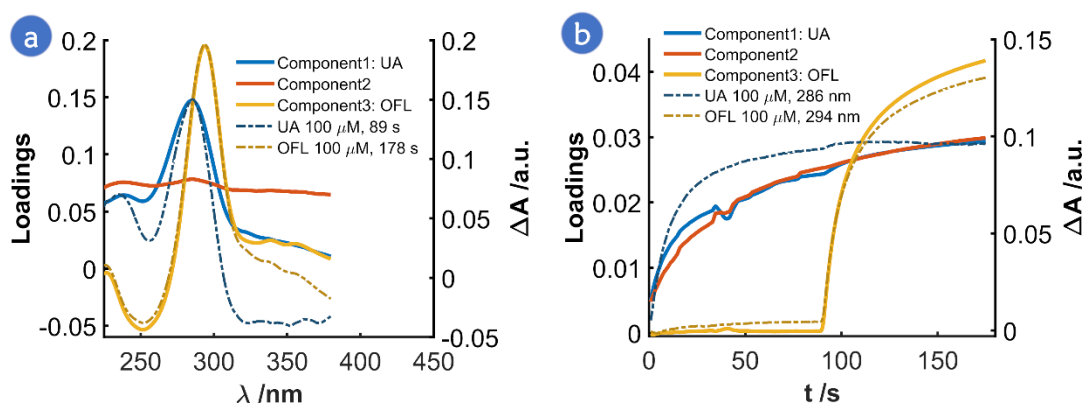


Figure 6-23. Loadings of each component obtained with the PARAFAC model versus (a) the wavelength and (b) the time during the double potential step SEC experiments. Solid lines: loadings of the three components versus wavelength (a) and time (b). Dashed lines: UA and OFL spectra (a) and UA and OFL chronoabsorptograms (b) of samples E03 and E02, respectively.

This PARAFAC model allows the quantification of OFL-enriched urine samples. Two calibration curves were constructed, by correlating the loadings of the 1st and 3rd component with the concentrations of UA and OFL in the samples listed in Table 6-7, respectively. The calibration curves and the figures of merit are shown in Figure 6-24 and in Table 6-9, respectively. Least median squares regression was performed to detect outliers, obtaining that the experiment corresponding to sample E04 was an outlier. Once this point is removed a Least Square Regression was performed for the two set of data, loadings of the 3rd component versus OFL concentration (Figure 6-24a) and loadings of 1st component versus UA concentration (Figure 6-24b).

The figures of merit of the two calibration models obtained indicate the good correlation achieved between loadings of the 3rd component and OFL concentration, with coefficient of determination near 1 (R^2), low residual standard deviation (S_{yx}) and low limit of detection (LOD). However, the UA linear regression model does not supply as good results, with a worse R^2 value and higher S_{yx} and LOD values, indicating that the complexity of the urine matrix affects significantly the UA determination.

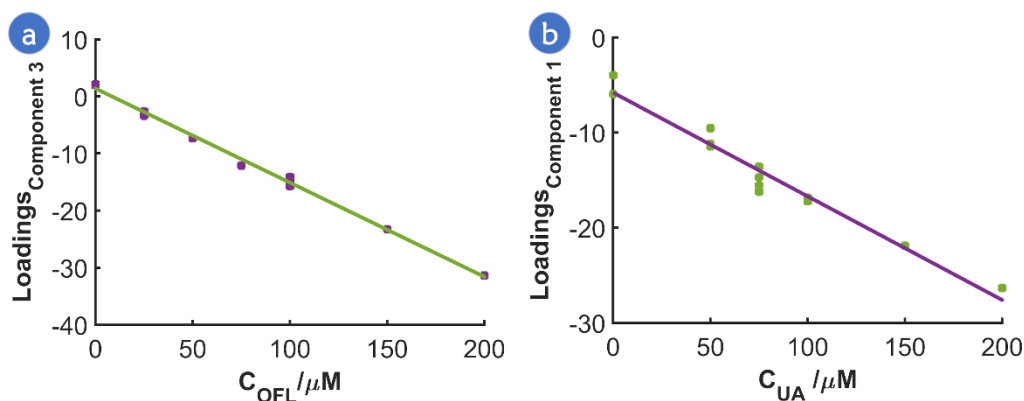


Figure 6-24. Calibration curves with the loadings of the PARAFAC model constructed versus (a) OFL concentration and (b) UA concentration.

Table 6-9. Regression parameters obtained from the determination of OFL and UA in mixtures of these molecules with the loadings of the PARAFAC model constructed.

Molecule	Calibration model	R^2	Sensitivity (μM^{-1})	S_{yx}	LOD (μM)
OFL	Loadings component 3 vs. C_{OFL}	0.9957	-0.1649	0.66	13.22
UA	Loadings component 1 vs. C_{UA}	0.9625	-0.1089	1.22	37.02

R^2 : Determination coefficient. S_{yx} : residual standard deviation. LOD: limit of detection.

Finally, the test OFL-spiked urine sample (T2) was spectroelectrochemically measured following the same MAD protocol described for the mixture samples listed in Table 6-7. Let us recall that this sample was prepared adding OFL in 10 mL of urine reaching a concentration of 855 μM . Next, this solution was dissolved in 0.1 M KCl and in BR buffer, giving a nominal concentration of 42.75 μM of OFL (T2).

The OFL concentration of this test urine solution was estimated with the PARAFAC calibration model presented in Figure 6-24a and Table 6-9. A very good accuracy is obtained in the prediction of OFL concentration, $41.9 \pm 2.5 \mu M$, 98.1 % of recovery with a 3.8 % of relative standard deviation (%RSD) and a relative error of 2.0 %. The %RSD value also indicates that the procedure followed is very repeatable. Therefore, the OFL concentration in the initial solution (T2) is $838 \pm 50 \mu M$. These results demonstrate that the PARAFAC model perfectly fulfills the main objective of this work which is the determination of OFL in a test urine sample spiked with this target molecule.

6.3.4. Conclusions

Development of new SEC devices becomes critical because the fields of application of this technique are growing, being chemical analysis one of the most attractive applications for SEC. In this work, we present a new SEC cell that allows the sensitive and reproducible quantification of OFL in urine samples without any pretreatment, only by performing a simple dilution. This SEC cell facilitates the study and quantification of molecules which can modify the WE surface during different oxidation or reduction processes. In this work, the matrix of all the test solutions was urine, which has not been previously pretreated. The different interfering compounds present in urine affect the electrochemical and/or spectroscopic determination of OFL when only univariate models are used. To overcome this drawback, multivariate statistical tools have been used, selecting in this case PARAFAC as the best tool to determine OFL in urine samples. The fitted PARAFAC model has allowed us to properly deconvolve the contribution of OFL and UA to the global SEC signal. Once this objective was achieved, the determination of OFL has been carried out by correlating the loadings of one of the components of the PARAFAC model with the OFL concentration, yielding very good figures of merit. From these results, it can be deduced that the combination of the sensitivity and reproducibility of a good SEC cell and set-up, with the specificity of the spectroscopic methods when the test molecule participates in an electrochemical reaction, even in a complex matrix, leads to develop useful, versatile and reliable methods to quantify urinary biomarkers.

6.4. Derivative UV/Vis spectroelectrochemistry in a thin-layer regime: deconvolution and simultaneous quantification of ascorbic acid, dopamine and uric acid.

This sub-Section will address the study of a complex system by UV/Vis SEC in a thin-layer regime and parallel configuration. Specifically, dopamine, ascorbic acid and uric acid will be quantified in a complex mixture where these components are in random concentrations. The quantification of these molecules is becoming very important due to the fundamental role they play in human metabolism, which makes them really important in the analytical and biomedical fields. However, this is not a straightforward task due to the fact that all the aforementioned compounds are at the same time highly interfering with each other: they coexist in the human body, they oxidize at close potentials and their UV/Vis absorption bands overlap. Hence, this study proposes the combination of derivative SEC with the suitable chemometric tools, such as PARAFAC, in order to quantify all these molecules. As it is demonstrated, the use of derivative UV/Vis together with PARAFAC is a powerful analytical tool when it comes to solve complex matrices, since it allows to separate the contribution of each molecule, being an effective solution to many analytical challenges. This has been published in [*Analytical and Bioanalytical Chemistry*, 2020, 412, 6329-6339](#).¹²⁴

6.4.1. Introduction

Dopamine (DA) is a neurotransmitter that participates in the regulation of physiological processes and can be found in the brain. Low levels of DA have been found in patients with Parkinson's disease and high levels in patients with schizophrenia.^{6,188} Meanwhile, ascorbic acid (AA) is an essential vitamin in the human diet that can be found in the brain in the presence of different neurotransmitters. Low levels of AA are related, for example, to scurvy.^{166,225} Lastly, uric acid (UA) is a natural antioxidant. High levels of UA in blood can promote the development of some diseases, such as gout or anemia.^{225,234} On the other hand, a low level of UA is associated with Parkinson's disease.²³⁴ Undoubtedly, these compounds of biological interest play a fundamental role in human metabolism and their inappropriate amounts are associated with some diseases. For this reason, the study and quantification of these molecules is essential and is becoming increasingly important, particularly in biomedical chemistry and in neurochemistry fields.

Up to now, mainly electrochemical methods have been used to carry out the simultaneous quantification of DA, AA and UA. However, the most important drawbacks of electrochemical methods to determine these compounds are their similar electro-oxidation potentials, the typical presence of the three compounds in the human body and the formation of by-products and side-products during their electrochemical reactions.³³⁴ Thus, due to the high interference that these compounds present, the electrode surface is usually modified with different materials that allow shifting the potentials at which these molecules are oxidized. Some of these modifications consist of using, for example, reduced graphene oxide (RGO),^{227,335} conductive polymers,³³⁶ RGO and conducting polymers nanocomposites,³³⁷ nitrogen doped graphene,²³⁸ carbon nanotubes (CNTs) and nanoparticles,³³⁸ ternary nanocompounds³³⁹ or hexagonal boron nitride.³⁴⁰ It should be highlighted that the use of modified electrodes achieves increased selectivity and sensitivity when compared with bare electrodes, but the modification process can be complex, time-consuming and tedious.³³⁴ In order to overcome these drawbacks, in this work a new method is proposed that combines electrochemistry and spectroscopy together with the appropriate statistical tools, using pristine electrodes without any type of modification in all experiments.

Spectroelectrochemistry (SEC) simultaneously provides the electrochemical and spectroscopic information about a system that undergoes an oxidation-reduction process.^{6,7,65,284} Normal and parallel configurations are the two main optical arrangements for UV/Vis molecular absorption SEC and differ mainly in the direction in which the radiation beam passes through the solution adjacent to the electrode. In the first case, the light beam strikes perpendicular to the electrode surface, while in the second one, it comes parallel to the electrode surface.^{7,57,58,66} In the present case, a parallel configuration is selected due to the several advantages that this arrangement presents with respect to the normal configuration, such as a longer optical path, which results in a better sensitivity and lower limits of detection for soluble compounds.^{42,65,123,129} Taking into account the diffusion regime, SEC in a thin-layer regime^{52,341,342} provides the electrochemical and spectroscopic information about the solution changes that occur only in a small confined space adjacent to the working electrode where it is electrolyzed. Consequently, it is much easier to completely electrolyze the reactant species. UV/Vis thin-layer SEC is gaining importance in many research fields, highlighting its significance in the study of different compounds.^{52,342,343} It should be emphasized that the importance of SEC for analytical

purposes is growing year by year,^{6,40,42,344–346} although it is still taking the first steps in this field trying to show its great potential, sometimes with the help of appropriate statistical tools.

In this work, we propose the simultaneous SEC determination of DA, AA and UA in mixtures that include these three molecules, randomly varying their concentrations in order to demonstrate the robustness of the analytical methodology, using bare carbon screen-printed electrodes (SPEs) and without any pretreatment of the sample or modification of the working electrode surface. In most of the works found in literature, to test the effectiveness of a new electroanalytical method, the concentration of two of these compounds is fixed and the other is determined, or the concentration of the three compounds is increased simultaneously. Undoubtedly, this situation is unrealistic. For this reason, the development of new techniques and/or methodologies should be implemented to allow the determination of these compounds in mixtures with random concentrations in solution. In this way, the determination of real samples can be carried out, where the concentration of these biomolecules can vary significantly depending on the matrix. DA, AA and UA show similarities not only in their oxidation potentials, but also in the position and shape of the absorption spectra of their oxidized forms. Given the high similarities between their spectra, we propose the derivative UV/Vis absorption SEC with respect to the wavelength as an analytical tool to study these mixtures. Derivative spectroscopy is based on plotting the first, second or higher derivative of the absorbance with respect to the wavelength.³⁴⁷ This technique could be the solution to many analytical challenges, such as resolution of complex matrices, due to the effective improvement of resolution.³⁴⁸ Thus, it would allow us to separate compounds with overlapping spectra or to remove the contribution of interfering species.³⁴⁹ Moreover, the power of derivative spectroscopy can be increased substantially when coupled with electrochemistry, a technique known as derivative SEC. This technique, performing the derivative of the absorbance with respect to time or potential, has been used to study and understand redox reactions of several systems^{250,350,351} and to characterize thin electrochromic films.²⁵¹ Furthermore, the quantification of acetylsalicylic acid in presence of AA and UA has been reported using a modified electrode and the derivative of the absorbance with respect to potential at a characteristic wavelength as the quantitative signal.⁴¹

In this work, we propose the use of the derivative of the absorbance with respect to the wavelength as an analytical signal in time-resolved experiments where multiple spectra

were acquired during a cyclic voltammograms (CV). The whole set of derivative spectroelectrochemical data is statistically analyzed using multivariate chemometric tools that help to unravel this complex set of data. In particular, in this work, parallel factor analysis (PARAFAC)^{248,352,353} is chosen because this set of data is intrinsically trilinear; for each sample studied with the three analytes at different concentrations, absorbance depends both on the potential applied and on the wavelength.

Therefore, the main objective of our work is the development of a new methodology that combines the evolution of the derivative of the absorbance with respect to the wavelength during an electrochemical experiment with PARAFAC as a statistical tool for solving complex problems. This methodology is demonstrated, as a proof of concept, with the simultaneous determination of DA, AA and UA using commercial SPEs without any modification.

6.4.2. Materials and methods

6.4.2.1. *Reagents and materials*

Dopamine (DA, 3-Hydroxytyramine hydrochloride, 99 %, Acros Organics), ascorbic acid (AA, L(+)-Ascorbic acid, ACS reagent, Acros Organics), uric acid (UA, 99 +%, Acros Organics) and HCl (37%, VWR) were used as received without further purification. All solutions were prepared daily using high-quality water (18.2 M Ω cm resistivity at 25 °C, Milli-Q Direct 8, Millipore). All experiments were performed at room temperature using SPEs (DRP-110, Metrohm-DropSens). Each SPE has a 4 mm diameter disk screen-printed carbon working electrode (WE), a carbon counter electrode (CE) and a silver pseudoreference electrode (RE).

6.4.2.2. *Instrumentation*

SEC experiments were carried out using a customized SPELEC instrument (Metrohm-DropSens) controlled by DropView SPELEC software (Metrohm-DropSens).

6.4.2.3. *Experimental Set-up*

SEC experiments in parallel configuration under a semi-infinite diffusion regime were performed using devices previously developed by our research group.^{42,123} Two bare optical fibers (100 μ m in diameter, Ocean Optics) were perfectly aligned face to face and fixed with nail polish on the working electrode to carry out SEC experiments in parallel

configuration. The distance between the ends of the two bare optical fibers defined the optical path length. Therefore, it is very easy to change the distance between the fibers, which allows us to study compounds with very different molar absorption coefficients. In order to perform the corresponding experiments, a drop of solution (100 μL) was placed on the SPE, covering the three-electrode system and the ends of the bare optical fibers. Finally, one of the optical fibers was connected to the light source and the other one to the spectrometer.

On the other hand, SEC experiments in parallel arrangement and under a thin-layer diffusion regime were carried out with the same device, but placing a transparent quartz plate on the fibers and the WE to ensure the thin layer regime.⁵² A transparent plate is used because it allows us to follow the process. Two bare optical fiber pieces were placed at the ends of the electrode support to ensure that the quartz plate was completely parallel to the electrode surface, ensuring that the distance between the electrode and the quartz plate was always the same. The volume of the solution drop was 40 μL , and it was to be added on the electrode before placing the quartz plate. The thickness of the thin-layer can be controlled by the diameter of the fibers used. In this case, it was approximately 140 μm (100 μm corresponds to the diameter of the fiber and 20 μm to its cladding/coating materials).⁵² Parallel thin-layer SEC has important advantages over semi-infinite or normal configuration measurements. It is characterized by a high sensitivity due to the long optical pathway, it is possible to completely electrolyze the whole solution, and the use of optical transparent electrodes is not required.

It should be highlighted that in each experiment, the spectrum of the initial solution was taken as reference spectrum. The same SPE with the optical fibers fixed on it was used to measure all solutions in each calibration model. Optical signals were filtered using a Savitzky-Golay filter that enables the derivative signals to be obtained.

6.4.2.4. *PARAFAC analysis*

Matlab R2018a was used to carry out the PARAFAC analysis, using the N-way toolbox developed by Rasmus Bro.²⁵⁶

6.4.3. Results and discussion

6.4.3.1. *Comparison between UV/Vis absorption SEC under semi-infinite diffusion and thin-layer regime*

In order to demonstrate the better performance and higher sensitivity of parallel configuration under thin-layer diffusion regime with respect to semi-infinite diffusion regime some characteristic experiments are described below. UV/Vis absorption SEC experiments were performed in a parallel configuration under both regimes, with UA chosen as the model analyte. Cyclic voltammograms of $1 \cdot 10^{-4}$ M UA in acidic medium (0.1 M HCl) were recorded between +0.30 V and +0.80 V at $0.005 \text{ V} \cdot \text{s}^{-1}$, and at the same time the evolution and changes in the UV/Vis absorption spectra were registered every 200 ms. The oxidation of UA shows an irreversible anodic peak around +0.60 V in the two diffusion regimes (Figure 6-25a). Concomitantly, oxidation of UA shows the decrease of a band at 288 nm, assigned to the consumption of UA during the anodic scan.²³⁵ Figure 6-25b shows this process for the experiment performed under thin-layer diffusion regime.

Selecting the characteristic UA wavelength of 288 nm, Figure 6-25c depicts the corresponding voltabsorptograms in thin-layer and in semi-infinite diffusion regimes. In both voltabsorptograms, it is observed that in the anodic scan, the oxidation of UA starts at +0.55 V, decreasing the absorbance at 288 nm until the vertex potential, +0.80 V, due to the consumption of UA. Besides, in the backward scan, absorbance does not recover the starting zero value, due to the irreversible oxidation of UA. However, some differences can be observed in these two voltabsorptograms. On the one hand, in a thin-layer diffusion regime, a stationary state is reached during the reduction scan, indicating that practically all the UA present in the solution has been consumed. Consequently, in the backward scan, the absorbance reaches a constant value related to the amount of UA consumed during the oxidation. Meanwhile, in semi-infinite diffusion regime, a small increase of absorbance from +0.55 V onwards is observed during the cathodic scan. This is due to the diffusion of UA from the bulk solution to the area sampled by the optical fibers. Another significant difference is observed in Figure 6-25c. The absorbance value achieved in the thin-layer is ca. 36 % higher than that obtained in the semi-infinite configuration, because the diffusion is limited in the thin-layer arrangement, and the electrolysis of all UA occurs in the solution sampled by the optical fibers. This aspect confers higher sensitivity to the thin-layer diffusion regime configuration.

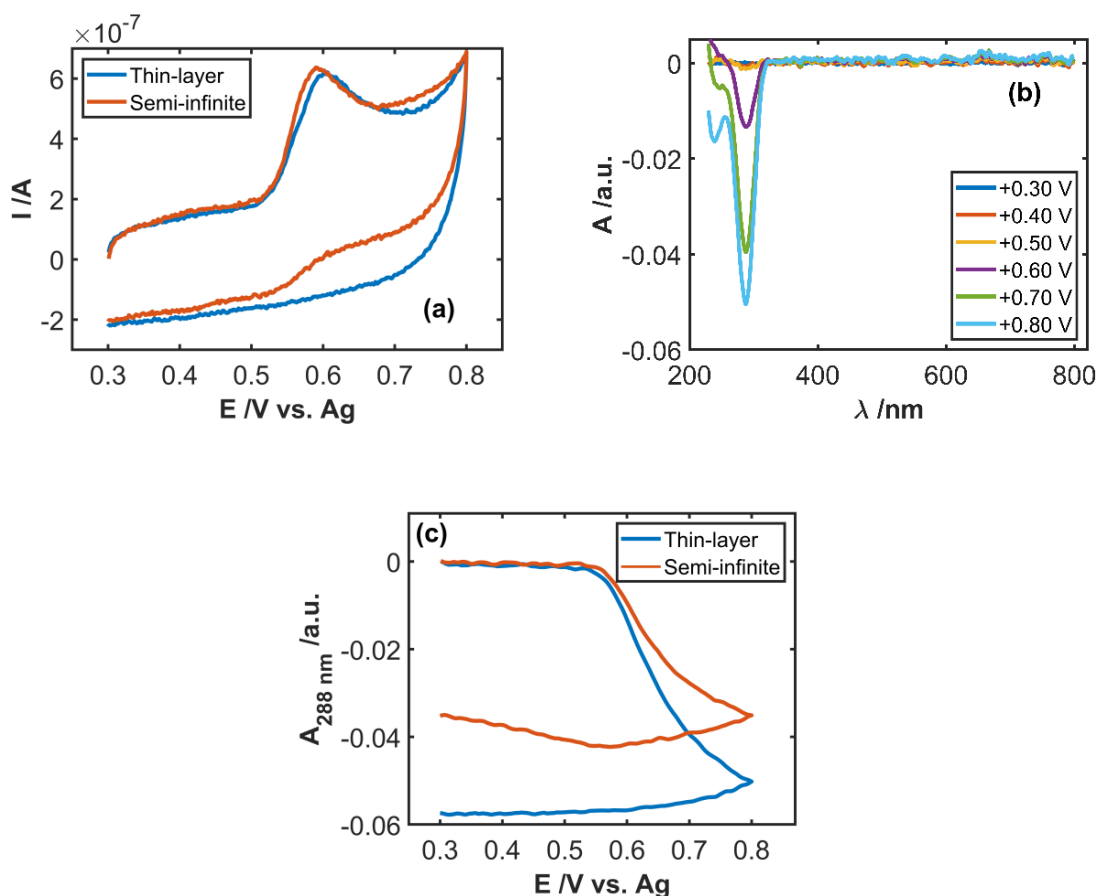


Figure 6-25. (a) CVs, (b) spectra evolution during anodic scan in a thin-layer diffusion regime, and (c) voltabsorptograms at 288 nm. Solution $1 \cdot 10^{-4}$ M UA in 0.1 M HCl. The potential is scanned between +0.30 V and +0.80 V at $0.005 \text{ V} \cdot \text{s}^{-1}$. Blue lines in (a) and (c) are related to signals in the thin-layer diffusion regime. Red lines in (a) and (c) are associated with signals in the semi-infinite diffusion regime.

Furthermore, to check the improvement in sensitivity when performing the experiments in a thin-layer regime rather than in semi-infinite diffusion, two calibration models were constructed using both configurations. A set of calibration samples with concentrations of UA between $1 \cdot 10^{-5}$ and $1 \cdot 10^{-4}$ M in 0.1 M HCl were measured by UV/Vis absorption SEC performing voltammetric and voltabsorptometric measurements between +0.30 and +0.80 V at $0.005 \text{ V} \cdot \text{s}^{-1}$, in both regimes. This range of concentrations was chosen because it is the concentration interval in which a linear correlation is achieved for both configurations. For each diffusion regime, two linear calibration models were constructed with the spectroelectrochemical data, one correlating the anodic peak current of the CV with UA concentration, while the other one correlates the absorbance values at 288 nm and at the vertex potential (+0.80 V) with the UA concentration. The main statistical indicators used to assess the quality of each calibration model, such as the coefficient of

determination (R^2), the sensitivity and the standard deviation of residuals of the linear regression (S_{yx}) are summarized in Table 6-10.

Table 6-10. Regression parameters obtained for the determination of UA by UV/Vis SEC in thin-layer and semi-infinite diffusion regimes.

	Analysis method ⁽¹⁾	R^2 ⁽²⁾	Sensitivity ⁽³⁾	S_{yx} ⁽⁴⁾
Thin-layer	Ip vs. [UA]	0.9900	$3.63 \cdot 10^{-3} \text{ A} \cdot \mu\text{M}^{-1}$	$1.33 \cdot 10^{-8}$
	$A_{288 \text{ nm}, +0.80 \text{ V}}$ vs. [UA]	0.9870	$-441.83 \text{ a.u.} \cdot \text{M}^{-1}$	$1.87 \cdot 10^{-3}$
Semi-infinite	Ip vs. [UA]	0.9903	$3.70 \cdot 10^{-3} \text{ A} \cdot \mu\text{M}^{-1}$	$1.29 \cdot 10^{-8}$
	$A_{288 \text{ nm}, +0.80 \text{ V}}$ vs. [UA]	0.9869	$-341.02 \text{ a.u.} \cdot \text{M}^{-1}$	$1.50 \cdot 10^{-3}$

⁽¹⁾ Concentration range: 10^{-5} - 10^{-4} M UA in 0.1 M HCl; electrochemical method correlates anodic peak current with UA concentration; spectroscopic method correlates absorbance at 288 nm and +0.80 V with UA concentration. All data are obtained during the same experiment and at the same time/potential.

⁽²⁾ Coefficient of determination.

⁽³⁾ Slope of the linear regression model.

⁽⁴⁾ Standard deviation of residuals.

From the electrochemical data, it can be concluded that the diffusion regime selected does not imply any difference; all statistical parameters are similar, even the sensitivity. However, from the spectroscopic data obtained simultaneously to the electrochemical data, one remarkable difference is found. The sensitivity in thin-layer experiments is 30% higher than in a semi-infinite diffusion regime. The increased sensitivity resulting from the ability to achieve total electrolysis of the analyte in a thin-layer regime, along with the limited diffusion from the bulk solution, makes UV/Vis absorption SEC in a thin-layer configuration a more suitable approach for quantitative analysis.

6.4.3.2. Simultaneous determination of DA, AA and UA with derivative UV/Vis absorption thin-layer SEC

After demonstrating the highest sensitivity of the thin-layer configuration from a quantitative point of view, the main objective of this work is to prove the great possibilities that UV/Vis absorption thin-layer SEC offers in the determination of very complex mixtures, from both electrochemical and spectroscopic points of view, as is the case of DA, AA and UA in acid medium. As was explained above, voltammetric and voltabsorptometric experiments were performed simultaneously to study and quantify the

electrochemical oxidation of these analytes. In this case, the potential was swept between 0.00 and +0.90 V at $0.005 \text{ V} \cdot \text{s}^{-1}$, and the integration time in the acquisition of spectra was 400 ms. Therefore, with this purpose, different calibration samples were prepared to construct a calibration model able to predict the concentration of test samples (Table 6-11). All solutions were prepared in 0.1 M HCl, varying the concentration of the three analytes between $1 \cdot 10^{-5}$ and $1 \cdot 10^{-4}$ M. The concentration of each compound in each sample was randomly chosen.

Table 6-11. Set of calibration and test samples used in UV/Vis absorption thin-layer SEC experiments to determine DA, AA and UA in acid medium.

Experiment Number	$C_{AA} \text{ (M)}$	$C_{DA} \text{ (M)}$	$C_{UA} \text{ (M)}$
E01	0	0	0
E02	$1 \cdot 10^{-4}$	0	0
E03	0	$1 \cdot 10^{-4}$	0
E04	$7 \cdot 10^{-5}$	$2 \cdot 10^{-5}$	$3 \cdot 10^{-5}$
E05 (test sample)	$9 \cdot 10^{-5}$	$5 \cdot 10^{-5}$	$4 \cdot 10^{-5}$
E06	$5 \cdot 10^{-5}$	$7 \cdot 10^{-5}$	$9 \cdot 10^{-5}$
E07	$2 \cdot 10^{-5}$	$9 \cdot 10^{-5}$	$5 \cdot 10^{-5}$
E08	$1 \cdot 10^{-5}$	$8 \cdot 10^{-5}$	$8 \cdot 10^{-5}$
E09	$5 \cdot 10^{-5}$	$7 \cdot 10^{-5}$	$9 \cdot 10^{-5}$
E10	$5 \cdot 10^{-5}$	$7 \cdot 10^{-5}$	$9 \cdot 10^{-5}$
E11	0	0	0
E12	0	0	$1 \cdot 10^{-4}$
E13	$1 \cdot 10^{-4}$	0	0
E14	0	$1 \cdot 10^{-4}$	0

Each experiment provides an electrochemical signal, a CV, and the simultaneous evolution of the spectroscopic signal along the CV. Figure 6-26a shows the CVs of aqueous solutions in 0.1 M HCl of DA ($1 \cdot 10^{-4}$ M), AA ($1 \cdot 10^{-4}$ M), UA ($1 \cdot 10^{-4}$ M) and of a solution mixture of the three molecules, which corresponds with the experiment E05 of Table 6-11.

These CVs show the redox behavior of these species individually and in a mixture. In acid media, AA is electrochemically oxidized to dehydroascorbic acid (DHA) by transfer of two protons and two electrons in a reversible electron transfer process coupled to an irreversible chemical reaction where DHA yields to an electro-inactive product after solvation.^{280,354} Consequently, in the CV of AA (Figure 6-26a, blue line), only one anodic peak is observed, at +0.45 V, related to the electrogeneration of DHA. At pH = 1 (HCl 0.1 M), DA is oxidized to dopaminequinone (DAQ) in a two-proton and two-electron quasi-reversible process,⁶⁵ so that its CV shows an anodic and a cathodic peak at +0.56 V and +0.22 V, respectively (Figure 6-26a, orange line). Lastly, UA shows an irreversible behavior as was indicated in Figure 6-25, with one anodic peak at +0.60 V (Figure 6-26a, green line). The electrochemical oxidation of UA yields di-imine species by a two-proton and two-electron reversible process. This di-imine species in aqueous solution is unstable, and after solvation it is transformed into an imine-alcohol, also unstable, that is hydrated to give uric acid-4,5-diol.^{235,236} The anodic peaks related to the oxidation of these three molecules emerge at very similar potentials, which indicates a high degree of overlap in mixture solutions of these three molecules. As can be observed in Figure 6-26a (magenta line), only one anodic peak at +0.58 V with a shoulder at +0.45 V is observed, making very difficult to determine any of these molecules in presence of the others using only the electrochemical signal.

However, it is more difficult to find two compounds with the same electrochemical and spectroscopic behavior. Figure 6-26b shows the absorption spectra of each solution at a potential of +0.90 V registered during the cyclic voltammetry experiment. In all cases, the spectrum of the initial solution with the corresponding analyte (DA, UA, AA or the mixture of them) was taken as reference in the absorptometric measurements. The spectrum of the AA solution (Figure 6-26b, blue line) shows a single negative absorption band at 245 nm, related to the consumption of AA during the anodic scan.⁴² In the same way, the electrooxidation of UA yields an absorption spectrum with a negative band at 288 nm (Figure 6-26b, green line), related to the consumption of UA.²³⁵ Finally, the spectrum related to the oxidation of DA in 0.1 M HCl (Figure 6-26b, orange line) shows two positive absorption bands peaking at 390 and 250 nm, characteristics of the *o*-quinone group of the DAQ electrogenerated.³⁵⁵ Additionally, a negative absorption band at 280 nm is observed related to the L_a and L_b transitions of the DA consumed during this oxidation process.³⁵⁶ As can be observed in Figure 6-26b (magenta line), the absorption

spectrum related to the oxidation of a mixture solution of the three analytes shows a number of overlapping bands related to all the molecules consumed and the DAQ, the only oxidation product observed in these experiments. From these signals, it can be concluded that the spectroscopic quantification of these compounds is also very complicated due to the substantial overlapping of the absorption bands between 200 and 310 nm. Only DA could be detected in the presence of AA and UA using the spectroscopic signals from the UV/Vis absorption spectroelectrochemical data, using the absorption band peaking at 390 nm.

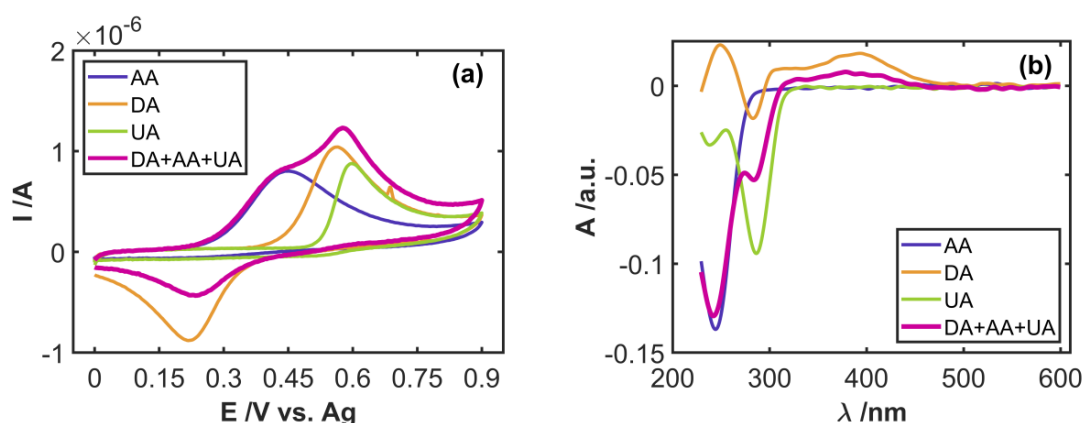


Figure 6-26. (a) CVs and (b) absorption spectra registered at the vertex potential (+0.90 V). Spectroelectrochemical responses recorded between 0.00 V and +0.90 V at $0.005 \text{ V}\cdot\text{s}^{-1}$ of $1\cdot 10^{-4} \text{ M}$ AA (blue line), $1\cdot 10^{-4} \text{ M}$ DA (orange line), $1\cdot 10^{-4} \text{ M}$ UA (green line) and of a complex mixture of $5\cdot 10^{-5} \text{ M}$ DA + $9\cdot 10^{-5} \text{ M}$ AA + $4\cdot 10^{-5} \text{ M}$ UA (magenta line) in 0.1 M HCl.

Nevertheless, the determination of all these molecules is essential, due to the fundamental role that they play in the human metabolism. For this purpose, a multivariate technique using PARAFAC and based on the derivative of the spectroscopic response is proposed, due to its ability to solve complex matrixes without the need to carry out any pretreatment or modification of the electrode surface. The reason for choosing the derivative of absorbance with respect to the wavelength as analytical signal in the quantification of mixtures of DA, AU and AA in 0.1 M HCl medium is that this transformation of the optical signals enables better discrimination of all absorptometric changes occurring during the voltammetric experiment. Figure 6-27a shows the non-derivative spectral changes registered during the oxidation of a mixture of these three molecules. As can be observed, a main absorption band decreases at 243 nm overlapped with a second absorption band peaking at 285 nm, while a small band increases around 390 nm. From these spectra, it is extremely difficult to distinguish the evolution of the different

molecules during this potential cycle. However, with the derivative of each spectrum with respect to the wavelength ($dA/d\lambda$, Figure 6-27b), some differences can be appreciated that will help to discriminate the evolution of each molecule. The comparison of the derivative of the spectrum at the vertex potential of pure samples with only one molecule and the derivative of the spectrum at the same potential of the solution mixture of the three compounds displays the possibilities that this transformation of the absorptometric signals offers (Figure 6-27c).

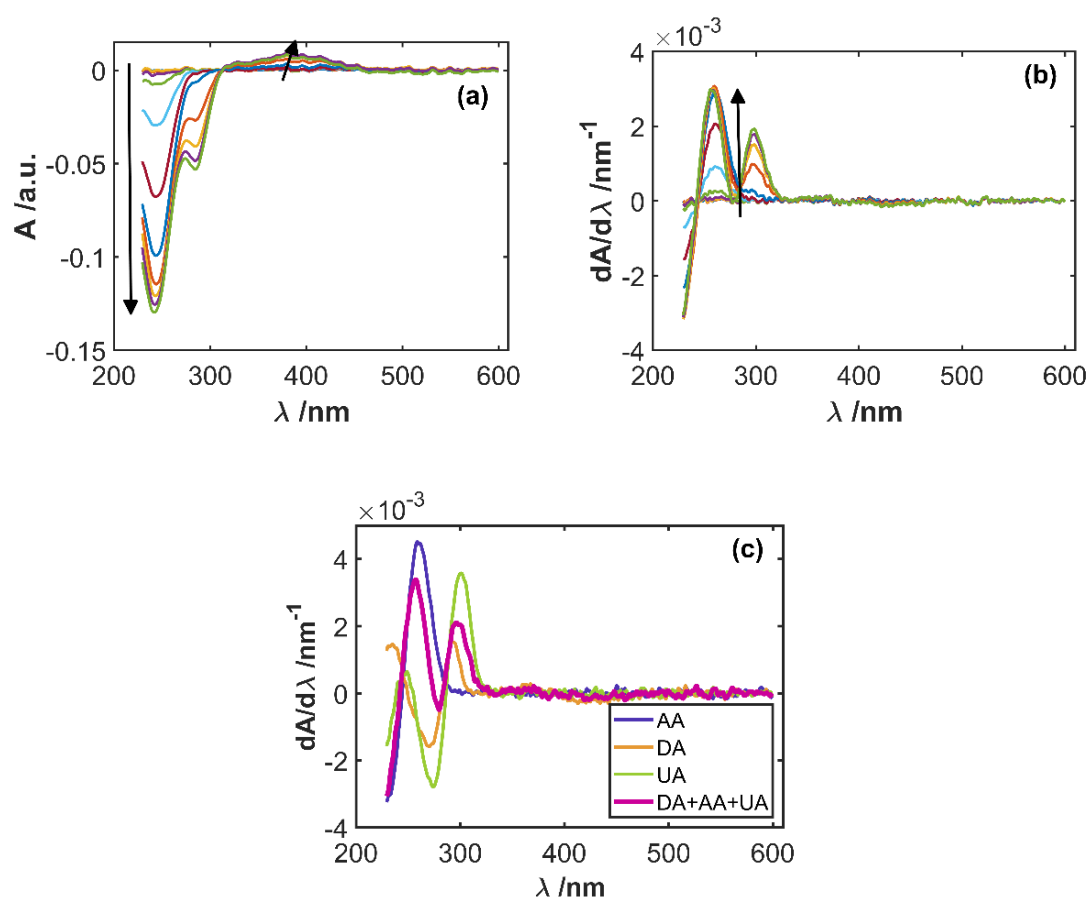


Figure 6-27. Evolution of (a) absorption spectra and (b) derivative spectra with respect to the wavelength between 0.00 V and +0.90 V at $0.005\text{ V}\cdot\text{s}^{-1}$ of a complex mixture of $5\cdot 10^{-5}\text{ M DA} + 9\cdot 10^{-5}\text{ M AA} + 4\cdot 10^{-5}\text{ M UA}$ in 0.1 M HCl. (c) Derivative spectra with respect to the wavelength at the vertex potential (+0.90 V) of $1\cdot 10^{-4}\text{ M AA}$ (blue line), $1\cdot 10^{-4}\text{ M DA}$ (orange line), $1\cdot 10^{-4}\text{ M UA}$ (green line) and of a solution mixture of $5\cdot 10^{-5}\text{ M DA} + 9\cdot 10^{-5}\text{ M AA} + 4\cdot 10^{-5}\text{ M UA}$ (magenta line) in 0.1 M HCl.

Given the interest in and advantages of using the derivative of absorbance with respect to the wavelength ($dA/d\lambda$) in the analysis of mixtures of molecules in a solution, we propose the use of these signals to resolve and quantify mixtures of AA, DA and UA with PARAFAC. The matrix used to construct the calibration model with PARAFAC consisted

of a three-dimensional data matrix with the values of $dA/d\lambda$ from +0.28 V in the forward scan to the end of the backward scan (0.00 V) and between 235 and 550 nm for each sample prepared and measured (Table 6-11). The selected potential window corresponds to the potentials at which some oxidation or reduction were observed both in the CV and in the spectra. Furthermore, the chosen wavelength window matched with the UV/Vis spectral region where significant changes of $dA/d\lambda$ took place. Hence, regions without analytical information about the mixture of AA, DA and UA were excluded. The matrix dimension is defined by the values of $dA/d\lambda$ at 761 potentials x 397 wavelengths x 14 concentrations, and corresponds with up to 4229638 values of $dA/d\lambda$ to construct the PARAFAC model. According to the three compounds present in the mixture (DA, AA and UA), three components were selected, one for each molecule. The outputs of the model were obtained after 42 iterations with a CORCONDIA value of 89.4 %. This value is the core consistency diagnostic that gives a clear-cut answer as to whether or not a model is appropriate. When the PARAFAC model is valid, the corcondia value is close to 100 %.²⁵⁵

Figure 6-28a and Figure 6-28b represent the loadings of the three components, which can be easily ascribed to one of the three molecules (DA, AA and UA), with respect to the range of potentials selected and to the range of wavelengths chosen to obtain the PARAFAC model, respectively. As can be seen in Figure 6-28a, the plot of the loadings vs. the potential applied shows that the component ascribed to AA shows its irreversible oxidation at lower potentials, from +0.30 V onward. Next, the component assigned to DA indicates the reversible oxidation of DA from +0.45 V onwards. Finally, the last component is related to the irreversible oxidation of UA that occurs from +0.55 V onwards. These processes of sequential oxidation of the three molecules are better observed in the plot of the derivative of the loadings with respect to time (Figure 6-28c), with anodic peaks at +0.45 V, +0.54 V and +0.60 V related to AA, DA and UA, respectively.

The comparison of the three signals in Figure 6-28c with the corresponding CVs registered with solutions prepared with only one of the molecules shows a really high similarity (Figure 6-29), taking into account that the loadings have been exclusively obtained from absorptometric values. Figure 6-29a shows a high overlapping between these two signals related to AA. The main differences can be found in the capacitive current observed in the CV, but not appreciated in the derivative of the loadings of the

first component. The similarity between the derivative of the loadings of the second component of the PARAFAC model and the CV of a solution of DA is very high, with the anodic and cathodic peaks of the two signals completely overlapped (Figure 6-29b). A higher similarity was observed in the derivative of the loadings of the third component and the CV of a solution of UA. In this case, the positions of the anodic peaks obtained with both the electrochemical and the optical responses are exactly the same (Figure 6-29c). It is worth noting that also in this Figure 6-29, the capacitive current is only observed in the CV.

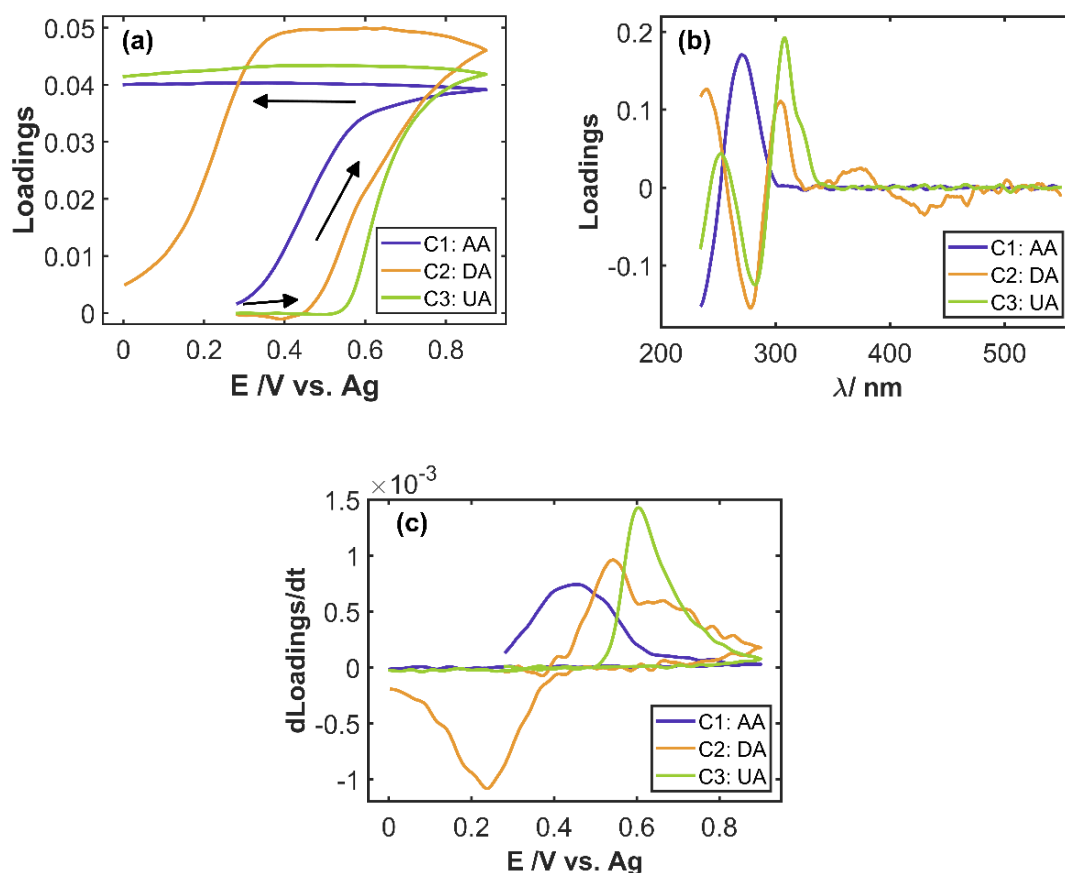


Figure 6-28. Loadings of each component versus (a) the potential and (b) the wavelength, after PARAFAC analysis. (c) Derivative of loadings with respect to time for each component. Blue line is the signal of the first component related to AA; orange line is the signal of the second component related to DA; green line is the signal of the third component related to UA.

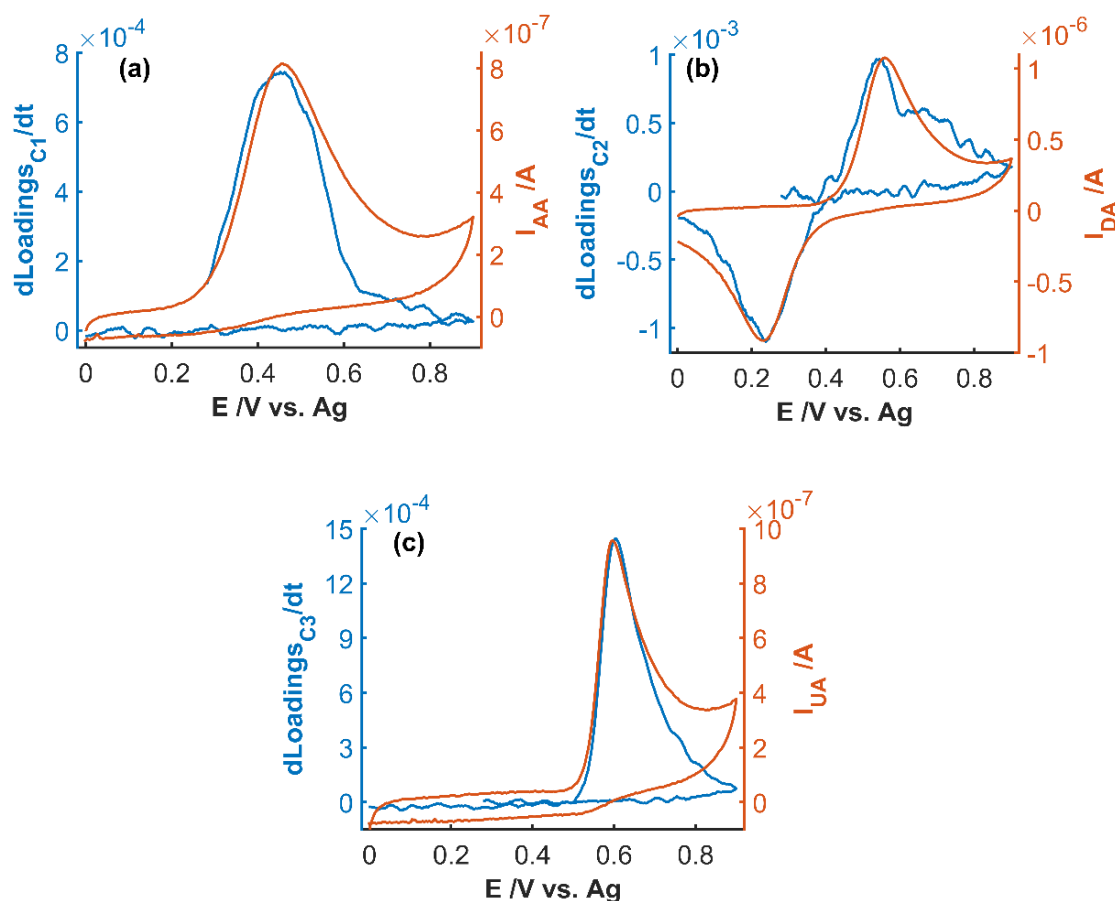


Figure 6-29. Comparison between the derivative of the loadings of each component from the PARAFAC model with respect to time and the CVs of (a) ascorbic acid, (b) dopamine, and (c) uric acid. The CVs correspond to solutions with only one of the molecules (a) $1 \cdot 10^{-4}$ M AA in 0.1 M HCl, (b) $1 \cdot 10^{-4}$ M DA in 0.1 M HCl, and (c) $1 \cdot 10^{-4}$ M UA in 0.1 M HCl. Experiments performed between 0.00 V and +0.90 V at $0.005 \text{ V} \cdot \text{s}^{-1}$. Blue lines correspond to the derivative of the loadings with respect to time. Orange lines correspond to the CVs of each molecule.

These similarities can also be appreciated between the signals in Figure 6-28b obtained from the PARAFAC model and the derivative of the absorbance with respect to the wavelength of solutions with only one of the molecules (Figure 6-30). Therefore, we can conclude that the PARAFAC model constructed with the values of the derivative of absorbance with respect to the wavelength enables the deconvolution of the optical signals related to each of the three molecules tested, i.e., AA, DA and UA.

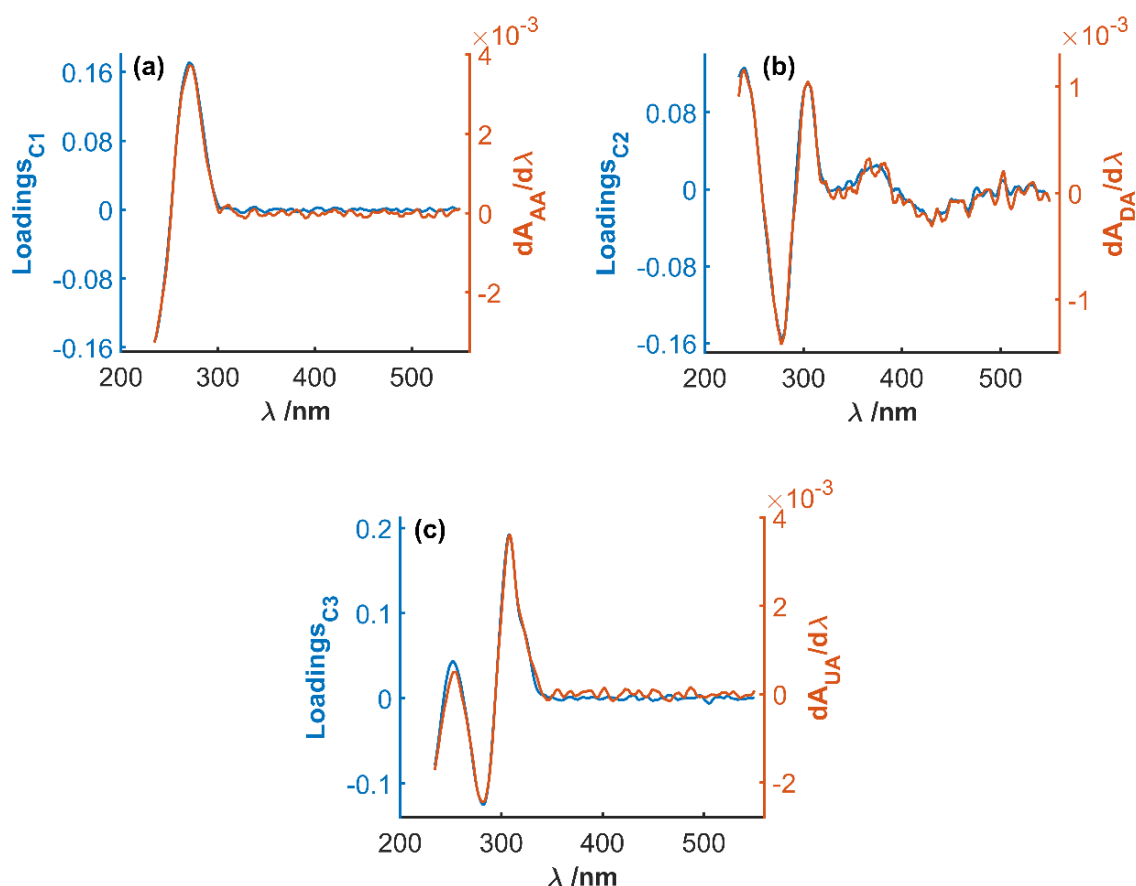


Figure 6-30. Comparison between the loadings of each component from the PARAFAC model and the derivative of the absorbance with respect to the wavelength at +0.90 V of (a) ascorbic acid, (b) dopamine, and (c) uric acid. The $dA/d\lambda$ corresponds to solutions with only one of the molecules (a) $1 \cdot 10^{-4}$ M AA in 0.1 M HCl, (b) $1 \cdot 10^{-4}$ M DA in 0.1 M HCl, and (c) $1 \cdot 10^{-4}$ M UA in 0.1 M HCl. Experiments performed between 0.00 V and +0.90 V at $0.005 \text{ V} \cdot \text{s}^{-1}$. Blue lines correspond to the loadings of each component. Orange lines correspond to the $dA/d\lambda$ of each molecule.

Once the optical signals are deconvolved, the determination of each molecule can be carried out. With this objective, three calibration curves were constructed using a linear least squares method, one for each molecule, correlating the values of the loadings for each component with the concentration of each specific molecule in the different mixtures tabulated in Table 6-11. Figure 6-31 and Table 6-12 show the figures of merit of these calibration models. In these calibration models, the sample E05, used as test sample, was excluded. The three calibration models constructed using the linear least squares method for the three components show the high relationship between the loadings of each component and the concentration of each one of the molecules.

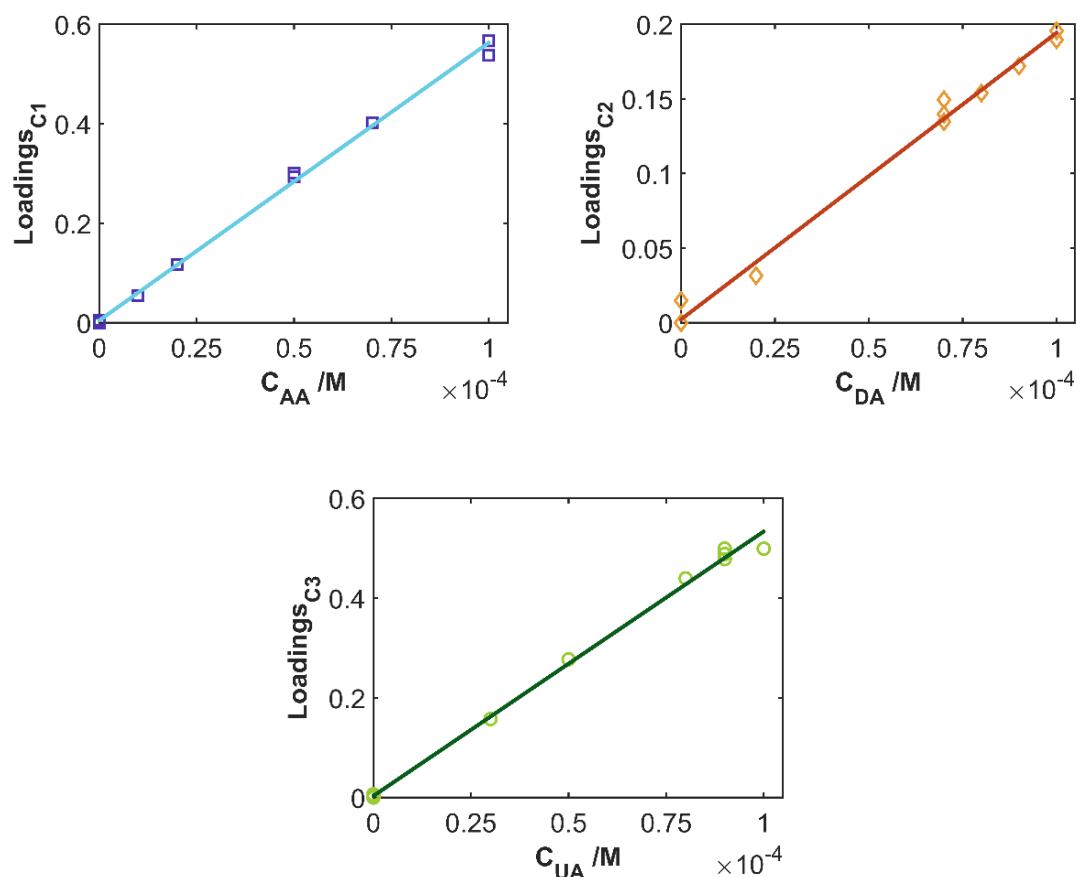


Figure 6-31. Linear regression models obtained with the loadings and the concentrations of (a) AA, (b) DA, and (c) UA.

High coefficients of determination (R^2) and low values of the standard deviation of residuals of the regressions (S_{yx}) were obtained for the three linear calibration models constructed for the molecules, indicating the suitable deconvolution of the raw signals with the PARAFAC model obtained.

The repeatability of the procedure was evaluated by replicating some samples three times. These replicated samples (E06, E09 and E10) can be found in Table 6-11. The repeatability was evaluated for each calibration model constructed for each molecule, obtaining %RSD values lower than 5.5 % in all cases, indicating that the procedure provides good repeatability.

The reproducibility of the procedure was demonstrated by performing three PARAFAC models with data collected on three different days, each using a different SPE with the optical fibers fixed on it. The set of experiments was always the same, as shown in Table 6-11. The values of the relative standard deviation of the slope indicate a high

reproducibility (Table 6-12, RSD_{slope} values). Lastly, the limits of detection were calculated, with values for the three molecules lower than 16 μM .

Table 6-12. Regression parameters obtained for the determination of AA, DA and UA in mixtures by UV/Vis SEC in thin-layer regime with the loadings of the PARAFAC model.

Molecule	Calibration model ⁽¹⁾	R^2 ⁽²⁾	Sensitivity ⁽³⁾ (M)	S_{yx} ⁽⁴⁾	% RSD_{slope} ⁽⁵⁾	LOD ⁽⁶⁾ (M)
AA	Loadings C1 vs. [AA]	0.9976	5582.7	$1.09 \cdot 10^{-2}$	0.3	$0.6 \cdot 10^{-5}$
DA	Loadings C2 vs. [DA]	0.9941	1920.7	$0.66 \cdot 10^{-2}$	8.7	$1.6 \cdot 10^{-5}$
UA	Loadings C3 vs. [UA]	0.9970	5301.6	$1.31 \cdot 10^{-2}$	11.8	$1.2 \cdot 10^{-5}$

⁽¹⁾ Correlation between the loadings obtained from the PARAFAC model for the three components and the concentrations of AA, DA or UA in samples tabulated in Table 6-11, except E05

⁽²⁾ Coefficient of determination.

⁽³⁾ Slope of the linear regression model.

⁽⁴⁾ Standard deviation of residuals.

⁽⁵⁾ Relative standard deviation of the slope ($n=3$).

⁽⁶⁾ Limit of detection.

Finally, the concentration of each molecule in a test sample (sample E05 in Table 6-11) was estimated with each of the linear calibration models constructed on three different days (Table 6-13). The estimated concentration of each molecule shows values with a relative error lower than 3% (AA and UA) and 9% (DA). These values indicate that the procedure followed and the linear models developed allow a suitable assessment with a high precision of the unknown concentrations of AA, DA and UA in a test mixture. Furthermore, the values of the relative standard deviation of the estimated concentrations (% RSD_{conc}) for each molecule with each one of the three linear calibration models are lower than 5 %, denoting a high reproducibility.

The results presented, both in the deconvolution of the raw spectroelectrochemical signals and in the simultaneous quantification of DA, AA and UA in a mixture, demonstrate that the strategy proposed has been successfully implemented. The combination of derivative UV/Vis absorption SEC with thin-layer regime measurements and multivariate

chemometric tools such as PARAFAC has allowed us to simultaneously determine these three molecules, which show high overlapping both in the electrochemical and in the absorptiometric signals.

Table 6-13. Estimation of the concentration of AA, DA and UA in a test sample.

Molecule	[Real] ⁽¹⁾ (M)	[Estimated] ⁽²⁾ (M)	CI ⁽³⁾ (M)	%RE ⁽⁴⁾	%RSD _{conc} ⁽⁵⁾
AA	$9 \cdot 10^{-5}$	$9.1 \cdot 10^{-5}$	$[8.3 - 9.9] \cdot 10^{-5}$	0.8	3.6
DA	$5 \cdot 10^{-5}$	$4.6 \cdot 10^{-5}$	$[4.1 - 5.1] \cdot 10^{-5}$	8.1	4.1
UA	$4 \cdot 10^{-5}$	$3.9 \cdot 10^{-5}$	$[3.5 - 4.3] \cdot 10^{-5}$	2.0	4.3

⁽¹⁾ Real concentration of each molecule in the test sample (E05, Table 6-11).

⁽²⁾ Media of the estimated concentration of each molecule of the test mixture sample with the constructed linear calibration models (Table 6-12).

⁽³⁾ Confidence interval, $\alpha = 0.05$.

⁽⁴⁾ Relative error calculated as $|[Estimated] - [Real]| \cdot 100 / [Real]$.

⁽⁵⁾ Relative standard deviation of the concentration ($n=3$).

6.4.4. Conclusions

The analysis of high overlapping signals where three different molecules are responsible of the changes of current or absorbance in spectroelectrochemical experiments is highly complicated. However, the development of strategies that allow the researchers to know the exact contribution of each molecule in a sample to the signals studied is highly interesting. In this work, the combination of derivative UV/Vis absorption spectroelectrochemistry data and suitable multivariate chemometric tools has allow us to successfully achieve this objective.

Thin-layer experiments, where the diffusion of reactants and products is limited, have been very helpful to improve the sensitivity of the methodology proposed. Long-optical path-way experiments, where the optical path way is delimited by the distance between the two optical fibers, have also contributed to increase this sensitivity.

The oxidation of AA, DA, and UA in mixture samples supplies high overlapping signals both in the CVs and in the absorption spectra. The best strategy to study this kind of samples has demonstrated to be the use of the derivative of the absorbance with respect to the wavelength because it allows a much better differentiation of the range of potentials at which each molecule is oxidized. Additionally, the use of PARAFAC, a multivariate

chemometric tool useful in these kinds of problems due to the trilinear character of spectroelectrochemical data, has allowed to successfully deconvolve the pristine spectroelectrochemical signals and to quantify DA, AA and UA simultaneously, yielding to micromolar limits of detection. The sensitivity of the method is clearly dependent on the molecule studied, being in this case much more sensitive to AA and UA than to DA.

Nevertheless, instrumental improvements should help to obtain better limits of detection in the near future, making SEC a very interesting technique for quantitative analysis because of its intrinsic trilinear character. Developments in electrochemistry instrumentation helped in the past to improve limits of detection and, in the same way, developments in optical equipment should help SEC. Detectors with better signal-to-noise ratio, more stable light sources and better optical fibers could help to highly improve limits of detection, allowing applying the methodology presented in this work to more complex problems.

The results presented here show only one of the multiple possibilities that the synergy between spectroelectrochemical data and chemometric tools has in solving complex chemical problems, avoiding tough and time-consuming strategies of optimization and fabrication of chemically modified electrodes.

6.5. Triple Spectroelectrochemistry. Simultaneous UV/Vis absorption, photoluminescence and Raman Spectroelectrochemistry.

6.5.1. Introduction

Spectroelectrochemistry (SEC) provides a huge amount of information in a single experiment, since it permits to follow a system from two different points of view: the one provided by the electrochemistry and the one obtained from spectroscopy.^{43,117,124,281} Additionally, technical improvements can be used to simultaneous recording spectroscopic signals of different nature. This is of great interest, as it is a powerful tool for studying all the chemical processes taking place in an electrode process, providing key information for many fields of research, as for example, the improvement of analytical protocols in multiple areas such as medical, pharmacological, food, or environmental analysis.

Several SEC studies combining two independent spectroscopic techniques have been described in the past. Different devices have been proposed to perform bidimensional UV/Vis absorption SEC,^{58,66,257} and to study simultaneously chemical systems in fluorescence (PL) and UV/Vis absorption¹¹⁹ or in Raman scattering and UV/Vis absorption.^{65,118} Nevertheless, to the best of our knowledge, the simultaneous coupling of three independent spectroscopic techniques has not yet been performed.

In this work, a novel design that allows the simple and reproducible coupling of *operando* SEC measurements of UV/Vis absorption, PL and Raman scattering is proposed. This new set-up focuses on the wealth of information that all these techniques combined can provide in the study of a chemical system. UV/Vis SEC supplies information related to all the compounds present or electrogenerated at the electrode/solution interface and in the diffusion layer, as well as possible reactions with interfering substances. PL-SEC allows us to identify fluorescent compounds involved in the electrode process. And finally, Raman SEC provides vibrational information and surface sensitive information of the analytes, thanks to the use of *Surface Enhanced Raman Scattering* (SERS) phenomenon.

All these signals have been demonstrated to be helpful not only in the study of the electrochemical process but also in the quantification of the molecule of interest.^{281,357,358} In the best scenario, quantification could be achieved with four different signals obtained simultaneously: the electrochemical signal, and three spectroscopic signals (UV/Vis, PL and Raman spectra). This would imply the self-validation of the results obtained in the determination.

For the characterization of complex systems, such electrochemical processes, usually the realization of SEC experiments separately is the most used strategy. However, the simultaneous combination of analytical techniques is a superior strategy, since it ensures the study of the same chemical system, avoiding any influence of irreproducibility among experiments, for example when the electrode is modified using methodologies with poor reproducibility. The coupling of all these techniques allows studies that could not be carried out with them separately. As a proof of concept, in this work we study the SEC performance of gold nanoparticles deposited on an electrode. Drop-casting is a method that stands out for its irreproducibility. Therefore, the individual study of different drop-casted NP films, cannot guarantee that the same substrate provides correlated information between different spectroscopic techniques.

6.5.2. Experimental Section

6.5.2.1. *Reagents and materials*

In order to carry out the appropriate experiments to validate the designed cell, tris(2,2'-bipyridyl) ruthenium (II) chloride hexahydrate ($\text{Ru}(\text{bpy})_3^{2+}$, Acros Organics) solutions were prepared in 0.1 M KNO_3 (Sigma-Aldrich) as supporting electrolyte. Ofloxacin (OFL, Sigma-Aldrich) solutions were prepared in 0.1 M Britton-Robinson (BR) as supporting electrolyte. The BR buffer solution contains boric acid (H_3BO_3 , Panreac), phosphoric acid (H_3PO_4 , 85 %, Panreac), acetic acid (HAc, VWR Chemicals) and sodium hydroxide (NaOH, Acros Organics). The pH of this solution was adjusted to 4.25.

The gold nanoparticle (Au-NP) dispersion was prepared using the following reagents: gold (III) chloride (HAuCl_4 , Sigma-Aldrich), tri-Sodium citrate dihydrate (Merck) and sodium hydroxide (NaOH, Acros Organics).

All reagents were used as provided without additional purification and all solutions were prepared in high-quality ultrapure deionized water (18.2 M Ω cm resistivity at 25 °C, Milli-Q Direct 8, Millipore). All analyses were conducted at room temperature.

6.5.2.2. *Synthesis of gold-nanoparticles*

A well-known protocol has been used to synthesize Au-NPs.^{359–361} Quasi-spherical 60 nm Au-NPs have been synthesized using a solution $2.5 \cdot 10^{-4}$ M of HAuCl₄. This solution was heated to boiling and then 0.375 mL of $3.4 \cdot 10^{-2}$ M citric acid was added. Twenty seconds after the addition of citrate, the solution turns a dark lilac color, at which point the synthesis of the Au-NPs begins. This solution is left on the hot plate to boil for 15 minutes. After this time, it is removed from the plate, and it is left to cool. Once the Au-NPs have been synthesized, it is important to apply a cleaning protocol, which will consist of adding NaOH to the dispersion, which will cause the precipitation of the NPs. The supernatant is then removed, taking care not to remove the NPs that have precipitated at the bottom of the dispersion. This process is repeated two additional times.

6.5.2.3. *Electrode modification*

It is a well-known fact that Raman spectroscopy signal is characterized by the low intensity of the spectral bands. Therefore, to overcome this drawback, modify gold screen-printed electrodes (Au-SPE, DRP-220AT, Metrohm-DropSens) were used to carry out the experimental work. The working electrode is modified by the drop-casting method, that is by dropping the aqueous solution of the quasi-spherical 60 nm Au-NPs in order to create a SERS substrate able to amplify the Raman signal.

In this sense, the WE will be modified with 60 nm spherical Au-NPs, whose synthesis has been explained in the previous Section. For this purpose, 5 μ L of the NP dispersion are deposited on the WE and once the drop has dried, a second 5 μ L aliquot is added on top, replicating the first drop. This process is repeated one more time. In this way, the electrode is modified by adding 3 drops of 5 μ L of a dispersion of quasi-spherical Au-NPs. To accelerate the drying process of the drop, a nitrogen stream is applied.

6.5.2.4. *Instrumentation and software*

SEC experiments were performed using three customized SPELEC instruments (Metrohm-DropSens) synchronized with each other and controlled by DropView

SPELEC software (Metrohm-DropSens, Version: 3.4 19KZ07). For *in-situ* time-resolved UV/Vis SEC the customized SPELEC instrument includes a potentiostat, a halogen-deuterium light, and a spectrometer. The customized PL-SPELEC instrument consists of a potentiostat, a spectrometer and an external LED. To carry out the PL-SEC, an UV-LED with a maximum emission at 310 nm was selected for following both systems, because it was close to the absorption maximum bands. In addition, the use of this LED allows detecting the fluorescence of $\text{Ru}(\text{bpy})_3^{2+}$ and OFL without interference from the excitation light. The SPELEC-Raman instrument includes a potentiostat, a spectrometer and a 785.18 nm laser source (laser power = 203 mW). A schematic representation of how these devices have been integrated is shown in the Figure 6-32.

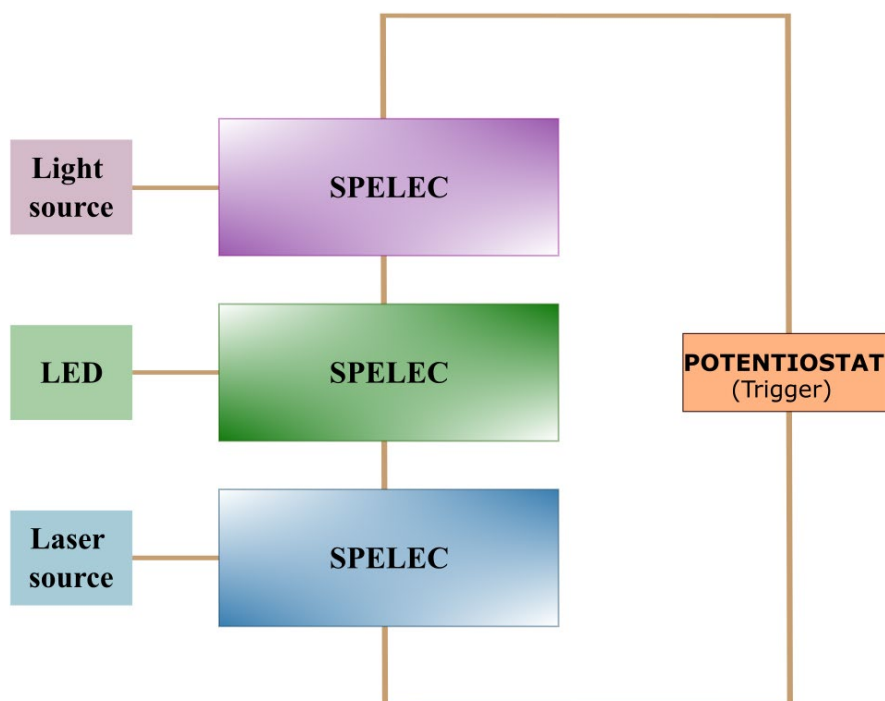


Figure 6-32. A schematic representation of how the different SPELEC instruments are connected. The Raman-SPELEC is the one that controls the electrochemistry of the experiment and all three are connected to each other.

The use of filters is essential to avoid saturating the CCD detectors of the spectrometers that record the absorbance and fluorescence signals with the light coming from the LED or laser source. For registering the UV/Vis absorption a band-pass filter (Ocean Optics) that allows only those signals whose wavelengths are in the range 300-500 nm thereby attenuating signals outside this spectral range is required. The use of this type of filters is necessary to prevent the spectrometer that records the absorbance signal from being

saturated with light from the LED or laser source. So, the filter is connected to one of the bare optical fibers and to another commercial fiber (Ocean Optics, QP450-1-XSR) which is connected to the spectrometer. In the case of PL, a notch-filter (StopLine® Notch-filter, Semrock), which attenuates the signal of 785.18 cm^{-1} , is connected to one of the bare optical fibers and to another optical fiber (Ocean Optics, QP600-025-VIS). The notch-filter is required to minimize the light provided by the Raman probe, since without the filter, the spectrometer used in PL measurements is saturated by the Rayleigh scattering.

6.5.2.5. Design and fabrication of the device for UV/Vis-PL-Raman SEC measurements.

The cell designed to carry out SEC experiments in which UV/Vis, PL and Raman spectroscopic changes are simultaneously registered during an electrochemical process is shown in Figure 6-33.

This cell consists of three main parts, as it is shown in Figure 6-33b. The piece placed at the bottom (I) has been created using UV sensitive 405 nm resin (ANYCUBIC) with a 3D printer (Photon Mono SE, ANYCUBIC). This piece has four magnets at the corners and is designed to accommodate a SPE in a recess. The second piece (II) is made of PMMA using a high-precision CO₂ laser cutting machine, and it was also created with four magnets located at the corners, which facilitates assembly with the lower part. The design of piece II was inspired in a similar cell reported in a previous article (Section 6.3).³²² This piece was designed to hold two 100 μm bare optical fibers (Ocean Optics), which will be used for UV/Vis measurements in parallel arrangement. The fibers were fixed to two pillars of 2.6 mm diameter; these pillars are attached to the piece II by means of a rectangular recess of 2.0 mm wide, leaving the central part of piece II. Structure of piece II facilitates the simultaneous use of the Raman probe and the fibers selected to perform the fluorescence measurements. The space between the two optical fibers fixed on the cell to carry out UV/Vis absorption measurements in parallel configuration is measured with ImageJ,³³¹ so the optical pathway is always known. In this work, the optical pathway for the Ru(bpy)₃²⁺ experiments is 0.40 mm, whereas the OFL experiments were performed maintaining an optical pathways of 0.68 mm. Two metal nuts on the left side were added to the piece II, in order to be able to incorporate the third piece (III), which has two magnets in the lower part, so it is easily fixed to the previous one. This third piece (21.0 x 26.0 x 30.5 mm) is made of PMMA and has been designed

with a diagonal recess that facilitates the incorporation of the optical fibers required to perform the PL experiments in the side configuration. Nail polish was used to fix the optical fibers in both parts (II and III), to ensure the stability of optical signals un UV/Vis and PL experiments.

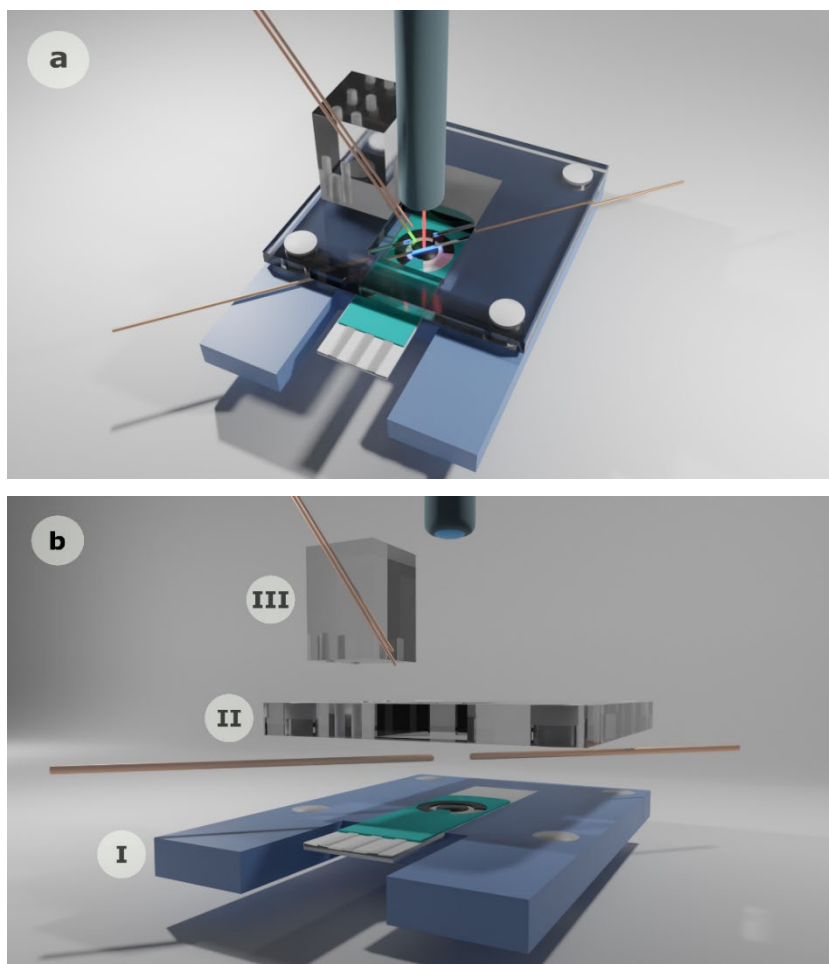


Figure 6-33. (a) Schematic image of the SEC cell assembled and (b) detailed schematic view of each part of the cell. (I) lower piece, (II) middle piece and (III) upper piece.

6.5.3. Results and discussion

6.5.3.1. Validation of the UV/Vis-PL-Raman SEC with $\text{Ru}(\text{bpy})_3^{2+}$.

The first step consists of the validation of the new multi-SEC cell. It will be demonstrated that this cell can have a great potential in the study of processes where, at the same time that a potential is applied, the behavior of the system under study can be followed by performing three spectroscopic techniques (UV/Vis, PL and Raman). For this purpose, a $1 \cdot 10^{-3}$ M $\text{Ru}(\text{bpy})_3^{2+}$ solution in 0.1 M KNO_3 was measured. This compound was selected

since it is a well-known reference system for SEC. It has been reported that $\text{Ru}(\text{bpy})_3^{2+}$ undergoes a reversible electrochemical oxidation-reduction process with the transfer of one proton and one electron.⁴⁴ Several studies also report UV/Vis,^{362,363} PL^{362,363} and Raman spectra^{364–366} of the reduced and oxidized form of this coordination complex.

Thus, $\text{Ru}(\text{bpy})_3^{2+}$ was measured in an Au-SPE modified with Au-NPs. A cyclic voltammetry was performed between -0.10 V and +1.10 V, starting at +0.60 V in the anodic direction and at $0.02 \text{ V}\cdot\text{s}^{-1}$. The evolution of UV/Vis, PL and Raman spectra of the species in the solution and on the electrode surface were registered simultaneously. All experiments were carried out selecting 1000 ms as the integration time for the acquisition of all spectra. It should also be noted that the spectrum at the OCP has been used as reference spectrum for absorbance measurements.

The evolution of the spectra with potential/time are shown in the Figure 6-34. The UV/Vis spectra (Figure 6-34a) are characterized by a positive absorption band at 307 nm and other negative band at 448 nm, which is accompanied by a little shoulder at 350 nm. Since the registered spectral changes are compared with the initial solution, it is possible to observe negative absorption bands corresponding to the disappearance of $\text{Ru}(\text{bpy})_3^{2+}$ species and positives ones related to the generation of $\text{Ru}(\text{bpy})_3^{3+}$. The absorption band at 307 nm in Figure 6-34a is due to the electrogenerated oxidation product ($\text{Ru}(\text{bpy})_3^{3+}$) and is assigned to a ligand $\pi-\pi^*$ transition (LC) centered in the heterocyclic ligand,^{44,362,367–370} while the negative absorption band centered at 448 nm is related to the $\text{Ru}(\text{bpy})_3^{2+}$ disappearance, and is assigned to a metal-to-ligand ($d-\pi^*$) charge transfer (MLCT) process.^{367,368,370} The little shoulder at 350 nm is attributed to the MC d-d transition of the $\text{Ru}(\text{bpy})_3^{2+}$. The evolution of the PL spectrum (Figure 6-34b) represents the fluorescence variation with respect the initial one. As it can be seen it exhibits a band at 625 nm attributed to emission from the MLCT triplet excited state ($^3\text{MLCT}$) to the ground state.^{44,362} Regarding the Raman spectrum (Figure 6-34c), several peaks can be observed, corresponding with the classical reported $\text{Ru}(\text{bpy})_3^{2+}$ SERS spectra. Table 6-14 summarizes the Raman band assignment for this experiment.^{366,371}

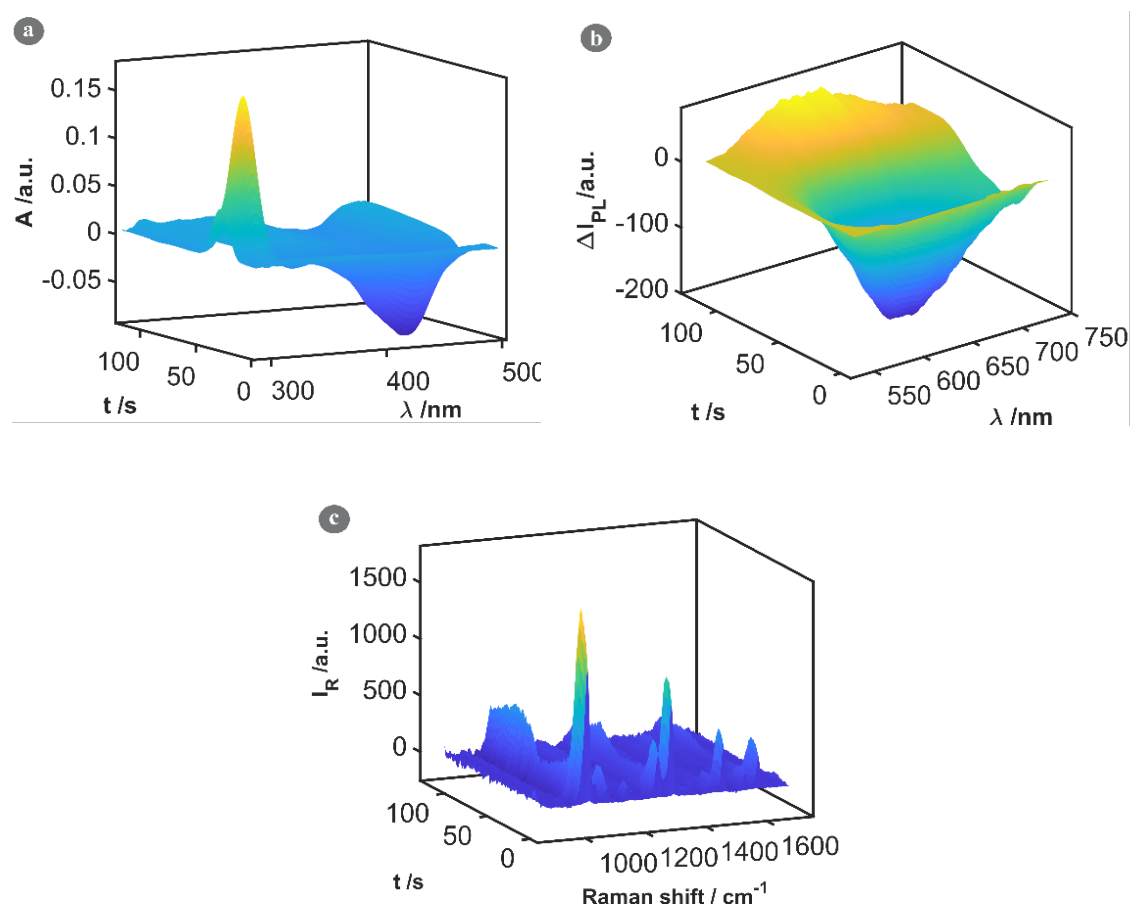


Figure 6-34. Spectra evolution of $1 \cdot 10^{-3}$ M $\text{Ru}(\text{bpy})_3^{2+}$ in 0.1 M KNO_3 between -0.10 V and +1.10 V, starting at +0.60 V in the anodic direction at $0.02 \text{ V} \cdot \text{s}^{-1}$. (a) UV-Vis, (b) PL and (c) Raman.

Table 6-14. Band assignments of Raman spectrum for $\text{Ru}(\text{bpy})_3^{2+}$ in 0.1 M KNO_3 during the SEC experiment, shown in Figure 6-34c.

EC-SERS bands for $\text{Ru}(\text{bpy})_3^{2+}$	Mode assignments ³⁶⁶
1038	Ru-N stretch, ring breathing
1273	C-H wag, C-N stretch
1318	2-2' C-C stretch, C-H wag
1487	C-C stretch, C-H wag
1560	C-C stretch, C-H wag
1602	C-C stretch, C-H wag

Figure 6-35 shows the cyclic voltammogram (CV) and the evolution of the most characteristic bands for each spectroscopic signal with respect to the potential/time.

The CV (Figure 6-35a) shows one reversible oxidation-reduction process at +0.94 V. The anodic peak (O_1) that reaches its maximum around +0.98 V indicates the oxidation of the $\text{Ru}(\text{bpy})_3^{2+}$ to $\text{Ru}(\text{bpy})_3^{3+}$.^{370,372–375} During the backward sweep, two reduction peaks (R_1 and R_2) are observed. The first cathodic peak (R_1 , +0.90 V) is associated to the reduction of $\text{Ru}(\text{bpy})_3^{3+}$ to $\text{Ru}(\text{bpy})_3^{2+}$.^{370,372–375} A second cathodic peak (R_2) appears in the CV, which is related to the reduction of the Au-NPs deposited on the WE surface.³⁶⁶ Therefore, it can be deduced that during the oxidation of the complex, also the oxidation of the Au-NPs is taking place, but the electrochemical signal does not provide a direct evidence of this information.

The cyclic voltabsorptogram (CVA) at 307 nm (evolution of absorbance with potential, Figure 6-35b) shows how from +0.85 V onwards the absorbances increases until the system reaches a potential of +1.10 V during the backward scan, which matches with the oxidation/reduction of the coordination complex studied. This signal has a similar behavior to the CV (Figure 6-35a), except that the second reduction peak is not observed, and therefore, it is no related to any electrochemical process suffered by the $\text{Ru}(\text{II})$ complex. This also demonstrates that redox process observed at +0.94 V is due to the reversible oxidation of $\text{Ru}(\text{bpy})_3^{2+}$. The cyclic voltaFluorogram at 625 nm (evolution of PL with potential) depicted in the Figure 6-35c confirms the same reversible redox behavior; first, the fluorescence decreases from +0.80 V onwards due to the oxidation process and the generation of $\text{Ru}(\text{bpy})_3^{3+}$; in the backward scan when the applied potential is cathodic enough to regenerate $\text{Ru}(\text{bpy})_3^{2+}$ the fluorescence increases recovering the starting value.^{44,362} In the cyclic voltaRamagram (CVR) at 1038 cm^{-1} (evolution of Raman intensity with the potential applied, Figure 6-35d) Raman intensity increases until +0.80 V, related to the interaction of $\text{Ru}(\text{bpy})_3^{2+}$ with the Au-NPs on the WE surface. The decay of the Raman intensity from +0.80 V onwards coincides with the oxidation onset potential shown in the CV, due to the oxidation of both, Au-NPs and $\text{Ru}(\text{bpy})_3^{2+}$. This Raman intensity decreases until the reduction of gold complexes and oxides takes place to generate new Au-NPs (around +0.45 V), where a slight increase of the signal is observed due to the formation of the SERS substrate. As can be appreciated in Figure 6-34c, the Raman spectrum shows several characteristic peaks, however the peak selected for this representation is the one appearing at 1038 cm^{-1} because it is the one which reaches the highest Raman intensity.

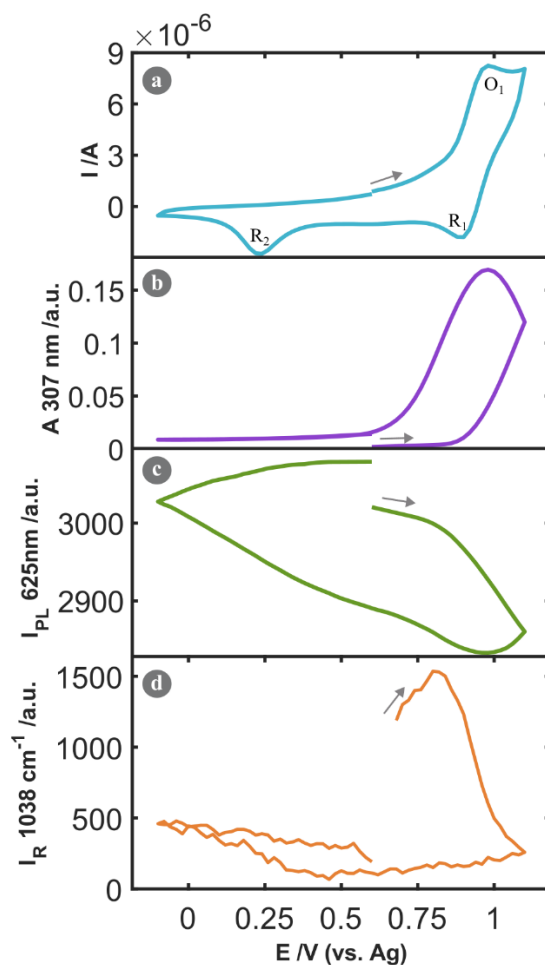


Figure 6-35. SEAS experiment of $1 \cdot 10^{-3}$ M $\text{Ru}(\text{bpy})_3^{2+}$ in 0.1 M KNO_3 between the vertex potentials -0.10 V and +1.10 V, starting at +0.60 V in the anodic direction at $0.02 \text{ V} \cdot \text{s}^{-1}$. (a) cyclic voltammogram (b) cyclic voltabsorptogram (CVA) at 307 nm, (c) cyclic voltafluorogram (CVF) at 625 nm and (d) cyclic voltaRamanogram (CVR) at 1038 cm^{-1} .

With this experiment, we have been demonstrated the correct synchronization between the electrochemical and spectroscopic signals.

6.5.3.2. Application of the cell in the study of a compound of interest in the medical field.

Experiments with $\text{Ru}(\text{bpy})_3^{2+}$ allowed us to demonstrate that synchronized information from four different points of view can be achieved with this cell. Nevertheless, the main challenge is to be able to apply this cell to study different systems of interest, proposing in this case the analysis of ofloxacin (OFL), a commonly used antibiotic.

For this purpose, a $5 \cdot 10^{-4}$ M OFL solution in Britton-Robinson buffer (BR, pH = 4.25) was prepared in order to perform SEAS experiments while maintaining the above

experimental conditions. In the same manner, cyclic voltammetry was selected as electrochemical technique. In this case, changes that occur between the two vertex potentials, 0.00 V and +1.10 V, starting at +0.60 V in the cathodic direction were recorded. Two scans were registered at $0.02 \text{ V} \cdot \text{s}^{-1}$ and the spectrum at OCP was selected as reference for UV/Vis absorption spectroscopy. All spectra were registered every 1000 ms.

In the Figure 6-36 is shown the contour plot for each spectroscopic technique. In this case, the UV/Vis absorption representation (Figure 6-36a) is characterized by a negative band at 298 nm, which corresponds to a $\pi \rightarrow \pi^*$ electronic transition.³²² The PL spectra (Figure 6-36b) show a band at 518 nm and it is accompanied by a little shoulder at 425 nm.³⁷⁶ Lastly, the evolution of Raman spectra (Figure 6-36c) is dominated by two peaks at 1397 and 1618 cm^{-1} . The first one is due to the symmetric stretching mode of COO^- , and the second one is caused by the CC tensile vibration of the quinolone aromatic ring.³⁷⁷

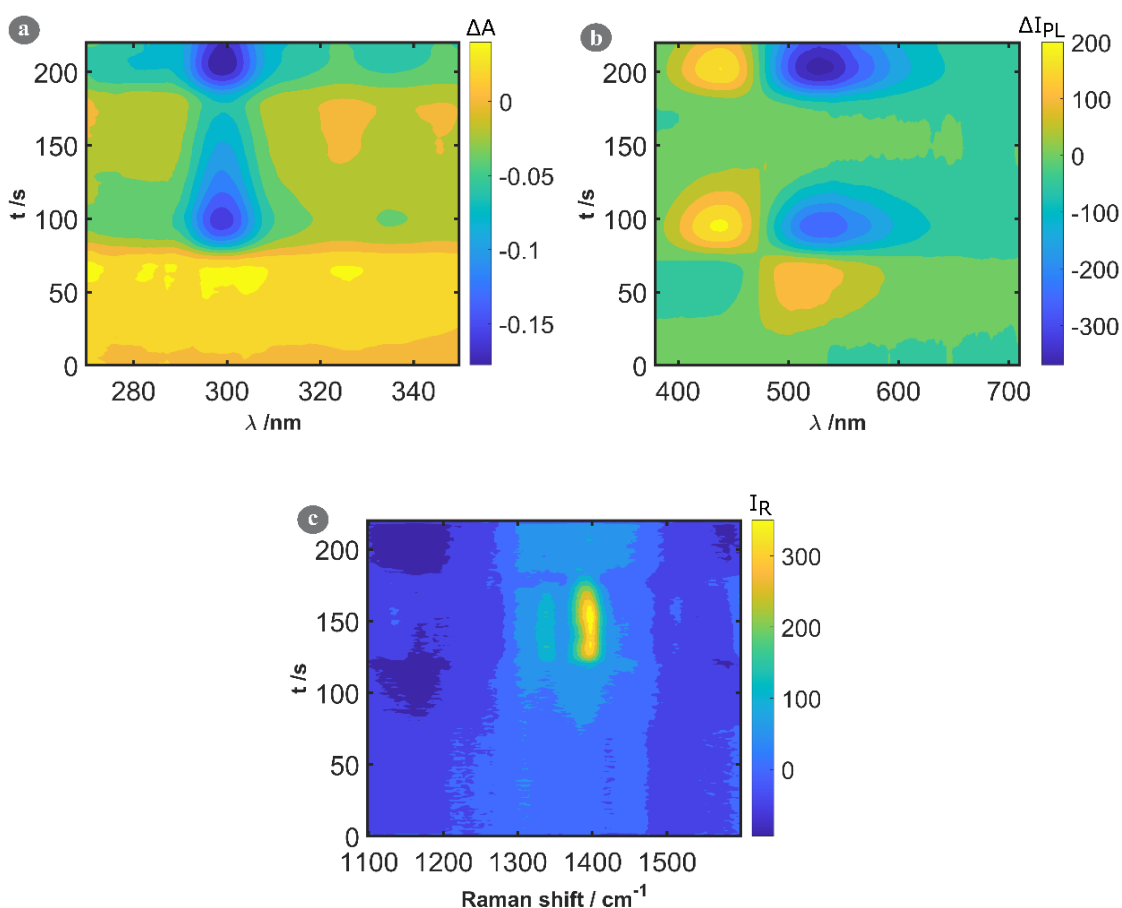


Figure 6-36. Spectra evolution of $1 \cdot 10^{-3} \text{ M}$ OFL in BR at $0.02 \text{ V} \cdot \text{s}^{-1}$ between 0.00 V and +1.10 V, starting at +0.60 V in the cathodic direction. 2 cycles are represented. (a) UV/Vis, (b) PL and (c) Raman.

Figure 6-37a shows the corresponding CV, where during the first cathodic scan no reduction peak is observed. When the potential is reversed in the anodic direction a first oxidation peak (O_1) at +0.90 V due to the oxidation of the Au-NPs and OFL is observed. The OFL is irreversibly oxidized, as was demonstrated in previous works (Section 6.3).³²² With regard to the second cycle, a cathodic peak (R_2) is observed at +0.28 V, corresponding to the reduction of Au-NPs, oxidized at potentials above +0.95 V. During the second anodic scan the potential at which OFL and Au-NPs is oxidized (O_2) is shifted to higher potentials, peaking at +0.94 V.

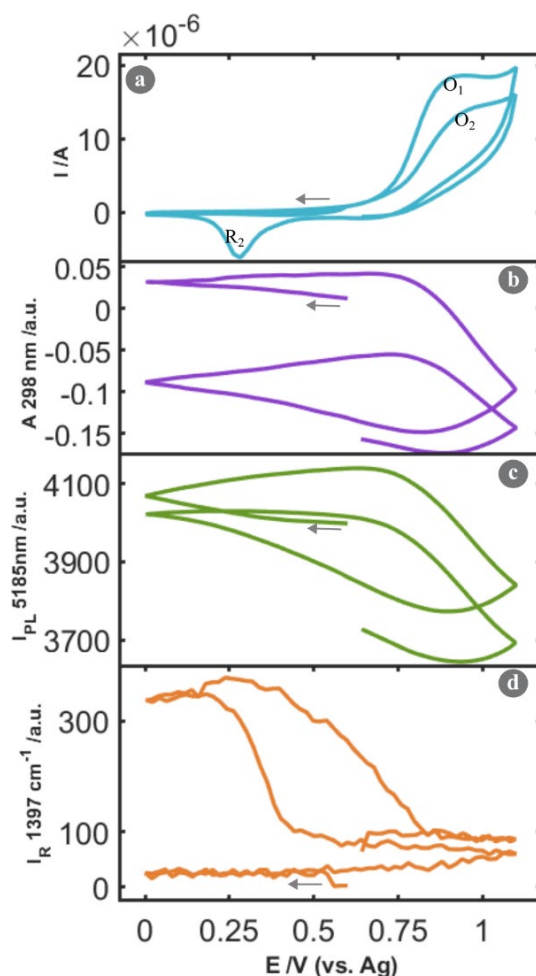


Figure 6-37. SEC experiment of $5 \cdot 10^{-3}$ M OFL in BR between the vertex potentials 0.00 V and +1.10 V, starting at +0.60 V in the cathodic direction at $0.02 \text{ V} \cdot \text{s}^{-1}$. (a) cyclic voltammogram (b) cyclic voltabsorptogram at 298 nm, (c) cyclic voltafluorogram at 518 nm and (d) cyclic voltaRamangram at 1397 cm^{-1} .

The cyclic voltammogram (CVA) at 298 nm (Figure 6-37b) and the cyclic voltafluorogram (CVF) at 518 nm (Figure 6-37c) show the same behavior; as it is expected, in the first potential, when the oxidation onset potential (+0.75 V) is reached,

the absorbance and the fluorescence decrease due to the consumption and irreversible oxidation of OFL. In the second cathodic scan, when the potential of +0.75 V is reached both signals (CVA at 298 nm and CVF at 518 nm) increase, due to the diffusion of OFL from the bulk solution to the diffusion layer interrogated by the optical probes. Finally, in the second anodic scan, the behavior is similar to the first scan, observing a decrease of both signals during the oxidation of OFL. Lastly, the cyclic voltammogram at 1397 cm^{-1} (Figure 6-37d) shows how the Raman intensity does not increase until the Au-NPs are reduced in the second cathodic scan. So, with the Raman signal we have demonstrated that if the experiment is started towards reduction, amplification of the Raman signal does not occur. In other words, initial Au-NPs are not acting as SERS substrate because the OFL Raman spectrum is not well-defined. However, when Au-NPs are first oxidized and then reduced, the corresponding Raman spectrum evolves clearly, demonstrating that the reconstruction of Au-NPs surface plays a key role in the activation of the SERS phenomenon. This reconstruction suggests a new experimental pathway for the detection of this family of antibiotics by SERS.

Four independent signals have been obtained in a single experiment, helping us to understand the chemical processes that takes place in an electrochemical reaction, providing a full-picture of the electrode process. While the UV/Vis and PL optical signals show changes throughout the redox process of OFL, the Raman signal demonstrates that an activation of the SERS substrate is needed without prior oxidation and subsequent reduction of the Au-NPs, no substrate capable of amplifying the Raman signal is created, and therefore no $\text{Ru}(\text{bpy})_3^{2+}$ or OFL spectrum is observed up to that point.

6.5.4. Conclusions

All these improvements, which allow the coupling of electrochemistry with three spectroscopic techniques (UV/Vis, PL and Raman), facilitate the understanding of all the chemical processes taking place in an electrochemical reaction. This is a great advantage, since it is a useful starting point to develop analytical protocols for the identification and quantification of molecules of interest in multiple areas of interest.

6.6. Triple Spectroelectrochemistry. Simultaneous UV/Vis absorption in Normal and Parallel Configuration and Raman Spectroelectrochemistry.

6.6.1. Introduction

In the previous Section, the combination of three spectroscopic techniques (UV/Vis absorption, PL and Raman) to perform SEC experiments has been described, being the first time where three spectroscopic techniques are combined with electrochemistry. However, due to the great diversity of techniques, many other combinations are possible. Therefore, this Section will focus on experiments combining simultaneously UV/Vis absorption in normal configuration, UV/Vis absorption in parallel configuration and Raman scattering with electrochemistry, demonstrating the wealth of information that can be extracted from such experiments. Sampling a system by UV/Vis absorption spectroscopy in the two typical configurations, normal and parallel respect to the electrode, provides information on the changes occurring both at the electrode surface and in the diffusion layer. In addition, Raman scattering will provide very sensitive information about both the electrode surface and the analytes interacting with it, allowing to obtain vibrational information about the compounds involved in the redox process.

Thus, in this Section a cell which allows the practical implementation of this triple SEC technique will be proposed. It should be noted that this research is still in the development phase, so only the design and testing stages of the cell have been carried out experimentally. Therefore, these first stages will be described in detail below, and later, in an extra Section (Future perspectives), we will point out systems in which it could be of great interest to apply this technique.

6.6.2. Experimental Section

6.6.2.1. *Reagents and materials*

To validate the designed cell, *o*-tolidine (Aldrich) solutions in 0.5 M acetic acid (HAc, VWR Chemicals) and 1 M perchloric acid (HClO₄, Panreac) were prepared. In addition, it should be borne in mind that Raman experiments require the WE to be modified with appropriate nanostructures to detect the *o*-tolidine. For this purpose, an AuNPs dispersion

was prepared using the following reagents: gold (III) chloride (HAuCl_4 , Sigma-Aldrich), tri-Sodium citrate dihydrate (Merck) and sodium hydroxide (NaOH , Acros Organics).

All reagents were used as provided without additional purification and all solutions were prepared in high-quality ultrapure deionized water ($18.2 \text{ M}\Omega \text{ cm}$ resistivity at 25°C , Milli-Q Direct 8, Millipore). All analyses were conducted at room temperature.

6.6.2.2. *Synthesis of gold nanoparticles*

A well-known protocol has been used to synthesize Au-NPs.^{359–361} 60 nm quasi-spherical Au-NPs were chemically synthesized using a solution $2.5 \cdot 10^{-4} \text{ M}$ of HAuCl_4 , following the synthesis protocol previously described in Section 6.5.

6.6.2.3. *Electrode modification*

SERS substrates capable of amplifying the Raman signal were fabricated modifying gold screen-printed electrodes (Au-SPE, DRP-220AT, Metrohm-DropSens). Analogously to what was explained in Section 6.5, the working electrode (WE) of the SPEs has been modified with Au-NPs, keeping the same protocol described in Section 6.5.

6.6.2.4. *Instrumentation and software*

Three customized SPELEC instrument (Metrohm-DropSens) synchronized with each other and controlled by DropView SPELEC software (Metrohm-DropSens, Version: 3.4 19KZ07) were used for performing the SEC experiments that will be described in this Section. In this instance, two of the spectroscopic signals correspond to UV/Vis absorption spectroscopy and were registering using only one halogen-deuterium light. This is only feasible with a bifurcated fiber; capable of splitting the light beam coming from the light source into two light beams, allowing the system to be sampled in the two different configurations. Nevertheless, two different light sources could be used, but using a single light source, any change in the light power will be affect in the same way to the two absorption experiments. Notwithstanding the requirement for a single source, the use of two customized UV/Vis SPELEC is irreplaceable, as two spectrophotometers and one potentiostat are still necessary. In this case, the experiments in parallel configuration were recorded with a spectrometer capable of recording wavelengths from 200 nm to 1000 nm, while the spectrometer used to record spectra obtained with the normal configuration goes from 250 nm to 1000 nm. *In-situ* time-resolved Raman experiments were recorded using

a SPELEC-Raman, which includes a potentiostat, a spectrometer and a 785.18 nm laser source.

The coupling of so many techniques in such a small area as the WE of an SPE has the great disadvantage that one of the optical signals is likely to saturate the detectors of the spectrometers responsible for recording the other optical signals, i.e., that these spectrophotometers receive an excessively high number of photons at certain wavelengths. In particular, the laser source is very prone to saturate the two spectrometers that record the absorbance signals. To cope with this handicap, a band-pass filter (Ocean Optics) is placed between the bare optical fiber collecting the beam in the parallel arrangement and a commercial fiber (Ocean Optics, QP450-1-XSR), which is directly connected to the spectrometer. Moreover, a notch-filter (StopLine® Notch-filter, Semrock) is placed between the 600 μm bare optical fiber collecting the beam from the normal configuration and an optical fiber (Ocean Optics, QP600-025-VIS), which is directly connected to the second spectrometer.

6.6.2.5. *Description of the set-up*

The cell developed by our research group¹¹⁸ to perform simultaneously SEC experiments registering two spectroscopic techniques, UV/Vis absorption spectroscopy in the normal configuration and Raman spectroscopy, and the electrochemical signal (Figure 6-38) has been modified to include a third spectroscopic signal corresponding to UV/Vis absorption spectroscopy in parallel configuration.

The cell consists of two main parts: a lower piece with four magnets at the corners and a recess to fit the SPE; and an upper piece with two magnets located at the top corners, which facilitates its assembly with the lower part. This upper piece has a semicircle cutout in the middle that allows to introduce the Raman probe. Two 600 μm bare optical fibers are attached to this second piece in order to record the spectroscopic signal corresponding to the normal arrangement. These fibers are placed in a side configuration with a near 90° angle between them.

The lower piece has been modified to perform simultaneously the measurements in parallel configuration. As a preliminary step, two 100 μm optical fibers have been directly attached to the WE. As might be expected, the attachment of the optical fibers to the WE has advantages and disadvantages. On the one hand, it is worth noting that we start from the basis of an easy-to-implement assembly, since it has already been used by our research

group,¹¹⁸ with the novelty of adding two optical fibers aligned on the WE. The alignment of the optical fibers directly on the WE has also already been tested in simple UV/Vis SEC experiments.²⁸³ Relying on these facts, the joint implementation is expected to provide good results. On the other hand, the alignment and fixation of the optical fiber on the WE have some limitations, especially when performing Raman experiments in which the WE is modified to create a SERS substrate, where the use of an SPE is mandatory for each measurement. In this regard, the proposed design is only of great applicability for the study of systems from a qualitative point of view, in which reproducibility is not a requirement.

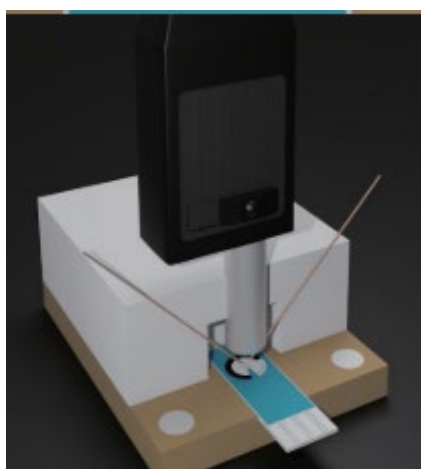


Figure 6-38. Schematic of the cell reported in literature and used for UV/Vis-Raman SEC. *Reproduced with permission from ref.¹¹⁸ Copyright 2022 Nano Research.*

Consequently, the following sub-Section will be dedicated to the validation of the cell performing simultaneously UV/Vis SEC in both arrangements together with Raman SEC using a typical SEC test molecule.

6.6.3. Results and discussion

6.6.3.1. Validation of the cell

A SEC system reference, such *o*-tolidine, was selected to validate this cell. For this purpose, a $5 \cdot 10^{-5}$ M *o*-tolidine solution in 0.5 M HAc and in 1 M HClO₄ was prepared and measured using a Au-SPE modified with Au-NPs. Cyclic voltammetry (CV) was selected as the electrochemistry technique, recording the cyclic voltammograms at $0.05 \text{ V} \cdot \text{s}^{-1}$ between +0.20 V and +0.40 V, starting at +0.20 V in the anodic direction. Simultaneously, spectroscopic information is recorded. It should be noted, that the

integration time for the acquisition of Raman spectra is 1000 ms and 500 ms for both types of UV/Vis absorption spectra. In addition, the spectrum at the OCP has been used as reference spectrum for absorbance measurements.

Figure 6-39 shows the three optical responses obtained during the experiment. Both UV/Vis spectra (Figure 6-39a and Figure 6-39b) are characterized by a positive absorption band peaking at 437 nm due to the electrogenerated oxidation product, *o*-tolidinium cation.⁶⁷ The main difference between them lies in the absorbance values reached. Higher absorbance values are registered when the sample is tested in a direction parallel to the WE surface, which is due to the larger optical pathway. Regarding the Raman spectrum (Figure 6-39c), several peaks can be observed, which are consistent with the classical reported *o*-tolidine spectra.³⁷⁸

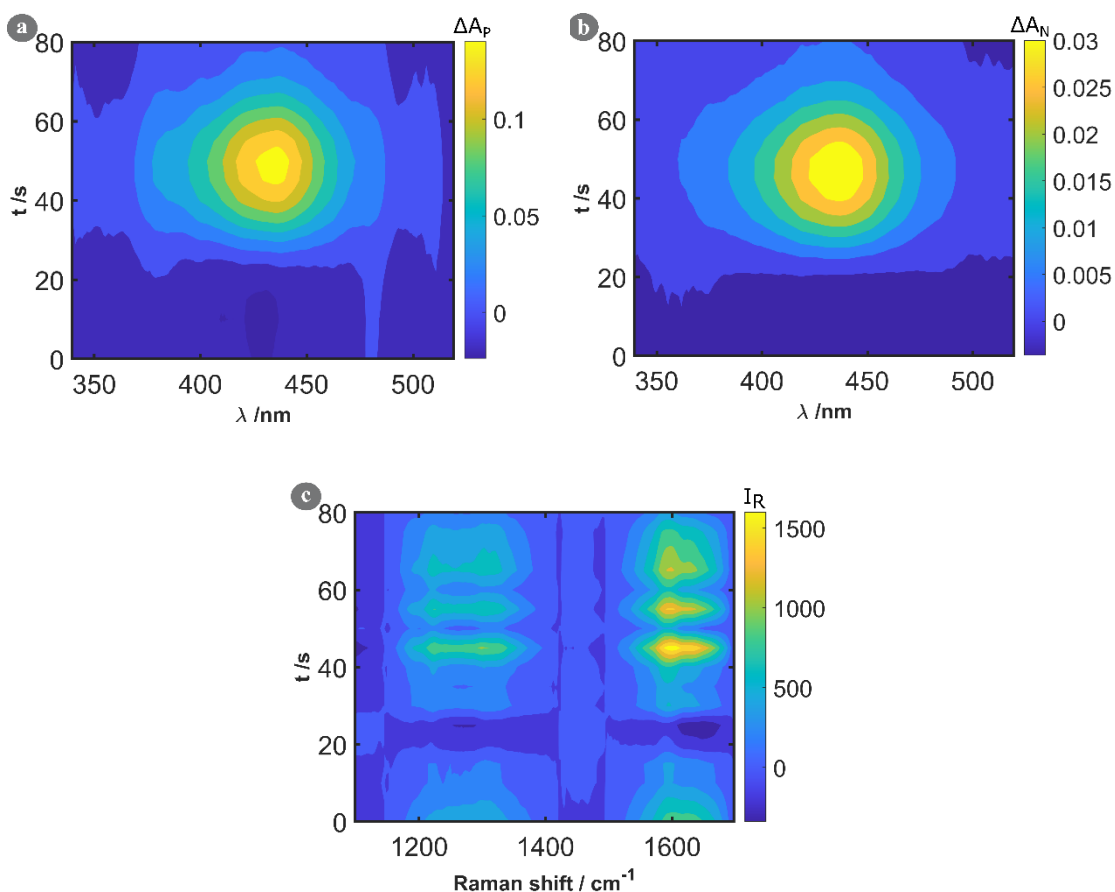


Figure 6-39. Spectra evolution experiments of $5 \cdot 10^{-5}$ M *o*-tolidine in 0.5 M HAc and in 1 M HClO₄ between +0.20 V and +0.40 V at $0.05 \text{ V} \cdot \text{s}^{-1}$. (a) Parallel UV/Vis, (b) Normal UV/Vis and (c) Raman signals.

Figure 6-40 shows the electrochemical signal and the evolution of the most characteristic band of each spectroscopic signal with respect the potential/time. Figure 6-40a depicts

the CV, where an anodic peak, that reaches its maximum at +0.35 V, and a cathodic peak around +0.30 V are observed, indicating that this is a reversible oxidation-reduction process in which two electrons and two protons are exchanged. Figure 6-40b and Figure 6-40c show the cyclic voltabsorptograms (CVA) at 437 nm, in which the absorbance increases from +0.30 V onwards in the anodic scan due to the *o*-tolidine oxidation and the electrogeneration of *o*-tolidinium cation, and the absorbance decreases from +0.35 V onwards in the backward scan due to the reduction of *o*-tolidinium cation to *o*-tolidine.

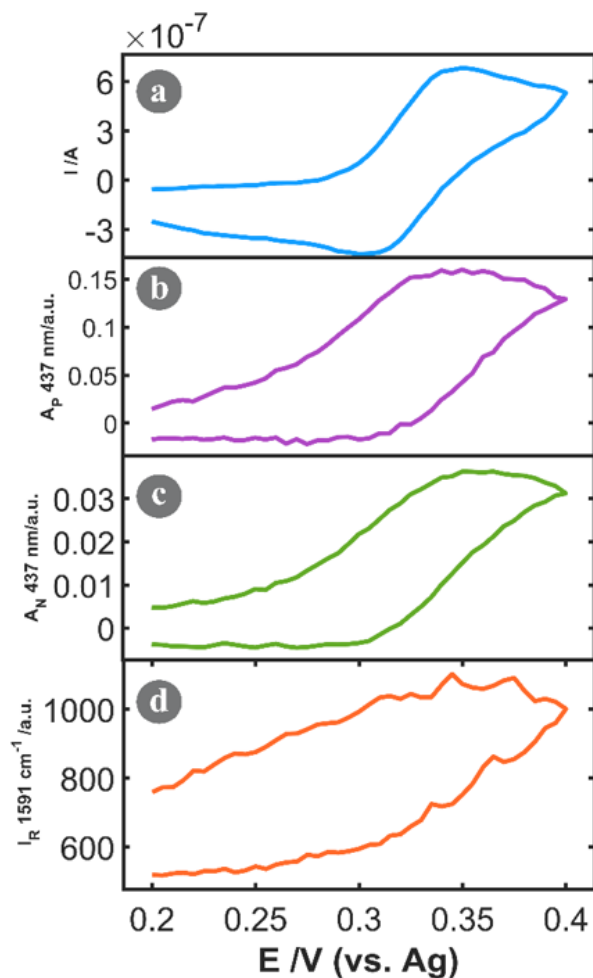


Figure 6-40. SEC experiment of $5 \cdot 10^{-5}$ M *o*-tolidine in 0.5 M HAc and in 1 M HClO₄ between +0.20 V and +0.40 V at $0.05 \text{ V} \cdot \text{s}^{-1}$. (a) cyclic voltammogram, (b) parallel CVA at 437 nm, (c) normal CVA at 437 nm and (d) VoltaRamogram at 1591 cm^{-1} .

Lastly, the evolution of the Raman intensity at 1591 cm^{-1} during experiment is represented in the so-called cyclic voltaRamogram³⁷⁹ (Figure 6-40d), which follows a pattern similar to the cyclic voltabsorptograms shown in Figure 6-40b and Figure 6-40c. The increase in the Raman intensity (from +0.30 V onwards) coincides with the oxidation of *o*-tolidine

and lasts until the potential of +0.35 V in the backward scan is reached, at which time a decay of Raman intensity occurs due to the reduction of *o*-tolidinium cation.

In summary, the electrogeneration of *o*-tolidinium cation by oxidation of *o*-tolidine leads to an increase in both absorbance and Raman intensity. When this product is reduced, the optical responses decrease due to its disappearance in the diffusion layer. This demonstrates that both the oxidation and reduction peaks observed in the CV are due to the reversible oxidation of *o*-tolidine.

As proof of concept, the correct synchronization and correlation between the electrochemical and spectroscopic signals has been demonstrated. Furthermore, the same experiment has been repeated on different modified Au-SPE, corroborating the same behavior in all cases.

It should be noted that this has been raised as a departure point for future research, and that the reproducibility of this experiment cannot be evaluated, since the fibers must be fixed for each experiment on a different electrode, not being able to preserve a constant optical pathway. In other words, considering the features of the cell proposed here, its use in quantitative analysis is inadvisable, since maintaining the optical pathway between measurements is an arduous task.

To circumvent the problem arising from the parallel configuration, an additional piece made of PMMA has been recently proposed. This piece has been designed with the final purpose of being added, in a simple way, to the set-up described in Section 6.6.2.5. This piece contains two metal nuts and two pillars of 2.6 mm diameter, that are kept attached to the rest of the part by a little recess. The metal nuts allow the easy assembly with the lower part, and the two optical fibers are aligned with nail polish on the two pillars.

6.6.4. Conclusions and future perspectives

To sum up, so far, a new design to perform simultaneously UV/Vis absorption SEC in both configurations and Raman SEC has been proposed. In this case, the last breakthrough which consists in aligning the fibers directly on the PMMA piece has not been tested, unlike the preceding design which has been assessed with *o*-tolidine. In any case, it is intended to apply this design to the study of systems of interest. As a starting point, it is envisaged to characterize silver hexacyanoferrate (II) substrates.

7. FINAL CONCLUSIONS

The main conclusions drawn from the results of this Thesis are presented below:

1. The capability of UV/Vis absorption SEC for quantitative analysis has been demonstrated. This technique cannot be as sensitive as electrochemical techniques but it is a very robust technique which allows to obtain reliable results in complex matrices and in presence of interferents.
2. A correct selection of the optical configuration is necessary to obtain reliable results in UV/Vis absorption SEC. For this purpose, a study of epinephrine in presence of ascorbic acid was performed. BSEC experiments demonstrated that the appropriate optical arrangement to carry out this determination is the parallel arrangement. Using this configuration, the determination of epinephrine in the presence of ascorbic acid has been successfully performed.
3. UV/Vis SEC has been applied to quantify the amount of folic acid in a pharmaceutical sample (Yodocefol®), demonstrating that interfering compounds in this complex matrix do not affect the quantification. Although UV/Vis SEC provides simultaneously the electrochemical and the spectroscopic signal, the determination is only possible with the spectroscopic data obtained during the oxidation of the folic acid.
4. Different SEC cells have been developed for analytical purposes, demonstrating that a good cell design is fundamental to obtain reproducible results. A new SEC cell working in semi-infinite diffusion and parallel configuration has been developed and used to quantify ofloxacin by parallel UV/Vis SEC in a urine matrix, where uric acid is a highly interfering compound. Nevertheless, PARAFAC, a very powerful multivariate chemometric tool, allows us to deconvolve the contribution of both compounds, leading to a versatile and reliable method to quantify ofloxacin in urine without the need for any pretreatment.
5. The trilinear character of SEC data is very useful to solve complex mixtures allowing the determination of the different components of the sample. Dopamine, ascorbic acid and uric acid have been simultaneously quantified in mixture samples by combining derivative UV/Vis SEC with PARAFAC. In addition, thin-layer experiments in parallel configuration have also been crucial in this quantification, since the diffusion layer limitation and the long-optical path-way allow improving the sensitivity of the method

6. The combination of SEC techniques provides a full-picture of the processes taking place in the electrode/solution interface. A new SEC cell to perform UV/Vis, PL and Raman SEC simultaneously has been designed, developed and tested with a reference system. Furthermore, this cell has been applied to the study of oxidation of ofloxacin, demonstrating that obtaining four signals in the same experiment can be very helpful, both to study complex systems and to perform analysis with the different signals, validating the obtained results.
7. Firsts steps to perform double UV/Vis absorption and Raman SEC have been established. Future improvements are in mind that will allow this technique to be applied to the study of systems of interest.

8. FUTURE PERSPECTIVES

A Thesis is not an endpoint but the beginning of new challenges. Once demonstrated the capabilities of SEC for analysis, the next stage should be related to the development of the automatization of this type of measurements. In this Thesis, a number of different cells have been developed which most of them could be coupled with auto-sampling instrumentation. In this way, repetitive measurements could be performed to use this powerful analytical technique in routine analysis. Another alternative could be the use of SEC flow cells. The good performance of SEC based on optical fibers could be combined with flow cells or, even, microfluidic devices to develop lab-on-a-chip analysis, creating much powerful devices.

Another starting point in this Thesis is related to the combination of different spectroscopies in a single experiment. This multi-technique approach has been shown as a proof of concept in this work. Undoubtedly, this type of combinations can be applied in several fields of chemistry and biochemistry. In close proximity to the research carried out in our labs, the use of SEC has been demonstrated to be very useful to understand the generation of enhancing Raman substrates, and a lot of work in this field should be done in the near future. Also, applying this combination of techniques in the field of electrocatalysis is very attractive from different points of view. The first one is related to the detection of reactants, products and/or intermediates in the electrocatalytic reaction. The second one, but non-less important, is the operando analysis of the evolution of the catalyzers during these important processes. It is well-known that much of the catalyzers, usually nanostructures, evolves during the electrocatalytic reactions. Therefore, obtaining a full picture of the processes taking place on the modified electrode should provide very valuable information to improve the yield of the processes and the quality of the catalyzers.

Moreover, this combination of techniques should be useful in many other fields of chemistry, like the understanding of chemical reactions coupled with the electrochemical processes or the understanding of many biological processes, especially, those related to biosensors.

Therefore, I hope to see a growing interest on Spectroelectrochemistry in the next years.

9. REFERENCES

- (1) Mantell, J.; Zaromb, S. Inert Electrode Behavior of Tin Oxide-Coated Glass on Repeated Plating-Depositing Cycling in Concentrated NaI-AgI Solutions. *J. Electrochem. Soc.* **1962**, *109* (10), 992–993. <https://doi.org/10.1149/1.2425221>.
- (2) Cooper, W. A Transparent Semiconducting Indicator Electrode. *Nature* **1962**, *194* (4828), 569–570. <https://doi.org/10.1038/194569b0>.
- (3) Kuwana, T.; Darlington, R. K.; Leedy, D. W. Electrochemical Studies Using Conducting Glass Indicator Electrodes. *Anal. Chem.* **1964**, *36* (10), 2023–2025. <https://doi.org/10.1021/ac60216a003>.
- (4) Kuwana, T.; Epstein, B.; Seo, E. T. Electrochemical Generation of Solution Luminiscence. *J. Phys. Chem.* **1963**, *67* (10), 2243–2244. <https://doi.org/10.1021/j100804a516>.
- (5) Lopez-Palacios, J. Avances En Espectroelectroquímica. *Abstr. book. XXVI Reun. Bien. Grup. Electroquímica la RSEQ - VII Iber. Meet. Electrochem.* **2004**.
- (6) Gonzalez-Dieguez, N.; Colina, A.; Lopez-Palacios, J.; Heras, A. Spectroelectrochemistry at Screen-Printed Electrodes: Determination of Dopamine. *Anal. Chem.* **2012**, *84* (21), 9146–9153. <https://doi.org/10.1021/ac3018444>.
- (7) Garoz-Ruiz, J.; Perales-Rondon, J. V. J. V. J. V. J. V.; Heras, A.; Colina, A.; Garoz-Ruiz, J.; Perales-Rondon, J. V.; Heras, A.; Colina, A.; Garoz-Ruiz, J.; Perales-Rondon, J. V. J. V. J. V.; Heras, A.; Colina, A. Spectroelectrochemical Sensing: Current Trends and Challenges. *Electroanalysis* **2019**, *31* (7), 1254–1278. <https://doi.org/10.1002/elan.201900075>.
- (8) Zhai, Y.; Zhu, Z.; Zhou, S.; Zhu, C.; Dong, S. Recent Advances in Spectroelectrochemistry. *Nanoscale* **2018**, *10* (7), 3089–3111. <https://doi.org/10.1039/c7nr07803j>.
- (9) Belding, S. R.; Limon-Petersen, J. G.; Dickinson, E. J. F. F.; Compton, R. G.; Limon-Petersen, J. G.; Dickinson, E. J. F. F.; Compton, R. G. Cyclic Voltammetry in the Absence of Excess Supporting Electrolyte Offers Extra Kinetic and Mechanistic Insights: Comproportionation of Anthraquinone and the Anthraquinone Dianion in Acetonitrile. *Angew. Chemie - Int. Ed.* **2010**, *49* (48), 9242–9245. <https://doi.org/10.1002/anie.201004874>.
- (10) O'Mullane, A. P. *Electrochemistry*; Elsevier, 2013. <https://doi.org/10.1016/B978-0-12-409547-2.05344-0>.
- (11) Zhu, X.; Shi, L. *Electrochemistry*; Elsevier Inc., 2018; Vol. 1. <https://doi.org/10.1016/B978-0-12-815053-5.00009-X>.

- (12) Rackus, D. G.; Shamsi, M. H.; Wheeler, A. R. Electrochemistry, Biosensors and Microfluidics: A Convergence of Fields. *Chem. Soc. Rev.* **2015**, *44* (15), 5320–5340. <https://doi.org/10.1039/c4cs00369a>.
- (13) Lines, A. M.; Wang, Z.; Clark, S. B.; Bryan, S. A. Electrochemistry and Spectroelectrochemistry of Luminescent Europium Complexes. *Electroanalysis* **2016**, *28* (9), 2109–2117. <https://doi.org/10.1002/elan.201600034>.
- (14) Kaim, W.; Schwederski, B.; Dogan, A.; Fiedler, J.; Kuehl, C. J.; Stang, P. J. Metalla-Supramolecular Rectangles as Electron Reservoirs for Multielectron Reduction and Oxidation. *Inorg. Chem.* **2002**, *41* (15), 4025–4028. <https://doi.org/10.1021/ic020122n>.
- (15) Izquierdo, D.; Ferraresi-Curotto, V.; Heras, A.; Pis-Diez, R.; Gonzalez-Baro, A. C. C.; Colina, A. Bidimensional Spectroelectrochemistry: Application of a New Device in the Study of a *o*-Vanillin-Copper(II) Complex. *Electrochim. Acta* **2017**, *245*, 79–87. <https://doi.org/10.1016/j.electacta.2017.05.105>.
- (16) Dendisová, M.; Havránek, L.; Ončák, M.; Matějka, P. In Situ SERS Study of Azobenzene Derivative Formation from 4-Aminobenzenethiol on Gold, Silver, and Copper Nanostructured Surfaces: What Is the Role of Applied Potential and Used Metal? *J. Phys. Chem. C* **2013**, *117*, 21245–21253. <https://doi.org/10.1021/jp4040985>.
- (17) Liang, Z.; Kang, M.; Payne, G. F.; Wang, X.; Sun, R. Probing Energy and Electron Transfer Mechanisms in Fluorescence Quenching of Biomass Carbon Quantum Dots. *ACS Appl. Mater. Interfaces* **2016**, *8*, 17478–17488. <https://doi.org/10.1021/acsami.6b04826>.
- (18) Pigani, L.; Heras, A.; Colina, Á.; Seeber, R.; López-Palacios, J. Electropolymerisation of 3,4-Ethylenedioxythiophene in Aqueous Solutions. *Electrochem. commun.* **2004**, *6* (11), 1192–1198. <https://doi.org/10.1016/j.elecom.2004.09.021>.
- (19) Ledwon, P.; Lapkowski, M. The Role of Electrochemical and Spectroelectrochemical Techniques in the Preparation and Characterization of Conjugated Polymers: From Polyaniline to Modern Organic Semiconductors. *Polymers (Basel)*. **2022**, *14*, 4173. <https://doi.org/10.3390/polym14194173>.
- (20) Ibañez, D.; Romero, E. C.; Heras, A.; Colina, A. Dynamic Raman Spectroelectrochemistry of Single Walled Carbon Nanotubes Modified Electrodes Using a Langmuir-Schaefer Method. *Electrochim. Acta* **2014**, *129*, 171–176. <https://doi.org/10.1016/j.electacta.2014.02.094>.
- (21) Shiraishi, T.; Shiraki, T.; Nakashima, N. Substituent Effects on the Redox States of Locally Functionalized Single-Walled Carbon Nanotubes Revealed by in Situ Photoluminescence

- Spectroelectrochemistry. *Nanoscale* **2017**, *9* (43), 16900–16907. <https://doi.org/10.1039/c7nr05480g>.
- (22) Martínez-Periñán, E.; Bravo, I.; Mediavilla, M.; Revenga-Parra, M.; Mateo-Martí, E.; Pariente, F.; Lorenzo, E. Spectroelectrochemical Operando Method for Monitoring a Phenothiazine Electrografting Process on Amide Functionalized C-Nanodots/Au Hybrid Electrodes. *Electrochim. Acta* **2019**, *298*, 950–959. <https://doi.org/10.1016/j.electacta.2018.12.106>.
- (23) Wang, B.; Likodimos, V.; Fielding, A. J.; Dryfe, R. A. W. In Situ Electron Paramagnetic Resonance Spectroelectrochemical Study of Graphene-Based Supercapacitors: Comparison between Chemically Reduced Graphene Oxide and Nitrogen-Doped Reduced Graphene Oxide. *Carbon N. Y.* **2020**, *160*, 236–246. <https://doi.org/10.1016/j.carbon.2019.12.045>.
- (24) Nunes, W. G.; Pires, B. M.; Thaines, E. H. N. S.; Pereira, G. M. A.; da Silva, L. M.; Freitas, R. G.; Zanin, H. Operando Raman Spectroelectrochemical Study of Polyaniline Degradation: A Joint Experimental and Theoretical Analysis. *J. Energy Storage* **2022**, *55*, 105770. <https://doi.org/10.1016/j.est.2022.105770>.
- (25) Cave, E. A.; Olson, J. Z.; Schlenker, C. W. Ion-Pairing Dynamics Revealed by Kinetically Resolved in Situ FTIR Spectroelectrochemistry during Lithium-Ion Storage. *ACS Appl. Mater. Interfaces* **2021**, *13* (41), 48546–48554. <https://doi.org/10.1021/acsami.1c11964>.
- (26) Gümrükçü Köse, G.; Karaoğlu, G. K.; Erdağ Maden, Y.; Koca, A. Novel Silicon Phthalocyanine Photosensitizers Containing Carboxylic Acid Based Axial Anchoring Groups: Electrochemistry, Spectroelectrochemistry, and Dye Sensitized Solar Cell Performance. *Dye. Pigment.* **2022**, *207* (110686). <https://doi.org/10.1016/j.dyepig.2022.110686>.
- (27) D'Amario, L.; Jiang, R.; Cappel, U. B.; Gibson, E. A.; Boschloo, G.; Rensmo, H.; Sun, L.; Hammarström, L.; Tian, H. Chemical and Physical Reduction of High Valence Ni States in Mesoporous Nio Film for Solar Cell Application. *ACS Appl. Mater. Interfaces* **2017**, *9* (39), 33470–33477. <https://doi.org/10.1021/acsami.7b01532>.
- (28) Kumar, R.; Pillai, R. G.; Pekas, N.; Wu, Y.; McCreery, R. L. Spatially Resolved Raman Spectroelectrochemistry of Solid-State Polythiophene/Viologen Memory Devices. *J. Am. Chem. Soc.* **2012**, *134* (36), 14869–14876. <https://doi.org/10.1021/ja304458s>.
- (29) Fernandez-Blanco, C.; Heras, A.; Ruiz, V.; Colina, A. Spectroelectrochemical Synthesis of Gold Nanoparticles Using Cyclic Voltammetry in the Presence of a Protective Agent.

- RSC Adv.* **2014**, *4* (85), 45168–45173. <https://doi.org/10.1039/c4ra07451c>.
- (30) Fernández-Blanco, C.; Colina, Á.; Heras, A. UV/Vis Spectroelectrochemistry as a Tool for Monitoring the Fabrication of Sensors Based on Silver Nanoparticle Modified Electrodes. *Sensors* **2013**, *13* (5), 5700–5711. <https://doi.org/10.3390/s130505700>.
- (31) Fernández-Blanco, C.; Colina, A.; Heras, A.; Ruiz, V.; López-Palacios, J. Multipulse Strategies for the Electrosynthesis of Gold Nanoparticles Studied by UV/Vis Spectroelectrochemistry. *Electrochem. commun.* **2012**, *18*, 8–11. <https://doi.org/10.1016/j.elecom.2012.01.020>.
- (32) Takashima, T.; Hashimoto, K.; Nakamura, R. Mechanisms of pH-Dependent Activity for Water Oxidation to Molecular Oxygen by MnO₂ Electrocatalysts. *J. Am. Chem. Soc.* **2012**, *134* (3), 1519–1527. <https://doi.org/10.1021/ja206511w>.
- (33) Bozal-Ginesta, C.; Rao, R. R.; Mesa, C. A.; Liu, X.; Hillman, S. A. J.; Stephens, I. E. L.; Durrant, J. R. Redox-State Kinetics in Water-Oxidation IrO_x Electrocatalysts Measured by Operando Spectroelectrochemistry. *ACS Catal.* **2021**, *11* (24), 15013–15025. <https://doi.org/10.1021/acscatal.1c03290>.
- (34) Mesa, C. A.; Pastor, E.; Francàs, L. UV–Vis Operando Spectroelectrochemistry for (Photo)Electrocatalysis: Principles and Guidelines. *Curr. Opin. Electrochem.* **2022**, *35*, 101098. <https://doi.org/10.1016/j.coelec.2022.101098>.
- (35) Wahl, S.; El-Refaei, S. M.; Amsalem, P.; Buzanich, A. G.; Koch, N.; Pinna, N. Operando Diffuse Reflectance UV-Vis Spectroelectrochemistry for Investigating Oxygen Evolution Electrocatalysts. *Catal. Sci. Technol.* **2020**, *10* (2), 517–528. <https://doi.org/10.1039/c9cy02329a>.
- (36) Foo Lee, Y.; Jon, R. K. Absorption and Luminescence Spectroelectrochemical Characterization of a Highly Luminescent Rhenium(II) Complex. *J. Am. Chem. Soc.* **1994**, *116* (8), 3599–3600. <https://doi.org/10.1021/ja00087a056>.
- (37) López, I.; Le Poul, N. Low-Temperature Electrochemistry and Spectroelectrochemistry for Coordination Compounds. *Coord. Chem. Rev.* **2021**, *436*, 213823. <https://doi.org/10.1016/j.ccr.2021.213823>.
- (38) Taylor, J. O.; Pižl, M.; Klotz, M.; Rebarz, M.; McCusker, C. E.; McCusker, J. K.; Zálaiš, S.; Hartl, F.; Vlček, A. Optical and Infrared Spectroelectrochemical Studies of CN-Substituted Bipyridyl Complexes of Ruthenium(II). *Inorg. Chem.* **2021**, *60* (6), 3514–3523. <https://doi.org/10.1021/acs.inorgchem.0c03579>.
- (39) Kagilev, A. A.; Gafurov, Z. N.; Kantukov, A. O.; Mikhailov, I. K.; Yakhvarov, D. G. The

- Power of in Situ Spectroelectrochemistry for Redox Study of Organometallic and Coordination Compounds. *J. Solid State Electrochem.* **2024**, 28 (3–4), 897–910. <https://doi.org/10.1007/s10008-023-05765-7>.
- (40) Chen, W.; Liu, X.-Y. Y.; Qian, C.; Song, X.-N. N.; Li, W.-W. W.; Yu, H.-Q. Q. An UV-Vis Spectroelectrochemical Approach for Rapid Detection of Phenazines and Exploration of Their Redox Characteristics. *Biosens. Bioelectron.* **2015**, 64, 25–29. <https://doi.org/10.1016/j.bios.2014.08.032>.
- (41) Prado, T. M. d.; Cincotto, F. H.; Machado, S. A. S. Spectroelectrochemical Study of Acetylsalicylic Acid in Neutral Medium and Its Quantification in Clinical and Environmental Samples. *Electrochim. Acta* **2017**, 233, 105–112. <https://doi.org/10.1016/j.electacta.2017.03.038>.
- (42) Garoz-Ruiz, J.; Heras, A.; Colina, A. Direct Determination of Ascorbic Acid in a Grapefruit: Paving the Way for in Vivo Spectroelectrochemistry. *Anal. Chem.* **2017**, 89 (3), 1815–1822. <https://doi.org/10.1021/acs.analchem.6b04155>.
- (43) Hernandez, S.; Perales-Rondon, J. V.; Heras, A.; Colina, A. Determination of Uric Acid in Synthetic Urine by Using Electrochemical Surface Oxidation Enhanced Raman Scattering. *Anal. Chim. Acta* **2019**, 1085, 61–67. <https://doi.org/10.1016/j.aca.2019.07.057>.
- (44) Martín-Yerga, D.; Pérez-Junquera, A.; Hernández-Santos, D.; Fanjul-Bolado, P. Time-Resolved Luminescence Spectroelectrochemistry at Screen-Printed Electrodes: Following the Redox-Dependent Fluorescence of $[\text{Ru}(\text{Bpy})_3]^{2+}$. *Anal. Chem.* **2017**, 89 (20), 10649–10654. <https://doi.org/10.1021/acs.analchem.7b01734>.
- (45) Bumbrah, G. S.; Sharma, R. M. Raman Spectroscopy – Basic Principle, Instrumentation and Selected Applications for the Characterization of Drugs of Abuse. In *Egyptian Journal of Forensic Sciences*; The International Association of Law and Forensic Sciences (IALFS), 2016; Vol. 6, pp 209–215. <https://doi.org/10.1016/j.ejfs.2015.06.001>.
- (46) Bussy, U.; Giraudeau, P.; Silvestre, V.; Jaunet-Lahary, T.; Ferchaud-Roucher, V.; Krempf, M.; Akoka, S.; Tea, I.; Boujtita, M. In Situ NMR Spectroelectrochemistry for the Structure Elucidation of Unstable Intermediate Metabolites. *Anal. Bioanal. Chem.* **2013**, 405 (17), 5817–5824. <https://doi.org/10.1007/s00216-013-6977-z>.
- (47) Klod, S.; Ziegls, F.; Dunsch, L. In Situ NMR Spectroelectrochemistry of Higher Sensitivity by Large Scale Electrodes. *Anal. Chem.* **2009**, 81 (24), 10262–10267. <https://doi.org/10.1021/ac901641m>.

- (48) Long, Y. T.; Yu, Z. H.; Chen, H. Y. Determination of Coenzyme Q10 by in Situ EPR Spectroelectrochemistry. *Electrochem. commun.* **1999**, *1* (5), 194–196. [https://doi.org/10.1016/S1388-2481\(99\)00032-6](https://doi.org/10.1016/S1388-2481(99)00032-6).
- (49) Tarábek, J.; Kavan, L.; Kalbáč, M.; Raptá, P.; Zukalová, M.; Dunsch, L. In Situ EPR Spectroelectrochemistry of Single-Walled Carbon Nanotubes and C60 Fullerene Peapods. *Carbon N. Y.* **2006**, *44* (11), 2147–2154. <https://doi.org/10.1016/j.carbon.2006.03.014>.
- (50) Czastka, K.; Oughli, A. A.; Rüdiger, O.; DeBeer, S. Enzymatic X-Ray Absorption Spectroelectrochemistry. *Faraday Discuss.* **2022**, *234*, 214–231. <https://doi.org/10.1039/D1FD00079A>.
- (51) Tesch, M. F.; Simonov, A. N. Tracking the Transient: Real-Time Exploration of Electromaterials by Dynamic Single Energy X-Ray Spectroelectrochemistry. *Curr. Opin. Electrochem.* **2022**, *35*, 101038. <https://doi.org/10.1016/j.coelec.2022.101038>.
- (52) Garoz-Ruiz, J.; Guillen-Posteguillo, C.; Heras, A.; Colina, A. Simplifying the Assessment of Parameters of Electron-Transfer Reactions by Using Easy-to-Use Thin-Layer Spectroelectrochemistry Devices. *Electrochem. commun.* **2018**, *86*, 12–16. <https://doi.org/10.1016/j.elecom.2017.11.001>.
- (53) Bkhach, S.; Alévêque, O.; Morille, Y.; Breton, T.; Hudhomme, P.; Gautier, C.; Levillain, E. Absorption Spectroelectrochemistry on Mixed Perylenediimide-Based Self-Assembled Monolayers: Non-Linear Dependence of Absorbance versus Surface Coverage. *ChemElectroChem* **2017**, *4* (3), 601–606. <https://doi.org/10.1002/celc.201600770>.
- (54) Heras, A.; Colina, A.; Ruiz, V.; López-Palacios, J. UV-Visible Spectroelectrochemical Detection of Side-Reactions in the Hexacyanoferrate(III)/(II) Electrode Process. *Electroanalysis* **2003**, *15* (8), 702–708. <https://doi.org/10.1002/elan.200390088>.
- (55) Zhangyu, Y.; Tiande, G.; Mei, Q. Long Optical Path, Single Potential Step Chronoabsorptometric Determination of Heterogeneous Electron-Transfer Kinetic Parameters of Quasi-Reversible Processes. *Anal. Chem.* **1994**, *66*, 497–502. <https://doi.org/10.1021/ac00076a013>.
- (56) Molina, A.; Laborda, E.; Gómez-Gil, J. M.; Martínez-Ortiz, F.; Compton, R. G. Analytical Solutions for the Study of Homogeneous First-Order Chemical Kinetics via UV–Vis Spectroelectrochemistry. *J. Electroanal. Chem.* **2018**, *819*, 202–213. <https://doi.org/10.1016/j.jelechem.2017.10.031>.
- (57) Colina, A.; López-Palacios, J.; Heras, A.; Ruiz, V.; Fuente, L. Digital Simulation Model for Bidimensional Spectroelectrochemistry. *J. Electroanal. Chem.* **2003**, *553*, 87–95.

- [https://doi.org/10.1016/S0022-0728\(03\)00288-2](https://doi.org/10.1016/S0022-0728(03)00288-2).
- (58) Garoz-Ruiz, J.; Heras, A.; Palmero, S.; Colina, A. Development of a Novel Bidimensional Spectroelectrochemistry Cell Using Transfer Single-Walled Carbon Nanotubes Films as Optically Transparent Electrodes. *Anal. Chem.* **2015**, *87* (12), 6233–6239. <https://doi.org/10.1021/acs.analchem.5b00923>.
 - (59) Dai, Y.; Swain, G. M.; Porter, M. D.; Zak, J. New Horizons in Spectroelectrochemical Measurements: Optically Transparent Carbon Electrodes. *Anal. Chem.* **2008**, *80* (1), 14–22. <https://doi.org/10.1021/ac085996r>.
 - (60) Kuwana, T.; Heineman, W. R. Study of Electrogenerated Reactants Using Optically Transparent Electrodes. *Acc. Chem. Res.* **1976**, *9* (7), 241–248. <https://doi.org/10.1021/ar50103a001>.
 - (61) Heineman, W. R.; Kuwana, T. Mercury-Platinum Optically Transparent Electrode. *Anal. Chem.* **1971**, *43* (8), 1075–1078. <https://doi.org/10.1021/ac60303a007>.
 - (62) Sgobba, V.; Guldi, D. M. Carbon Nanotubes—Electronic/Electrochemical Properties and Application for Nanoelectronics and Photonics. *Chem. Soc. Rev.* **2009**, *38*, 165–184. <https://doi.org/10.1039/b802652c>.
 - (63) Kavan, L.; Dunsch, L. Spectroelectrochemistry of Carbon Nanotubes. *ChemPhysChem* **2011**, *12*, 47–55. <https://doi.org/10.1002/cphc.201000882>.
 - (64) Szewczyk, J.; Aguilar-Ferrer, D.; Coy, E. Polydopamine Films: Electrochemical Growth and Sensing Applications. *Eur. Polym. J.* **2022**, *174*, 111346. <https://doi.org/10.1016/j.eurpolymj.2022.111346>.
 - (65) Ibañez, D.; Garoz-Ruiz, J.; Heras, A.; Colina, A. Simultaneous UV-Visible Absorption and Raman Spectroelectrochemistry. *Anal. Chem.* **2016**, *88* (16), 8210–8217. <https://doi.org/10.1021/acs.analchem.6b02008>.
 - (66) López-Palacios, J.; Colina, A.; Heras, A.; Ruiz, V.; Fuente, L. Bidimensional Spectroelectrochemistry. *Anal. Chem.* **2001**, *73* (13), 2883–2889. <https://doi.org/10.1021/ac0014459>.
 - (67) Orcajo, O.; Ventosa, E.; Martínez, A.; Colina, Á.; Heras, A.; Ruiz, V.; López-Palacios, J. A New Reflection–Transmission Bidimensional Spectroelectrochemistry Cell: Electrically Controlled Release of Chemicals from a Conducting Polymer. *J. Electroanal. Chem.* **2006**, *596* (2), 95–100. <https://doi.org/10.1016/j.jelechem.2006.07.026>.
 - (68) DeAngelis, T. P.; Heineman, W. R. An Electrochemical Experiment Using an Optically

- Transparent Thin Layer Electrode. *J. Chem. Educ.* **1976**, *53* (9), 594. <https://doi.org/10.1021/ed053p594>.
- (69) Heineman, W. R.; DeAngelis, T. P.; Goelz, J. F. Mercury Film Nickel Minigrid Optically Transparent Thin Layer Electrochemical Cell. *Anal. Chem.* **1975**, *47* (8), 1364–1369. <https://doi.org/10.1021/ac60358a046>.
- (70) Lin, X. Q.; Kadish, K. M. Vacuum-Tight Thin-Layer Spectroelectrochemical Cell with a Doublet Platinum Gauze Working Electrode. *Anal. Chem.* **1985**, *57* (7), 1498–1501. <https://doi.org/10.1021/ac00284a079>.
- (71) León, L.; Maraver, J. J.; Carbajo, J.; Mozo, J. D. Simple and Multi-Configurational Flow-Cell Detector for UV–Vis Spectroelectrochemical Measurements in Commercial Instruments. *Sensors Actuators B Chem.* **2013**, *186*, 263–269. <https://doi.org/10.1016/j.snb.2013.06.024>.
- (72) Perez-Estebanez, M.; Hernandez, S.; Perales-Rondon, J. V.; Gomez, E.; Heras, A.; Colina, A. Chemical Selectivity in Electrochemical Surface Oxidation Enhanced Raman Scattering. *Electrochim. Acta* **2020**, *353*, 136560. <https://doi.org/10.1016/j.electacta.2020.136560>.
- (73) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Lakowicz, J. R., Ed.; Springer US: Boston, MA, 2006; Vol. Thrid Edit. <https://doi.org/10.1007/978-0-387-46312-4>.
- (74) So, P. T.; Dong, C. Y. Fluorescence Spectrophotometry. In *Encyclopedia of Life Sciences*; Wiley, 2001; pp 187–190. <https://doi.org/10.1038/npg.els.0002978>.
- (75) Williams, R. T.; Bridges, J. W. Fluorescence of Solutions: A Review. *J. Clin. Pathol.* **1964**, *17* (4), 371–394. <https://doi.org/10.1136/jcp.17.4.371>.
- (76) Jonkman, J.; Brown, C. M.; Wright, G. D.; Anderson, K. I.; North, A. J. Tutorial: Guidance for Quantitative Confocal Microscopy. *Nat. Protoc.* **2020**, *15* (5), 1585–1611. <https://doi.org/10.1038/s41596-020-0313-9>.
- (77) Yang, Y.; Fan, S.; Webb, J. A.; Ma, Y.; Goyette, J.; Chen, X.; Gaus, K.; Tilley, R. D.; Gooding, J. J. Electrochemical Fluorescence Switching of Enhanced Green Fluorescent Protein. *Biosens. Bioelectron.* **2023**, *237*, 115467. <https://doi.org/10.1016/j.bios.2023.115467>.
- (78) Ibáñez, D.; Izquierdo-Bote, D.; Pérez-Junquera, A.; González-García, M. B.; Hernández-Santos, D.; Fanjul-Bolado, P. Raman and Fluorescence Spectroelectrochemical Monitoring of Resazurin-Resorufin Fluorogenic System. *Dye. Pigment.* **2020**, *172*, 107848. <https://doi.org/10.1016/j.dyepig.2019.107848>.

- (79) Miomandre, F.; Allain, C.; Clavier, G.; Audibert, J.-F.; Pansu, R. B.; Audebert, P.; Hartl, F. Coupling Thin Layer Electrochemistry with Epifluorescence Microscopy: An Expedient Way of Investigating Electrofluorochromism of Organic Dyes. *Electrochem. commun.* **2011**, *13* (6), 574–577. <https://doi.org/10.1016/j.elecom.2011.03.013>.
- (80) Lei, C.; Hu, D.; Ackerman, E. J. Single-Molecule Fluorescence Spectroelectrochemistry of Cresyl Violet. *Chem. Commun.* **2008**, 7345 (43), 5490–5492. <https://doi.org/10.1039/b812161c>.
- (81) Liu, X.; Hu, L.; Pan, N.; Grimaud, L.; Labbé, E.; Buriez, O.; Delacotte, J.; Lemaître, F.; Guille-Collignon, M. Coupling Electrochemistry and TIRF-Microscopy with the Fluorescent False Neurotransmitter FFN102 Supports the Fluorescence Signals during Single Vesicle Exocytosis Detection. *Biophys. Chem.* **2018**, 235, 48–55. <https://doi.org/10.1016/j.bpc.2018.02.004>.
- (82) Čížková, M.; Cattiaux, L.; Mallet, J. M.; Labbé, E.; Buriez, O. Electrochemical Switching Fluorescence Emission in Rhodamine Derivatives. *Electrochim. Acta* **2018**, *260*, 589–597. <https://doi.org/10.1016/j.electacta.2017.12.104>.
- (83) Wang, S. M.; Hwang, J.; Kim, E. Polyoxometalates as Promising Materials for Electrochromic Devices. *J. Mater. Chem. C* **2019**, *7* (26), 7828–7850. <https://doi.org/10.1039/c9tc01722d>.
- (84) Raman, C. V.; Krishnan, K. S. A New Type of Secondary Radiation [11]. *Nature* **1928**, *121* (3048), 501–502. <https://doi.org/10.1038/121501c0>.
- (85) Brosseau, C. L.; Colina, A.; Perales-Rondon, J. V.; Wilson, A. J.; Joshi, P. B.; Ren, B.; Wang, X. Electrochemical Surface-Enhanced Raman Spectroscopy. *Nat. Rev. Methods Prim.* **2023**, *3* (1), 79. <https://doi.org/10.1038/s43586-023-00263-6>.
- (86) Das, R. S.; Agrawal, Y. K. Raman Spectroscopy: Recent Advancements, Techniques and Applications. *Vib. Spectrosc.* **2011**, *57* (2), 163–176. <https://doi.org/10.1016/j.vibspec.2011.08.003>.
- (87) Koningstein, J. A.; Mortensen, O. S. Electronic Raman Spectra. III. Absolute Cross Sections for Electronic Raman and Rayleigh Scattering. *Phys. Rev.* **1968**, *168* (1), 75–78. <https://doi.org/10.1103/PhysRev.168.75>.
- (88) Maiman, T. H. Stimulated Optical Radiation in Ruby. *Nature* **1960**, *187* (4736), 493–494. <https://doi.org/10.1038/187493a0>.
- (89) Schlücker, S. Surface-Enhanced Raman Spectroscopy: Concepts and Chemical Applications. *Angew. Chemie Int. Ed.* **2014**, *53* (19), 4756–4795.

- <https://doi.org/10.1002/anie.201205748>.
- (90) Fleischmann, M.; Hendra, P. J.; McQuillan, A. J. Raman Spectra of Pyridine Adsorbed at a Silver Electrode. *Chem. Phys. Lett.* **1974**, *26* (2), 163–166. [https://doi.org/10.1016/0009-2614\(74\)85388-1](https://doi.org/10.1016/0009-2614(74)85388-1).
 - (91) Albrecht, M. G.; Creighton, J. A. Anomalous Intense Raman Spectra of Pyridine at a Silver Electrode. *J. Am. Chem. Soc.* **1977**, *99* (15), 5215–5217. <https://doi.org/10.1021/ja00457a071>.
 - (92) Langer, J.; de Aberasturi, D. J.; Aizpurua, J.; Alvarez-Puebla, R. A.; Augu  , B.; Baumberg, J. J.; Bazan, G. C.; Bell, S. E. J.; Boisen, A.; Brolo, A. G.; Choo, J.; Cialla-May, D.; Deckert, V.; Fabris, L.; Faulds, K.; Javier Garc  a de Abajo, F.; Goodacre, R.; Graham, D.; Haes, A. J.; Haynes, C. L.; Huck, C.; Itoh, T.; K  ll, M.; Kneipp, J.; Kotov, N. A.; Kuang, H.; Le Ru, E. C.; Lee, H. K.; Li, J. F.; Ling, X. Y.; Maier, S. A.; Mayerh  fer, T.; Moskovits, M.; Murakoshi, K.; Nam, J. M.; Nie, S.; Ozaki, Y.; Pastoriza-Santos, I.; Perez-Juste, J.; Popp, J.; Pucci, A.; Reich, S.; Ren, B.; Schatz, G. C.; Shegai, T.; Schl  cker, S.; Tay, L. L.; George Thomas, K.; Tian, Z. Q.; van Duyne, R. P.; Vo-Dinh, T.; Wang, Y.; Willets, K. A.; Xu, C.; Xu, H.; Xu, Y.; Yamamoto, Y. S.; Zhao, B.; Liz-Marz  n, L. M. Present and Future of Surface-Enhanced Raman Scattering. *ACS Nano* **2020**, *14* (1), 28–117. <https://doi.org/10.1021/acsnano.9b04224>.
 - (93) Zhao, L. L.; Jensen, L.; Schatz, G. C. Surface-Enhanced Raman Scattering of Pyrazine at the Junction between Two Ag 20 Nanoclusters. *Nano Lett.* **2006**, *6* (6), 1229–1234. <https://doi.org/10.1021/nl0607378>.
 - (94) Markina, N. E.; Ustinov, S. N.; Zakharevich, A. M.; Markin, A. V. Copper Nanoparticles for SERS-Based Determination of Some Cephalosporin Antibiotics in Spiked Human Urine. *Anal. Chim. Acta* **2020**, *1138*, 9–17. <https://doi.org/10.1016/j.aca.2020.09.016>.
 - (95) He, Y.; Xiao, S.; Dong, T.; Nie, P. Gold Nanoparticles with Different Particle Sizes for the Quantitative Determination of Chlorpyrifos Residues in Soil by SERS. *Int. J. Mol. Sci.* **2019**, *20* (11), 2817. <https://doi.org/10.3390/ijms20112817>.
 - (96) Luo, H.; Wang, X.; Huang, Y.; Lai, K.; Rasco, B. A.; Fan, Y. Rapid and Sensitive Surface-enhanced Raman Spectroscopy (SERS) Method Combined with Gold Nanoparticles for Determination of Paraquat in Apple Juice. *J. Sci. Food Agric.* **2018**, *98* (10), 3892–3898. <https://doi.org/10.1002/jsfa.8906>.
 - (97) Sha, M. Y.; Xu, H.; Penn, S. G.; Cromer, R. SERS Nanoparticles: A New Optical Detection Modality for Cancer Diagnosis. *Nanomedicine* **2007**, *2* (5), 725–734.

- <https://doi.org/10.2217/17435889.2.5.725>.
- (98) Lane, L. A.; Qian, X.; Nie, S. SERS Nanoparticles in Medicine: From Label-Free Detection to Spectroscopic Tagging. *Chem. Rev.* **2015**, *115* (19), 10489–10529. <https://doi.org/10.1021/acs.chemrev.5b00265>.
 - (99) Zhao, L.; Blackburn, J.; Brosseau, C. L. Quantitative Detection of Uric Acid by Electrochemical-Surface Enhanced Raman Spectroscopy Using a Multilayered Au/Ag Substrate. *Anal. Chem.* **2015**, *87* (1), 441–447. <https://doi.org/10.1021/ac503967s>.
 - (100) Bindesri, S. D.; Alhatab, D. S.; Brosseau, C. L. Development of an Electrochemical Surface-Enhanced Raman Spectroscopy (EC-SERS) Fabric-Based Plasmonic Sensor for Point-of-Care Diagnostics. *Analyst* **2018**, *143* (17), 4128–4135. <https://doi.org/10.1039/C8AN01117F>.
 - (101) Mousavi, S. M.; Hashemi, S. A.; Rahmanian, V.; Kalashgrani, M. Y.; Gholami, A.; Omidifar, N.; Chiang, W.-H. Highly Sensitive Flexible SERS-Based Sensing Platform for Detection of COVID-19. *Biosensors* **2022**, *12* (7), 466. <https://doi.org/10.3390/bios12070466>.
 - (102) Leong, S. X.; Leong, Y. X.; Tan, E. X.; Sim, H. Y. F.; Koh, C. S. L.; Lee, Y. H.; Chong, C.; Ng, L. S.; Chen, J. R. T.; Pang, D. W. C.; Nguyen, L. B. T.; Boong, S. K.; Han, X.; Kao, Y.-C.; Chua, Y. H.; Phan-Quang, G. C.; Phang, I. Y.; Lee, H. K.; Abdad, M. Y.; Tan, N. S.; Ling, X. Y. Noninvasive and Point-of-Care Surface-Enhanced Raman Scattering (SERS)-Based Breathalyzer for Mass Screening of Coronavirus Disease 2019 (COVID-19) under 5 Min. *ACS Nano* **2022**, *16* (2), 2629–2639. <https://doi.org/10.1021/acsnano.1c09371>.
 - (103) Cailletaud, J.; De Bleye, C.; Dumont, E.; Sacré, P.-Y.; Netchacovitch, L.; Gut, Y.; Boiret, M.; Ginot, Y.-M.; Hubert, P.; Ziemons, E. Critical Review of Surface-Enhanced Raman Spectroscopy Applications in the Pharmaceutical Field. *J. Pharm. Biomed. Anal.* **2018**, *147*, 458–472. <https://doi.org/10.1016/j.jpba.2017.06.056>.
 - (104) Pang, S.; Yang, T.; He, L. Review of Surface Enhanced Raman Spectroscopic (SERS) Detection of Synthetic Chemical Pesticides. *TrAC Trends Anal. Chem.* **2016**, *85*, 73–82. <https://doi.org/10.1016/j.trac.2016.06.017>.
 - (105) Ibáñez, D.; González-García, M. B.; Hernández-Santos, D.; Fanjul-Bolado, P. Detection of Dithiocarbamate, Chloronicotinyl and Organophosphate Pesticides by Electrochemical Activation of SERS Features of Screen-Printed Electrodes. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2021**, *248*, 119174. <https://doi.org/10.1016/j.saa.2020.119174>.
 - (106) Moldovan, R.; Milenko, K.; Vereshchagina, E.; Iacob, B.-C.; Schneider, K.; Farcău, C.;

- Bodoki, E. EC-SERS Detection of Thiabendazole in Apple Juice Using Activated Screen-Printed Electrodes. *Food Chem.* **2023**, *405*, 134713. <https://doi.org/10.1016/j.foodchem.2022.134713>.
- (107) Markin, A. V.; Arzhanukhina, A. I.; Markina, N. E.; Goryacheva, I. Y. Analytical Performance of Electrochemical Surface-Enhanced Raman Spectroscopy: A Critical Review. *TrAC Trends Anal. Chem.* **2022**, *157*, 116776. <https://doi.org/10.1016/j.trac.2022.116776>.
- (108) Bao, Z. Y.; Lei, D. Y.; Jiang, R.; Liu, X.; Dai, J.; Wang, J.; Chan, H. L. W.; Tsang, Y. H. Bifunctional Au@Pt Core–Shell Nanostructures for in Situ Monitoring of Catalytic Reactions by Surface-Enhanced Raman Scattering Spectroscopy. *Nanoscale* **2014**, *6* (15), 9063–9070. <https://doi.org/10.1039/C4NR00770K>.
- (109) Bharati, M. S. S.; Soma, V. R. Flexible SERS Substrates for Hazardous Materials Detection: Recent Advances. *Opto-Electronic Adv.* **2021**, *4* (11), 210048. <https://doi.org/10.29026/oea.2021.210048>.
- (110) Saleh, T. A.; Al-Shalalfeh, M. M.; Onawole, A. T.; Al-Saadi, A. A. Ultra-Trace Detection of Methimazole by Surface-Enhanced Raman Spectroscopy Using Gold Substrate. *Vib. Spectrosc.* **2017**, *90*, 96–103. <https://doi.org/10.1016/j.vibspec.2017.03.009>.
- (111) Ishida, H.; Fukuda, H.; Katagiri, G.; Ishitani, A. An Application of Surface-Enhanced Raman Scattering to the Surface Characterization of Carbon Materials. *Appl. Spectrosc.* **1986**, *40* (3), 322–330. <https://doi.org/10.1366/0003702864509295>.
- (112) Ruiz, V.; Colina, Á.; Heras, A.; López-Palacios, J.; Seeber, R. Bidimensional Spectroelectrochemistry Applied to the Electrosynthesis and Characterization of Conducting Polymers: Study of Poly[4,4'-Bis(Butylthio)-2,2'-Bithiophene]. *Helv. Chim. Acta* **2001**, *84* (12), 3628–3642. [https://doi.org/10.1002/1522-2675\(20011219\)84:12<3628::AID-HLCA3628>3.0.CO;2-0](https://doi.org/10.1002/1522-2675(20011219)84:12<3628::AID-HLCA3628>3.0.CO;2-0).
- (113) Ruiz, V.; Colina, Á.; Heras, A.; López-Palacios, J.; Seeber, R. Bidimensional Chronoabsorptometric Study of Electropolymerisation of 4,4'-Bis(2-Methylbutylthio)-2,2'-Bithiophene. *Electrochem. commun.* **2002**, *4* (5), 451–456. [https://doi.org/10.1016/S1388-2481\(02\)00325-9](https://doi.org/10.1016/S1388-2481(02)00325-9).
- (114) López-Palacios, J.; Heras, A.; Colina, Á.; Ruiz, V. Bidimensional Spectroelectrochemical Study on Electrogeneration of Soluble Prussian Blue from Hexacyanoferrate(II) Solutions. *Electrochim. Acta* **2004**, *49* (7), 1027–1033. <https://doi.org/10.1016/j.electacta.2003.10.013>.

- (115) Ruiz, V.; Colina, Á.; Heras, A.; López-Palacios, J. Study of Electrochemical Stability of Conducting Polymers by Bidimensional Spectroelectrochemistry: P- and n-Doping of Poly(4,4'-Bis(Butylthio)-2,2'-Bithiophene) Films. *Polym. Degrad. Stab.* **2006**, *91* (12), 3117–3123. <https://doi.org/10.1016/j.polymdegradstab.2006.07.023>.
- (116) López-Palacios, J.; Muñoz, E.; Heras, M. A.; Colina, Á.; Ruiz, V. Study of Polyaniline Films Degradation by Thin-Layer Bidimensional Spectroelectrochemistry. *Electrochim. Acta* **2006**, *52* (1), 234–239. <https://doi.org/10.1016/j.electacta.2006.04.061>.
- (117) Garoz-Ruiz, J.; Heras, A.; Colina, A. Simultaneous Study of Different Regions of an Electrode Surface with a Novel Spectroelectrochemistry Platform. *Electrochem. commun.* **2018**, *90*, 73–77. <https://doi.org/10.1016/j.elecom.2018.04.006>.
- (118) Hernandez, S.; Perales-Rondon, J. V.; Heras, A.; Colina, A. Simultaneous Raman and Reflection UV/Vis Absorption Spectroelectrochemistry. *Nano Res.* **2022**, *15* (6), 5340–5346. <https://doi.org/10.1007/s12274-022-4137-5>.
- (119) Hernandez, S.; Perez-Estebanez, M.; Cheuquepan, W.; Perales-Rondon, J. V.; Heras, A.; Colina, A. Raman, UV–Vis Absorption, and Fluorescence Spectroelectrochemistry for Studying the Enhancement of the Raman Scattering Using Nanocrystals Activated by Metal Cations. *Anal. Chem.* **2023**, *95* (44), 16070–16078. <https://doi.org/10.1021/acs.analchem.3c01172>.
- (120) Perales-Rondon, J. V.; Hernandez, S.; Gonzalez-Baro, A. C.; Heras, A.; Colina, A. Simultaneous Scanning Electrochemical Microscopy and UV–Vis Absorption Spectroelectrochemistry. *Anal. Chem.* **2023**, *95* (28), 10532–10539. <https://doi.org/10.1021/acs.analchem.2c05468>.
- (121) Cui, H.; Wu, L.; Chen, J.; Lin, X. Multi-Mode in Situ Spectroelectrochemical Studies of Redox Pathways of Adrenaline. *J. Electroanal. Chem.* **2001**, *504* (2), 195–200. [https://doi.org/10.1016/S0022-0728\(01\)00444-2](https://doi.org/10.1016/S0022-0728(01)00444-2).
- (122) Kim, S. H.; Lee, J. W.; Yeo, I.-H. Spectroelectrochemical and Electrochemical Behavior of Epinephrine at a Gold Electrode. *Electrochim. Acta* **2000**, *45* (18), 2889–2895. [https://doi.org/10.1016/S0013-4686\(00\)00364-9](https://doi.org/10.1016/S0013-4686(00)00364-9).
- (123) Garoz-Ruiz, J.; Guillen-Posteguillo, C.; Colina, A.; Heras, A. Application of Spectroelectroanalysis for the Quantitative Determination of Mixtures of Compounds with Highly Overlapping Signals. *Talanta* **2019**, *195* (December 2018), 815–821. <https://doi.org/10.1016/j.talanta.2018.12.002>.
- (124) Olmo, F.; Garoz-Ruiz, J.; Colina, A.; Heras, A. Derivative UV/Vis

- Spectroelectrochemistry in a Thin-Layer Regime: Deconvolution and Simultaneous Quantification of Ascorbic Acid, Dopamine and Uric Acid. *Anal. Bioanal. Chem.* **2020**, *412* (24), 6329–6339. <https://doi.org/10.1007/s00216-020-02564-1>.
- (125) Liu, X.; Zhang, Z.; Cheng, G.; Dong, S. Spectroelectrochemical and Voltammetric Studies of L-DOPA. *Electroanalysis* **2003**, *15* (2), 103–107. <https://doi.org/10.1002/elan.200390009>.
- (126) Rein, F. N.; Rocha, R. C.; Toma, H. E. Catecholamine Complexes of Ruthenium–Edta and Their Redox Chemistry. *J. Inorg. Biochem.* **2001**, *85* (2–3), 155–166. [https://doi.org/10.1016/S0162-0134\(01\)00194-5](https://doi.org/10.1016/S0162-0134(01)00194-5).
- (127) Babaei, A.; McQuillan, A. J. In Situ Spectroelectrochemical Studies of the Decomposition of Hydroquinones on Platinum Electrodes in Dichloromethane Solutions. *J. Phys. Chem. B* **1997**, *101* (38), 7443–7447. <https://doi.org/10.1021/jp970131v>.
- (128) Zhang, X.-P.; Jiang, W.-L.; Cao, S.-H.; Sun, H.-J.; You, X.-Q.; Cai, S.-H.; Wang, J.-L.; Zhao, C.-S.; Wang, X.; Chen, Z.; Sun, S.-G. NMR Spectroelectrochemistry in Studies of Hydroquinone Oxidation by Polyaniline Thin Films. *Electrochim. Acta* **2018**, *273*, 300–306. <https://doi.org/10.1016/j.electacta.2018.04.048>.
- (129) Garoz-Ruiz, J.; Izquierdo, D.; Colina, A.; Palmero, S.; Heras, A. Optical Fiber Spectroelectrochemical Device for Detection of Catechol at Press-Transferred Single-Walled Carbon Nanotubes Electrodes. *Anal. Bioanal. Chem.* **2013**, *405* (11), 3593–3602. <https://doi.org/10.1007/s00216-013-6762-z>.
- (130) Alós, J.-I. I. Quinolonas. *Enferm. Infecc. Microbiol. Clin.* **2009**, *27* (5), 290–297. <https://doi.org/10.1016/j.eimc.2009.03.001>.
- (131) Brar, R. K.; Jyoti, U.; Patil, R. K.; Patil, H. C. Fluoroquinolone Antibiotics: An Overview. *Adesh Univ. J. Med. Sci. Res.* **2020**, *2* (1), 26–30. https://doi.org/10.25259/aujmsr_12_2020.
- (132) Zhu, L.; Santiago-Schübel, B.; Xiao, H.; Hollert, H.; Kueppers, S. Electrochemical Oxidation of Fluoroquinolone Antibiotics: Mechanism, Residual Antibacterial Activity and Toxicity Change. *Water Res.* **2016**, *102*, 52–62. <https://doi.org/10.1016/j.watres.2016.06.005>.
- (133) Drakopoulos, A. I.; Ioannou, P. C. Spectrofluorimetric Study of the Acid-Base Equilibria and Complexation Behavior of the Fluoroquinolone Antibiotics Ofloxacin, Norfloxacin, Ciprofloxacin and Pefloxacin in Aqueous Solution. *Anal. Chim. Acta* **1997**, *354* (1–3), 197–204. [https://doi.org/10.1016/S0003-2670\(97\)00465-0](https://doi.org/10.1016/S0003-2670(97)00465-0).

- (134) Riaz, L.; Mahmood, T.; Khalid, A.; Rashid, A.; Ahmed Siddique, M. B.; Kamal, A.; Coyne, M. S. Fluoroquinolones (FQs) in the Environment: A Review on Their Abundance, Sorption and Toxicity in Soil. *Chemosphere* **2018**, *191*, 704–720. <https://doi.org/10.1016/j.chemosphere.2017.10.092>.
- (135) Millanao, A. R.; Mora, A. Y.; Villagra, N. A.; Bucarey, S. A.; Hidalgo, A. A. Biological Effects of Quinolones: A Family of Broad-Spectrum Antimicrobial Agents. *Molecules* **2021**, *26* (23), 7153. <https://doi.org/10.3390/molecules26237153>.
- (136) Sharma, P. C.; Jain, A.; Jain, S. Fluoroquinolone Antibacterials: A Review on Chemistry, Microbiology and Therapeutic Prospects. *Acta Pol. Pharm. - Drug Res.* **2009**, *66* (6), 587–604.
- (137) Liu, T.; Xue, Q.; Jia, J.; Liu, F.; Zou, S.; Tang, R.; Chen, T.; Li, J.; Qian, Y. New Insights into the Effect of pH on the Mechanism of Ofloxacin Electrochemical Detection in Aqueous Solution. *Phys. Chem. Chem. Phys.* **2019**, *21* (29), 16282–16287. <https://doi.org/10.1039/C9CP03486B>.
- (138) Pi, Y.; Feng, J.; Sun, J.; Song, M.; Sun, J. Oxidation of Ofloxacin by Oxone/Co²⁺: Identification of Reaction Products and Pathways. *Environ. Sci. Pollut. Res.* **2014**, *21* (4), 3031–3040. <https://doi.org/10.1007/s11356-013-2220-x>.
- (139) Tian, Y.; Li, Q.; Zhang, M.; Nie, Y.; Tian, X.; Yang, C.; Li, Y. pH-Dependent Oxidation Mechanisms over FeCu Doped g-C₃N₄ for Ofloxacin Degradation via the Efficient Peroxymonosulfate Activation. *J. Clean. Prod.* **2021**, *315*, 1–10. <https://doi.org/10.1016/j.jclepro.2021.128207>.
- (140) Si, X.; Wei, Y.; Wang, C.; Li, L.; Ding, Y. A Sensitive Electrochemical Sensor for Ofloxacin Based on a Graphene/Zinc Oxide Composite Film. *Anal. Methods* **2018**, *10* (17), 1961–1967. <https://doi.org/10.1039/C8AY00127H>.
- (141) Rudnicki, K.; Sipa, K.; Brycht, M.; Borgul, P.; Skrzypek, S.; Poltorak, L. Electrochemical Sensing of Fluoroquinolone Antibiotics. *Trends Anal. Chem.* **2020**, *128*, 115907. <https://doi.org/10.1016/j.trac.2020.115907>.
- (142) Chen, T.-S. S.; Huang, K.-L. L.; Chen, J.-L. L. An Electrochemical Approach to Simultaneous Determination of Acetaminophen and Ofloxacin. *Bull. Environ. Contam. Toxicol.* **2012**, *89* (6), 1284–1288. <https://doi.org/10.1007/s00128-012-0833-2>.
- (143) Manjula, N.; Pulikkutty, S.; Chen, T.-W. W.; Chen, S.-M. M.; Liu, X. Hexagon Prism-Shaped Cerium Ferrite Embedded on GC Electrode for Electrochemical Detection of Antibiotic Drug Ofloxacin in Biological Sample. *Colloids Surfaces A Physicochem. Eng.*

- Asp.* **2021**, 627, 127129. <https://doi.org/10.1016/j.colsurfa.2021.127129>.
- (144) Jiang, Z.; Li, G.; Zhang, M. A Novel Electrochemical Sensor Based on SH- β -Cyclodextrin Functionalized Gold Nanoparticles/Reduced-Graphene Oxide Nanohybrids for Ultrasensitive Electrochemical Sensing of Acetaminophen and Ofloxacin. *Int. J. Electrochem. Sci.* **2017**, 12 (6), 5157–5173. <https://doi.org/10.20964/2017.06.28>.
- (145) Wu, F.; Xu, F.; Chen, L.; Jiang, B.; Sun, W.; Wei, X. Cuprous Oxide/Nitrogen-Doped Graphene Nanocomposites as Electrochemical Sensors for Ofloxacin Determination. *Chem. Res. Chinese Univ.* **2016**, 32 (3), 468–473. <https://doi.org/10.1007/s40242-016-5367-4>.
- (146) Elfiky, M.; Salahuddin, N.; Hassanein, A.; Matsuda, A.; Hattori, T. Detection of Antibiotic Ofloxacin Drug in Urine Using Electrochemical Sensor Based on Synergistic Effect of Different Morphological Carbon Materials. *Microchem. J.* **2019**, 146, 170–177. <https://doi.org/10.1016/j.microc.2018.12.034>.
- (147) Liu, X.; Huang, F.; Yu, Y.; Zhao, P.; Zhou, Y.; He, Y.; Xu, Y.; Zhang, Y. Ofloxacin Degradation over Cu–Ce Tyre Carbon Catalysts by the Microwave Assisted Persulfate Process. *Appl. Catal. B Environ.* **2019**, 253, 149–159. <https://doi.org/10.1016/j.apcatb.2019.04.047>.
- (148) Immanuel, C.; Hemanth Kumar, A. . Simple and Rapid High-Performance Liquid Chromatography Method for the Determination of Ofloxacin Concentrations in Plasma and Urine. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, 760 (1), 91–95. [https://doi.org/10.1016/S0378-4347\(01\)00260-2](https://doi.org/10.1016/S0378-4347(01)00260-2).
- (149) Garcia, M. A.; Solans, C.; Calvo, A.; Royo, M.; Hernandez, E.; Rey, R.; Bregante, M. A. Analysis of Ofloxacin in Plasma Samples by High-Performance Liquid Chromatography. *Chromatographia* **2002**, 55, 431–434. <https://doi.org/10.1007/BF02492272>.
- (150) Zeng, S.; Zhong, J.; Pan, L.; Li, Y. High-Performance Liquid Chromatography Separation and Quantitation of Ofloxacin Enantiomers in Rat Microsomes. *J. Chromatogr. B Biomed. Sci. Appl.* **1999**, 728 (1), 151–155. [https://doi.org/10.1016/S0378-4347\(99\)00085-7](https://doi.org/10.1016/S0378-4347(99)00085-7).
- (151) Horie, M.; Saito, K.; Nose, N.; Nakazawa, H. Simultaneous Determination of Benofloxacin, Danofloxacin, Enrofloxacin and Ofloxacin in Chicken Tissues by High-Performance Liquid Chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **1994**, 653 (1), 69–76. [https://doi.org/10.1016/0378-4347\(93\)E0428-S](https://doi.org/10.1016/0378-4347(93)E0428-S).
- (152) Attimarad, M.; Alnajjar, A. A Conventional HPLC-MS Method for the Simultaneous Determination of Ofloxacin and Cefixime in Plasma: Development and Validation. *J.*

- Basic Clin. Pharm.* **2013**, 4 (2), 36–41. <https://doi.org/10.4103/0976-0105.113606>.
- (153) Meredith, S. A.; Smith, P. J.; Norman, J.; Wiesner, L. An LC–MS/MS Method for the Determination of Ofloxacin in 20 µl Human Plasma. *J. Pharm. Biomed. Anal.* **2012**, 58 (1), 177–181. <https://doi.org/10.1016/j.jpba.2011.09.030>.
- (154) Plotas, P.; Anastasopoulos, C.; Makril, O. E.; Leotsinidis, M.; Georgakopoulos, C. D. A UPLC-MS Method for the Determination of Ofloxacin Concentrations in Aqueous Humor. *Anal. Chem. Insights* **2014**, 9, 27. <https://doi.org/10.4137/ACI.S13857>.
- (155) Hopkała, H.; Kowalczyk, D. Application of Derivative UV Spectrophotometry for the Determination of Ciprofloxacin, Norfloxacin and Ofloxacin in Tablets. *Acta Poloniae Pharmaceutica - Drug Research*. 2000, pp 3–13.
- (156) Attimarad, M. Simultaneous Determination of Ofloxacin and Flavoxate Hydrochloride by First-and Ratio First-Derivative UV Spectrophotometry. *J. Iran. Chem. Soc.* **2012**, 9 (4), 551–557. <https://doi.org/10.1007/s13738-012-0068-5>.
- (157) Lahouidak, S.; Soriano, M. L.; Salghi, R.; Zougagh, M.; Ríos, Á. Graphene Quantum Dots for Enhancement of Fluorimetric Detection Coupled to Capillary Electrophoresis for Detection of Ofloxacin. *Electrophoresis* **2019**, 40, 2336–2341. <https://doi.org/10.1002/elps.201900037>.
- (158) Francis, P. S.; Adcock, J. L. Chemiluminescence Methods for the Determination of Ofloxacin. *Anal. Chim. Acta* **2005**, 541, 3–12. <https://doi.org/10.1016/j.aca.2004.11.030>.
- (159) Bel'tyukova, S. V.; Malinka, E. V.; Liventsova, E. O. Determination of Ofloxacin and Norfloxacin in Wastewaters of Pharmaceutical Enterprises Using Sensitized Luminescence of Tb(III) Complexes. *J. Water Chem. Technol.* **2008**, 30 (1), 38–41. <https://doi.org/10.1007/s11962-008-1005-2>.
- (160) Kaur, R.; Kushwaha, J. P.; Singh, N. Electro-Oxidation of Ofloxacin Antibiotic by Dimensionally Stable Ti/RuO₂ Anode: Evaluation and Mechanistic Approach. *Chemosphere* **2018**, 193, 685–694. <https://doi.org/10.1016/j.chemosphere.2017.11.065>.
- (161) Yu, Y.; Huang, F.; He, Y.; Liu, X.; Song, C.; Xu, Y.; Zhang, Y. Heterogeneous Fenton-like Degradation of Ofloxacin over Sludge Derived Carbon as Catalysts: Mechanism and Performance. *Sci. Total Environ.* **2019**, 654, 942–947. <https://doi.org/10.1016/j.scitotenv.2018.11.156>.
- (162) Neugebauer, U.; Szeghalmi, A.; Schmitt, M.; Kiefer, W.; Popp, J.; Holzgrabe, U. Vibrational Spectroscopic Characterization of Fluoroquinolones. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **2005**, 61 (7), 1505–1517.

- <https://doi.org/10.1016/j.saa.2004.11.014>.
- (163) Gnegy, M. E. Catecholamines. In *Basic Neurochemistry*; Elsevier, 2012; pp 283–299. <https://doi.org/10.1016/B978-0-12-374947-5.00014-6>.
- (164) Li, F.; Cui, H.; Lin, X.-Q. Determination of Adrenaline by Using Inhibited Ru(Bpy)₃²⁺ Electrochemiluminescence. *Anal. Chim. Acta* **2002**, *471* (2), 187–194. [https://doi.org/10.1016/S0003-2670\(02\)00930-3](https://doi.org/10.1016/S0003-2670(02)00930-3).
- (165) Felix, F. S.; Yamashita, M.; Angnes, L. Epinephrine Quantification in Pharmaceutical Formulations Utilizing Plant Tissue Biosensors. *Biosens. Bioelectron.* **2006**, *21* (12), 2283–2289. <https://doi.org/10.1016/j.bios.2005.10.025>.
- (166) Jin, G.-P. P.; Peng, X.; Ding, Y.-F. F. The Electrochemical Modification of Clenbuterol for Biosensors of Dopamine, Norepinephrine, Adrenalin, Ascorbic Acid and Uric Acid at Paraffin-Impregnated Graphite Electrode. *Biosens. Bioelectron.* **2008**, *24* (4), 1031–1035. <https://doi.org/10.1016/j.bios.2008.06.028>.
- (167) Yang, G.-J. J.; Xu, J.-J. J.; Chen, H.-Y. Y. The Study of Redox Mechanism of Dobutamine at Different pH Media by Electrochemical and in Situ Spectroelectrochemical Methods. *Electrochim. Acta* **2004**, *49* (19), 3121–3127. <https://doi.org/10.1016/j.electacta.2004.02.026>.
- (168) Tao, Z.; Wang, G.; Goodisman, J.; Asefa, T. Accelerated Oxidation of Epinephrine by Silica Nanoparticles. *Langmuir* **2009**, *25* (17), 10183–10188. <https://doi.org/10.1021/la900958f>.
- (169) Bacil, R. P.; Garcia, P. H. M.; Serrano, S. H. P. New Insights on the Electrochemical Mechanism of Epinephrine on Glassy Carbon Electrode. *J. Electroanal. Chem.* **2022**, *908*, 116111. <https://doi.org/10.1016/j.jelechem.2022.116111>.
- (170) Chen, S.-M.; Peng, K.-T. The Electrochemical Properties of Dopamine, Epinephrine, Norepinephrine, and Their Electrocatalytic Reactions on Cobalt(II) Hexacyanoferrate Films. *J. Electroanal. Chem.* **2003**, *547* (2), 179–189. [https://doi.org/10.1016/S0022-0728\(03\)00220-1](https://doi.org/10.1016/S0022-0728(03)00220-1).
- (171) Hawley, M. D.; Tatawawadi, S. V.; Piekarski, S.; Adams, R. N. Electrochemical Studies of the Oxidation Pathways of Catecholamines. *J. Am. Chem. Soc.* **1967**, *89* (2), 447–450. <https://doi.org/10.1021/ja00978a051>.
- (172) Hansen, A. Validation of a High Performance Liquid Chromatography Analysis for the Determination of Noradrenaline and Adrenaline in Human Urine with an On-Line Sample Purification. *Talanta* **1999**, *50* (2), 367–379. <https://doi.org/10.1016/S0039->

- 9140(99)00029-6.
- (173) Thomas, A.; Geyer, H.; Mester, H. J.; Schänzer, W.; Zimmermann, E.; Thevis, M. Quantitative Determination of Adrenaline and Noradrenaline in Urine Using Liquid Chromatography-Tandem Mass Spectrometry. *Chromatographia* **2006**, *64* (9–10), 587–591. <https://doi.org/10.1365/s10337-006-0067-8>.
 - (174) Siddiqui, M. R.; Wabaidur, S. M.; Alothman, Z. A.; Rafiquee, M. Z. A.; Khan, M. A.; Sumbul, S. High Throughput Ultra Performance Liquid Chromatography - Mass Spectrometric Method for Determination of Adrenaline and Characterization of Its Stress Degradation Products: A Mechanistic Approach. *J. Chil. Chem. Soc.* **2018**, *63* (1), 3803–3810. <https://doi.org/10.4067/s0717-97072018000103803>.
 - (175) Al-Ameri, S. A. H. Spectrophotometric Determination of Adrenaline in Pharmaceutical Preparations. *Arab. J. Chem.* **2016**, *9*, S1000–S1004. <https://doi.org/10.1016/j.arabjc.2011.10.001>.
 - (176) Annersten, S.; Grönwall, A.; Köiw, E. The Fluorimetric Determination of Adrenaline in Blood Plasma. *Scand. J. Clin. Lab. Invest.* **1949**, *1* (1), 60–69. <https://doi.org/10.3109/00365514909065626>.
 - (177) Häggendal, J. An Improved Method for Fluorimetric Determination of Small Amounts of Adrenaline and Noradrenaline in Plasma and Tissues. *Acta Physiol. Scand.* **1963**, *59* (3), 242–254. <https://doi.org/10.1111/j.1748-1716.1963.tb02739.x>.
 - (178) Yu, C.; Tang, Y.; Han, X.; Zheng, X. Sensitive Assay for Catecholamines in Pharmaceutical Samples and Blood Plasma Using Flow Injection Chemiluminescence Analysis. *Anal. Sci.* **2006**, *22* (1), 25–28. <https://doi.org/10.2116/analsci.22.25>.
 - (179) Zhou, G.-J.; Zhang, G.-F.; Chen, H.-Y. Development of Integrated Chemiluminescence Flow Sensor for the Determination of Adrenaline and Isoprenaline. *Anal. Chim. Acta* **2002**, *463* (2), 257–263. [https://doi.org/10.1016/S0003-2670\(02\)00418-X](https://doi.org/10.1016/S0003-2670(02)00418-X).
 - (180) Britz-Mckibbin, P.; Kranack, A. R.; Paprica, A.; Chen, D. D. Y. Quantitative Assay for Epinephrine in Dental Anesthetic Solutions by Capillary Electrophoresis. *Analyst* **1998**, *123* (7), 1461–1463. <https://doi.org/10.1039/a800772a>.
 - (181) Britz-Mckibbin, P.; Wong, J.; Chen, D. D. Y. Analysis of Epinephrine from Fifteen Different Dental Anesthetic Formulations by Capillary Electrophoresis. *J. Chromatogr. A* **1999**, *853* (1–2), 535–540. [https://doi.org/10.1016/S0021-9673\(99\)00689-5](https://doi.org/10.1016/S0021-9673(99)00689-5).
 - (182) Dhanjai; Yu, N.; Mugo, S. M. A Flexible-Imprinted Capacitive Sensor for Rapid Detection of Adrenaline. *Talanta* **2019**, *204*, 602–606. <https://doi.org/10.1016/j.talanta.2019.06.016>.

- (183) Chandra H D, M.; Kumara Swamy, B. E. Development of Electrochemical Sensor for Adrenaline at Poly (Allura Red) Modified Carbon Paste Electrode: A Voltammetric Study. *Chem. Data Collect.* **2020**, 28, 100447. <https://doi.org/10.1016/j.cdc.2020.100447>.
- (184) Charithra, M. M.; Manjunatha, J. G. Electrochemical Sensing of Adrenaline Using Surface Modified Carbon Nanotube Paste Electrode. *Mater. Chem. Phys.* **2021**, 262, 124293. <https://doi.org/10.1016/j.matchemphys.2021.124293>.
- (185) Zaidi, S. A. Utilization of an Environmentally-Friendly Monomer for an Efficient and Sustainable Adrenaline Imprinted Electrochemical Sensor Using Graphene. *Electrochim. Acta* **2018**, 274, 370–377. <https://doi.org/10.1016/j.electacta.2018.04.119>.
- (186) Pruneanu, S.; Biris, A. S. R.; Pogacean, F.; Socaci, C.; Coros, M.; Rosu, M. C.; Watanabe, F.; Biris, A. S. R. The Influence of Uric and Ascorbic Acid on the Electrochemical Detection of Dopamine Using Graphene-Modified Electrodes. *Electrochim. Acta* **2015**, 154, 197–204. <https://doi.org/10.1016/j.electacta.2014.12.046>.
- (187) Garoz Ruiz, J.; Heras Vidaurre, A.; Colina Santamaría, Á. Multipurpose Spectroelectrochemistry: Paving the Way for In Vivo Measurements. *Tesis Dr. Univ. Burgos* **2017**.
- (188) Kim, Y. R.; Bong, S.; Kang, Y. J.; Yang, Y.; Mahajan, R. K.; Kim, J. S.; Kim, H. Electrochemical Detection of Dopamine in the Presence of Ascorbic Acid Using Graphene Modified Electrodes. *Biosens. Bioelectron.* **2010**, 25 (10), 2366–2369. <https://doi.org/10.1016/j.bios.2010.02.031>.
- (189) Chen, J.; Shi, Y.-P.; Liu, J.-Y. Determination of Noradrenaline and Dopamine in Chinese Herbal Extracts from Portulaca Oleracea L. by High-Performance Liquid Chromatography. *J. Chromatogr. A* **2003**, 1003 (1–2), 127–132. [https://doi.org/10.1016/S0021-9673\(03\)00786-6](https://doi.org/10.1016/S0021-9673(03)00786-6).
- (190) Wang, Y.; Fice, D. S.; Yeung, P. K. F. A Simple High-Performance Liquid Chromatography Assay for Simultaneous Determination of Plasma Norepinephrine, Epinephrine, Dopamine and 3,4-Dihydroxyphenyl Acetic Acid. *J. Pharm. Biomed. Anal.* **1999**, 21 (3), 519–525. [https://doi.org/10.1016/S0731-7085\(99\)00117-X](https://doi.org/10.1016/S0731-7085(99)00117-X).
- (191) Qi, D.; Zhang, Q.; Zhou, W.; Zhao, J.; Zhang, B.; Sha, Y.; Pang, Z. Quantification of Dopamine in Brain Microdialysates with High-Performance Liquid Chromatography-Tandem Mass Spectrometry. *Anal. Sci.* **2016**, 32 (4), 419–424. <https://doi.org/10.2116/analsci.32.419>.
- (192) Hows, M. E. P.; Lacroix, L.; Heidbreder, C.; Organ, A. J.; Shah, A. J. High-Performance

- Liquid Chromatography/Tandem Mass Spectrometric Assay for the Simultaneous Measurement of Dopamine, Norepinephrine, 5-Hydroxytryptamine and Cocaine in Biological Samples. *J. Neurosci. Methods* **2004**, *138* (1–2), 123–132. <https://doi.org/10.1016/j.jneumeth.2004.03.021>.
- (193) Yang, X.; Tian, F.; Wen, S.; Xu, H.; Zhang, L.; Zeng, J. Selective Determination of Dopamine in Pharmaceuticals and Human Urine Using Carbon Quantum Dots as a Fluorescent Probe. *Processes* **2021**, *9* (1), 170. <https://doi.org/10.3390/pr9010170>.
- (194) Liu, X.; Zhang, W.; Huang, L.; Hu, N.; Liu, W.; Liu, Y.; Li, S.; Yang, C.; Suo, Y.; Wang, J. Fluorometric Determination of Dopamine by Using Molybdenum Disulfide Quantum Dots. *Microchim. Acta* **2018**, *185* (4), 234. <https://doi.org/10.1007/s00604-018-2771-0>.
- (195) Abdolmohammad-Zadeh, H.; Zamani-Kalajahi, M. A Novel Chemiluminescent-Based Nano-Probe for Ultra-Trace Quantification of Dopamine in Human Plasma Samples. *Microchem. J.* **2020**, *155*, 104704. <https://doi.org/10.1016/j.microc.2020.104704>.
- (196) Olefirowicz, T. M.; Ewing, A. G. Dopamine Concentration in the Cytoplasmic Compartment of Single Neurons Determined by Capillary Electrophoresis. *J. Neurosci. Methods* **1990**, *34* (1–3), 11–15. [https://doi.org/10.1016/0165-0270\(90\)90036-F](https://doi.org/10.1016/0165-0270(90)90036-F).
- (197) Umapathi, S.; Masud, J.; Coleman, H.; Nath, M. Electrochemical Sensor Based on CuSe for Determination of Dopamine. *Microchim. Acta* **2020**, *187* (8), 440. <https://doi.org/10.1007/s00604-020-04405-5>.
- (198) Arumugasamy, S. K.; Govindaraju, S.; Yun, K. Electrochemical Sensor for Detecting Dopamine Using Graphene Quantum Dots Incorporated with Multiwall Carbon Nanotubes. *Appl. Surf. Sci.* **2020**, *508*, 145294. <https://doi.org/10.1016/j.apsusc.2020.145294>.
- (199) Li, J.; Xia, J.; Zhang, F.; Wang, Z.; Liu, Q. A Novel Electrochemical Sensor Based on Copper-based Metal-Organic Framework for the Determination of Dopamine. *J. Chinese Chem. Soc.* **2018**, *65* (6), 743–749. <https://doi.org/10.1002/jccs.201700410>.
- (200) Akbar, S.; Anwar, A.; Kanwal, Q. Electrochemical Determination of Folic Acid: A Short Review. *Anal. Biochem.* **2016**, *510*, 98–105. <https://doi.org/10.1016/j.ab.2016.07.002>.
- (201) Batra, B.; Narwal, V.; Kalra, V.; Sharma, M.; Rana, J. Folic Acid Biosensors: A Review. *Process Biochem.* **2020**, *92*, 343–354. <https://doi.org/10.1016/j.procbio.2020.01.025>.
- (202) Ferrazzi, E.; Tiso, G.; Di Martino, D. Folic Acid versus 5-Methyl Tetrahydrofolate Supplementation in Pregnancy. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2020**, *253*, 312–319. <https://doi.org/10.1016/j.ejogrb.2020.06.012>.

- (203) Shulpekova, Y.; Nechaev, V.; Kardasheva, S.; Sedova, A.; Kurbatova, A.; Bueverova, E.; Kopylov, A.; Malsagova, K.; Dlamini, J. C.; Ivashkin, V. The Concept of Folic Acid in Health and Disease. *Molecules* **2021**, *26* (12), 1–29. <https://doi.org/10.3390/molecules26123731>.
- (204) Kaye, A. D.; Jeha, G. M.; Pham, A. D.; Fuller, M. C.; Lerner, Z. I.; Sibley, G. T.; Cornett, E. M.; Urits, I.; Viswanath, O.; Kevil, C. G. Folic Acid Supplementation in Patients with Elevated Homocysteine Levels. *Adv. Ther.* **2020**, *37* (10), 4149–4164. <https://doi.org/10.1007/s12325-020-01474-z>.
- (205) Taruscio, D.; Carbone, P.; Granata, O.; Baldi, F.; Mantovani, A. Folic Acid and Primary Prevention of Birth Defects. *BioFactors* **2011**, *37* (4), 280–284. <https://doi.org/10.1002/biof.175>.
- (206) D’Souza, O. J.; Mascarenhas, R. J.; Satpati, A. K.; Detriche, S.; Mekhalif, Z.; Delhalle, J.; Dhason, A.; A, D. High Electrocatalytic Oxidation of Folic Acid at Carbon Paste Electrode Bulk Modified with Iron Nanoparticle-Decorated Multiwalled Carbon Nanotubes and Its Application in Food and Pharmaceutical Analysis. *Ionics (Kiel)*. **2017**, *23* (1), 201–212. <https://doi.org/10.1007/s11581-016-1806-y>.
- (207) Liu, H. Y.; Liu, S. M.; Zhang, Y. Z. Maternal Folic Acid Supplementation Mediates Offspring Health via DNA Methylation. *Reprod. Sci.* **2020**, *27* (4), 963–976. <https://doi.org/10.1007/s43032-020-00161-2>.
- (208) Modupe, O.; Maurras, J. B.; Diosady, L. L. A Spectrophotometric Method for Determining the Amount of Folic Acid in Fortified Salt. *J. Agric. Food Res.* **2020**, *2*, 100060. <https://doi.org/10.1016/j.jafr.2020.100060>.
- (209) Matias, R.; Ribeiro, P. R. S.; Sarraguça, M. C.; Lopes, J. A. A UV Spectrophotometric Method for the Determination of Folic Acid in Pharmaceutical Tablets and Dissolution Tests. *Anal. Methods* **2014**, *6* (9), 3065–3071. <https://doi.org/10.1039/c3ay41874j>.
- (210) Uysal, U. D.; Oncu-Kaya, E. M.; Tunçel, M. Determination of Folic Acid by CE in Various Cultivated Variety of Lentils. *Chromatographia* **2010**, *71* (7–8), 653–658. <https://doi.org/10.1365/s10337-010-1555-4>.
- (211) Rodríguez Flores, J.; Peñalvo, G. C.; Mansilla, A. E.; Gómez, M. J. R. Capillary Electrophoretic Determination of Methotrexate, Leucovorin and Folic Acid in Human Urine. *J. Chromatogr. B* **2005**, *819* (1), 141–147. <https://doi.org/10.1016/j.jchromb.2005.01.039>.
- (212) Hau Fung Cheung, R.; Hughes, J. G.; Marriott, P. J.; Small, D. M. Investigation of Folic

- Acid Stability in Fortified Instant Asian Noodles by Use of Capillary Electrophoresis. *Food Chem.* **2009**, *112* (2), 507–514. <https://doi.org/10.1016/j.foodchem.2008.05.110>.
- (213) Lebiezińska, A.; Dbrowska, M.; Szefer, P.; Marszał, M. High-Performance Liquid Chromatography Method for the Determination of Folic Acid in Fortified Food Products. *Toxicol. Mech. Methods* **2008**, *18* (6), 463–467. <https://doi.org/10.1080/15376510701623870>.
- (214) Vahteristo, L. T.; Ollilainen, V.; Koivistoinen, P. E.; Varo, P. Improvements in the Analysis of Reduced Folate Monoglutamates and Folic Acid in Food by High-Performance Liquid Chromatography. *J. Agric. Food Chem.* **1996**, *44* (2), 477–482. <https://doi.org/10.1021/jf9503467>.
- (215) Nelson, B. C.; Sharpless, K. E.; Sander, L. C. Quantitative Determination of Folic Acid in Multivitamin/Multielement Tablets Using Liquid Chromatography/Tandem Mass Spectrometry. *J. Chromatogr. A* **2006**, *1135* (2), 203–211. <https://doi.org/10.1016/j.chroma.2006.09.040>.
- (216) Zayed, A.; Bustami, R.; Alabsi, W.; El-Elimat, T. Development and Validation of a Rapid High-Performance Liquid Chromatography–Tandem Mass Spectrometric Method for Determination of Folic Acid in Human Plasma. *Pharmaceuticals* **2018**, *11* (2), 52. <https://doi.org/10.3390/ph11020052>.
- (217) Young, J. E.; Matyska, M. T.; Pesek, J. J. Liquid Chromatography/Mass Spectrometry Compatible Approaches for the Quantitation of Folic Acid in Fortified Juices and Cereals Using Aqueous Normal Phase Conditions. *J. Chromatogr. A* **2011**, *1218* (15), 2121–2126. <https://doi.org/10.1016/j.chroma.2010.09.025>.
- (218) Manzoori, J. L.; Jouyban, A.; Amjadi, M.; Soleymani, J. Spectrofluorimetric Determination of Folic Acid in Tablets and Urine Samples Using 1,10-phenanthroline-terbium Probe. *Luminescence* **2011**, *26* (2), 106–111. <https://doi.org/10.1002/bio.1191>.
- (219) Zhang, B.-T.; Zhao, L.; Lin, J.-M. Determination of Folic Acid by Chemiluminescence Based on Peroxomonosulfate-Cobalt(II) System. *Talanta* **2008**, *74* (5), 1154–1159. <https://doi.org/10.1016/j.talanta.2007.08.027>.
- (220) Chen, X.; Zhou, Q.; Zhang, T.; Wang, C.; Yu, Z.; Ahamada, H.; Bai, Z.; Huang, X. Development of a Sensitive Chemiluminescence Immunoassay for the Quantification of Folic Acid in Human Serum. *J. Anal. Methods Chem.* **2019**, *2019*, 1–7. <https://doi.org/10.1155/2019/5402903>.
- (221) GonçalvesdeAraújo, E.; Fernandes, N. S.; daSilvaSolon, L. G.; SoaresAragão, C. F.;

- Martinez-Huitle, C. A.; Gonçalves de Araújo, E.; Fernandes, N. S.; da Silva Solon, L. G.; Soares Aragão, C. F.; Martinez-Huitle, C. A. Voltammetric Determination of Folic Acid Using a Graphite Paste Electrode. *Electroanalysis* **2015**, 27 (2), 398–405. <https://doi.org/10.1002/elan.201400475>.
- (222) Manoj, D.; Ranjith Kumar, D.; Santhanalakshmi, J. Impact of CuO Nanoleaves on MWCNTs/GCE Nanocomposite Film Modified Electrode for the Electrochemical Oxidation of Folic Acid. *Appl. Nanosci.* **2012**, 2 (3), 223–230. <https://doi.org/10.1007/s13204-012-0093-9>.
- (223) Arvand, M.; Dehsaraei, M. A Simple and Efficient Electrochemical Sensor for Folic Acid Determination in Human Blood Plasma Based on Gold Nanoparticles–Modified Carbon Paste Electrode. *Mater. Sci. Eng. C* **2013**, 33 (6), 3474–3480. <https://doi.org/10.1016/j.msec.2013.04.037>.
- (224) Kanchana, P.; Sekar, C. Development of Electrochemical Folic Acid Sensor Based on Hydroxyapatite Nanoparticles. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2015**, 137, 58–65. <https://doi.org/10.1016/j.saa.2014.07.087>.
- (225) Zou, C.; Zhong, J.; Li, S.; Wang, H.; Wang, J.; Yan, B.; Du, Y. Fabrication of Reduced Graphene Oxide-Bimetallic PdAu Nanocomposites for the Electrochemical Determination of Ascorbic Acid, Dopamine, Uric Acid and Rutin. *J. Electroanal. Chem.* **2017**, 805, 110–119. <https://doi.org/10.1016/j.jelechem.2017.10.020>.
- (226) Yuan, J. P.; Chen, F. Degradation of Ascorbic Acid in Aqueous Solution. *J. Agric. Food Chem.* **1998**, 46 (12), 5078–5082. <https://doi.org/10.1021/jf9805404>.
- (227) Yang, L.; Liu, D.; Huang, J.; You, T. Simultaneous Determination of Dopamine, Ascorbic Acid and Uric Acid at Electrochemically Reduced Graphene Oxide Modified Electrode. *Sensors Actuators, B Chem.* **2014**, 193, 166–172. <https://doi.org/10.1016/j.snb.2013.11.104>.
- (228) Pancham, Y. P.; Suryawanshi Sanjay, S. UV-Spectrophotometric Method for Quantification of Ascorbic Acid in Bulk Powder. In *The Pharma Innovation Journal*; 2020; Vol. 9(5), pp 05–08.
- (229) Desai, A. P.; Desai, S. UV Spectroscopic Method for Determination of Vitamin C (Ascorbic Acid) Content in Different Fruits in South Gujarat Region. *Int. J. Environ. Sci. Nat. Resour.* **2019**, 22 (2), 41–44. <https://doi.org/10.19080/IJESNR.2019.21.556056>.
- (230) Porto, I. S. A.; Santos Neto, J. H.; dos Santos, L. O.; Gomes, A. A.; Ferreira, S. L. C. Determination of Ascorbic Acid in Natural Fruit Juices Using Digital Image Colorimetry.

- Microchem. J.* **2019**, *149*, 104031. <https://doi.org/10.1016/j.microc.2019.104031>.
- (231) Peng, J.; Ling, J.; Zhang, X.-Q.; Zhang, L.-Y.; Cao, Q.-E.; Ding, Z.-T. A Rapid, Sensitive and Selective Colorimetric Method for Detection of Ascorbic Acid. *Sensors Actuators B Chem.* **2015**, *221*, 708–716. <https://doi.org/10.1016/j.snb.2015.07.002>.
- (232) Chebrolu, K. K.; Jayaprakasha, G. K.; Yoo, K. S.; Jifon, J. L.; Patil, B. S. An Improved Sample Preparation Method for Quantification of Ascorbic Acid and Dehydroascorbic Acid by HPLC. *LWT - Food Sci. Technol.* **2012**, *47* (2), 443–449. <https://doi.org/10.1016/j.lwt.2012.02.004>.
- (233) Stan, M.; Soran, M. L.; Marutoiu, C. Extraction and HPLC Determination of the Ascorbic Acid Content of Three Indigenous Spice Plants. *J. Anal. Chem.* **2014**, *69* (10), 998–1002. <https://doi.org/10.1134/S106193481410013X>.
- (234) Huertas, I.; Jesús, S.; Lojo, J. A.; García-Gómez, F. J.; Cáceres-Redondo, M. T.; Oropesa-Ruiz, J. M.; Carrillo, F.; Vargas-Gonzalez, L.; Rodríguez, J. F. M.; Gómez-Garre, P.; García-Solís, D.; Mir, P. Lower Levels of Uric Acid and Striatal Dopamine in Non-Tremor Dominant Parkinson's Disease Subtype. *PLoS One* **2017**, *12* (3), 1–9. <https://doi.org/10.1371/journal.pone.0174644>.
- (235) Owens, J. L.; Marsh, H. A.; Dryhurst, G. Electrochemical Oxidation of Uric Acid and Xanthine. An Investigation by Cyclic Voltammetry, Double Potential Step Chronoamperometry and Thin-Layer Spectroelectrochemistry. *J. Electroanal. Chem.* **1978**, *91* (2), 231–247. [https://doi.org/10.1016/S0022-0728\(78\)80103-X](https://doi.org/10.1016/S0022-0728(78)80103-X).
- (236) Marsh, H. A.; Dryhurst, G. Enzymatic and Electrochemical Oxidation of Uric Acid. *J. Electroanal. Chem. Interfacial Electrochem.* **1979**, *95* (1), 81–90. [https://doi.org/10.1016/s0022-0728\(79\)80221-1](https://doi.org/10.1016/s0022-0728(79)80221-1).
- (237) Chen, J.-C.; Chung, H.-H.; Hsu, C.-T.; Tsai, D.-M.; Kumar, A. S.; Zen, J.-M. A Disposable Single-Use Electrochemical Sensor for the Detection of Uric Acid in Human Whole Blood. *Sensors Actuators B Chem.* **2005**, *110* (2), 364–369. <https://doi.org/10.1016/j.snb.2005.02.026>.
- (238) Sheng, Z.-H. H.; Zheng, X.-Q. Q.; Xu, J.-Y. Y.; Bao, W.-J. J.; Wang, F.-B. Bin; Xia, X.-H. H. Electrochemical Sensor Based on Nitrogen Doped Graphene: Simultaneous Determination of Ascorbic Acid, Dopamine and Uric Acid. *Biosens. Bioelectron.* **2012**, *34* (1), 125–131. <https://doi.org/10.1016/j.bios.2012.01.030>.
- (239) Chen, X.; Wu, G.; Cai, Z.; Oyama, M.; Chen, X. Advances in Enzyme-Free Electrochemical Sensors for Hydrogen Peroxide, Glucose, and Uric Acid. *Microchim.*

- Acta* **2014**, *181* (7–8), 689–705. <https://doi.org/10.1007/s00604-013-1098-0>.
- (240) Yang, Y.; Li, M.; Zhu, Z. A Novel Electrochemical Sensor Based on Carbon Nanotubes Array for Selective Detection of Dopamine or Uric Acid. *Talanta* **2019**, *201*, 295–300. <https://doi.org/10.1016/j.talanta.2019.03.096>.
- (241) Zuo, Y.; Wang, C.; Zhou, J.; Sachdeva, A.; Ruelos, V. C. Simultaneous Determination of Creatinine and Uric Acid in Human Urine by High-Performance Liquid Chromatography. *Anal. Sci.* **2008**, *24* (12), 1589–1592. <https://doi.org/10.2116/analsci.24.1589>.
- (242) Sakurai, T.; Irii, T.; Iwadate, K. Simultaneous Quantification of Urea, Uric Acid, and Creatinine in Human Urine by Liquid Chromatography/Mass Spectrometry. *Leg. Med.* **2022**, *55*, 102011. <https://doi.org/10.1016/j.legalmed.2021.102011>.
- (243) Gabriel, E. F. M.; Garcia, P. T.; Cardoso, T. M. G.; Lopes, F. M.; Martins, F. T.; Coltro, W. K. T. Highly Sensitive Colorimetric Detection of Glucose and Uric Acid in Biological Fluids Using Chitosan-Modified Paper Microfluidic Devices. *Analyst* **2016**, *141* (15), 4749–4756. <https://doi.org/10.1039/C6AN00430J>.
- (244) Li, F.; He, T.; Wu, S.; Peng, Z.; Qiu, P.; Tang, X. Visual and Colorimetric Detection of Uric Acid in Human Serum and Urine Using Chitosan Stabilized Gold Nanoparticles. *Microchem. J.* **2021**, *164*, 105987. <https://doi.org/10.1016/j.microc.2021.105987>.
- (245) Khajehsharifi, H.; Pourbasheer, E. Simultaneous Spectrophotometric Determination of Xanthine, Hypoxanthine and Uric Acid in Real Matrix by Orthogonal Signal Correction-Partial Least Squares. *J. Iran. Chem. Soc.* **2011**, *8* (4), 1113–1119. <https://doi.org/10.1007/BF03246569>.
- (246) Cialla, D.; März, A.; Böhme, R.; Theil, F.; Weber, K.; Schmitt, M.; Popp, J. Surface-Enhanced Raman Spectroscopy (SERS): Progress and Trends. *Anal. Bioanal. Chem.* **2012**, *403* (1), 27–54. <https://doi.org/10.1007/s00216-011-5631-x>.
- (247) Viets, C.; Hill, W. Laser Power Effects in SERS Spectroscopy at Thin Metal Films. *J. Phys. Chem. B* **2001**, *105* (27), 6330–6336. <https://doi.org/10.1021/jp004315c>.
- (248) Bro, R. PARAFAC. Tutorial and Applications. *Chemom. Intell. Lab. Syst.* **1997**, *38* (2), 149–171. [https://doi.org/10.1016/S0169-7439\(97\)00032-4](https://doi.org/10.1016/S0169-7439(97)00032-4).
- (249) Plieth, W.; Wilson, G. S.; de la Fe, C. G. Spectroelectrochemistry: A Survey of in Situ Spectroscopic Techniques. *Pure Appl. Chem.* **1998**, *70* (7), 1395–1414. <https://doi.org/10.1351/pac199870071395>.
- (250) Xie, Q.; Wei, W.; Nie, L.; Yao, S. Derivative Linear Sweep and Derivative Cyclic

- Voltabsorptometry of the Long-Path-Length Spectroelectrochemical Cell: The Single Reversible Electrode Reaction. *J. Electroanal. Chem.* **1993**, 348 (1–2), 29–47. [https://doi.org/10.1016/0022-0728\(93\)80121-W](https://doi.org/10.1016/0022-0728(93)80121-W).
- (251) Kulesza, P. J.; Zamponi, S.; Malik, M. A.; Miecznikowski, K.; Berrettoni, M.; Marassi, R. Spectroelectrochemical Identity of Prussian Blue Films in Various Electrolytes: Comparison of Time-Derivative Voltabsorptometric Responses with Conventional Cyclic Voltammetry. *J. Solid State Electrochem.* **1997**, 1 (1), 88–93. <https://doi.org/10.1007/s100080050027>.
- (252) Barclay, V. J.; Bonner, R. F.; Hamilton, I. P. Application of Wavelet Transforms to Experimental Spectra: Smoothing, Denoising, and Data Set Compression. *Anal. Chem.* **1997**, 69 (1), 78–90. <https://doi.org/10.1021/ac960638m>.
- (253) Savitzky, A.; Golay, M. J. E. Smoothing and Differentiation of Data by Simplified Least Squares Procedures. *Anal. Chem.* **1964**, 36 (8), 1627–1639. <https://doi.org/10.1021/ac60214a047>.
- (254) Lieber, C. A.; Mahadevan-Jansen, A. Automated Method for Subtraction of Fluorescence from Biological Raman Spectra. *Appl. Spectrosc.* **2003**, 57 (11), 1363–1367. <https://doi.org/10.1366/000370203322554518>.
- (255) Bro, R.; Kiers, H. A. L. A New Efficient Method for Determining the Number of Components in PARAFAC Models. *J. Chemom.* **2003**, 17 (5), 274–286. <https://doi.org/10.1002/cem.801>.
- (256) Andersson, C. A.; Bro, R. The N-Way Toolbox for MATLAB. *Chemom. Intell. Lab. Syst.* **2000**, 52 (1), 1–4. [https://doi.org/10.1016/S0169-7439\(00\)00071-X](https://doi.org/10.1016/S0169-7439(00)00071-X).
- (257) Olmo-Alonso, F.; Garoz-Ruiz, J.; Heras, A.; Colina, A. Normal or Parallel Configuration in Spectroelectrochemistry? Bidimensional Spectroelectroanalysis in Presence of an Antioxidant Compound. *J. Electroanal. Chem.* **2023**, 935, 117333. <https://doi.org/10.1016/j.jelechem.2023.117333>.
- (258) Lozeman, J. J. A.; Führer, P.; Olthuis, W.; Odijk, M. Spectroelectrochemistry, the Future of Visualizing Electrode Processes by Hyphenating Electrochemistry with Spectroscopic Techniques. *Analyst* **2020**, 145 (7), 2482–2509. <https://doi.org/10.1039/C9AN02105A>.
- (259) Roday, L.; González, J.; Molina, Á.; Laborda, E. Investigating Comproportionation in Multielectron Transfers via UV–Visible Spectroelectrochemistry: The Electroreduction of Anthraquinone-2-Sulfonate in Aqueous Media. *Anal. Chem.* **2022**, 94 (35), 12152–12158. <https://doi.org/10.1021/acs.analchem.2c02523>.

- (260) Gui, Y. P.; Kuwana, T. Long Optical Path Length Thin-Layer Spectroelectrochemistry: Hydrogenation of Adsorbed Aromatics by Coadsorbed Hydrogen Atoms at Platinum. *Langmuir* **1986**, 2 (4), 471–476. <https://doi.org/10.1021/la00070a017>.
- (261) Kusu, F.; Kuwana, T. Long Optical Path Length Thin-Layer Spectrochemistry Potential Dependence and Quantitation of 1 H -Purine-6-Amine Adsorbed on Gold. *Chem. Lett.* **1988**, 17 (3), 531–534. <https://doi.org/10.1246/cl.1988.531>.
- (262) Laborda, E.; García-Martínez, J.; Molina, A. Spectroelectrochemistry for the Study of Reversible Electrode Reactions with Complex Stoichiometries. *Electrochem. commun.* **2021**, 123, 106915. <https://doi.org/10.1016/j.elecom.2020.106915>.
- (263) Wang, W.; Grace, H. M.; Flowers, P. A. Simple Microfluidic Device for Spectroelectrochemistry. *Microchem. J.* **2022**, 181, 107718. <https://doi.org/10.1016/j.microc.2022.107718>.
- (264) Oliveira, L. G.; Lemos, S. G.; Frago, W. D. Simultaneous Determination of Benzenediol Isomers in Tap Water by Second-Order Calibration and Voltabsorptometry. *Electrochim. Acta* **2020**, 354, 136591. <https://doi.org/10.1016/j.electacta.2020.136591>.
- (265) Ghoorchian, A.; Afkhami, A.; Madrakian, T.; Rameshan, R.; Rameshan, C.; Hajian, A. Absorbance-Based Spectroelectrochemical Sensor for Determination of Ampyra Based on Electrochemical Preconcentration. *Sensors Actuators, B Chem.* **2020**, 324, 128723. <https://doi.org/10.1016/j.snb.2020.128723>.
- (266) Ali, H.; Verma, N. A Hybrid UV-Vis Spectroelectrochemical Approach for Measuring Folic Acid Using a Novel Ni-CNF/ITO Electrode. *Electrochim. Acta* **2022**, 428, 140920. <https://doi.org/10.1016/j.electacta.2022.140920>.
- (267) Sirin Ustabasi, G.; Bastos-Arrieta, J.; Pérez-Ràfols, C.; Serrano, N.; Díaz-Cruz, J. M. Considerations on the Use of Spectroelectrochemistry in Reflection Mode for Quantitative Analysis: Study of the Fe(III)/Fe(II) – Orthophenanthroline System. *Microchem. J.* **2022**, 181, 107678. <https://doi.org/10.1016/j.microc.2022.107678>.
- (268) Flatmark. Catecholamine Biosynthesis and Physiological Regulation in Neuroendocrine Cells. *Acta Physiol. Scand.* **2000**, 168 (1), 1–17. <https://doi.org/10.1046/j.1365-201x.2000.00596.x>.
- (269) Harley-Mason, J. 259. The Chemistry of Adrenochrome and Its Derivatives. *J. Chem. Soc.* **1950**, 1276. <https://doi.org/10.1039/jr9500001276>.
- (270) Hernández, P.; Sánchez, I.; Patón, F.; Hernández, L. Cyclic Voltammetry Determination of Epinephrine with a Carbon Fiber Ultramicroelectrode. *Talanta* **1998**, 46 (5), 985–991.

- [https://doi.org/10.1016/S0039-9140\(97\)00353-6](https://doi.org/10.1016/S0039-9140(97)00353-6).
- (271) Xue, K.-H.; Liu, J.-M.; Wei, R.-B.; Chen, S.-P. Electrochemical Behavior of Adrenaline at the Carbon Atom Wire Modified Electrode. *Chem. Phys.* **2006**, *327* (2–3), 319–326. <https://doi.org/10.1016/j.chemphys.2006.05.004>.
- (272) Shankar, S. S.; Shereema, R. M.; Rakhi, R. B. Electrochemical Determination of Adrenaline Using MXene/Graphite Composite Paste Electrodes. *ACS Appl. Mater. Interfaces* **2018**, *10* (50), 43343–43351. <https://doi.org/10.1021/acsami.8b11741>.
- (273) KIM, S. H.; YEO, I.-H. Spectroelectrochemical Studies on the Oxidation Pathway of Epinephrine. *Anal. Sci.* **1997**, *13*, 321–324. https://doi.org/10.2116/analsci.13.Supplement_321.
- (274) Salimi, A.; MamKhezri, H.; Hallaj, R. Simultaneous Determination of Ascorbic Acid, Uric Acid and Neurotransmitters with a Carbon Ceramic Electrode Prepared by Sol–Gel Technique. *Talanta* **2006**, *70* (4), 823–832. <https://doi.org/10.1016/j.talanta.2006.02.015>.
- (275) Nasirizadeh, N.; Shekari, Z.; Zare, H. R.; Reza Shishehbore, M.; Fakhari, A. R.; Ahmar, H. Electrosynthesis of an Imidazole Derivative and Its Application as a Bifunctional Electrocatalyst for Simultaneous Determination of Ascorbic Acid, Adrenaline, Acetaminophen, and Tryptophan at a Multi-Wall Carbon Nanotubes Modified Electrode Surface. *Biosens. Bioelectron.* **2013**, *41*, 608–614. <https://doi.org/10.1016/j.bios.2012.09.028>.
- (276) Wierzbicka, E.; Szultka-Młyńska, M.; Buszewski, B.; Sulka, G. D. Epinephrine Sensing at Nanostructured Au Electrode and Determination Its Oxidative Metabolism. *Sensors Actuators B Chem.* **2016**, *237*, 206–215. <https://doi.org/10.1016/j.snb.2016.06.073>.
- (277) Pradhan, P.; Mascarenhas, R. J.; Thomas, T.; Namboothiri, I. N. N.; D’Souza, O. J.; Mekhalif, Z. Electropolymerization of Bromothymol Blue on Carbon Paste Electrode Bulk Modified with Oxidized Multiwall Carbon Nanotubes and Its Application in Amperometric Sensing of Epinephrine in Pharmaceutical and Biological Samples. *J. Electroanal. Chem.* **2014**, *732*, 30–37. <https://doi.org/10.1016/j.jelechem.2014.08.023>.
- (278) Abdel-Hamid, R.; Newair, E. F. Adsorptive Stripping Voltammetric Determination of Gallic Acid Using an Electrochemical Sensor Based on Polypinephrine/Glassy Carbon Electrode and Its Determination in Black Tea Sample. *J. Electroanal. Chem.* **2013**, *704*, 32–37. <https://doi.org/10.1016/j.jelechem.2013.06.006>.
- (279) Gao, Z. F.; Li, T. T.; Xu, X. L.; Liu, Y. Y.; Luo, H. Q.; Li, N. B. Green Light-Emitting Polypinephrine-Based Fluorescent Organic Dots and Its Application in Intracellular

- Metal Ions Sensing. *Biosens. Bioelectron.* **2016**, *83*, 134–141. <https://doi.org/10.1016/j.bios.2016.04.041>.
- (280) Pisoschi, A. M.; Pop, A.; Serban, A. I.; Fafaneata, C. Electrochemical Methods for Ascorbic Acid Determination. *Electrochim. Acta* **2014**, *121*, 443–460. <https://doi.org/10.1016/j.electacta.2013.12.127>.
- (281) Olmo, F.; Rodriguez, A.; Colina, A.; Heras, A. UV/Vis Absorption Spectroelectrochemistry of Folic Acid. *J. Solid State Electrochem.* **2022**, *26* (1), 29–37. <https://doi.org/10.1007/s10008-021-05026-5>.
- (282) Paramo, A. E. A. E.; Palmero, S.; Heras, A.; Colina, A.; Ibañez, D. Development of Disposable Carbon Nanofibers Electrodes Supported on Filters. *Electroanalysis* **2016**, *28* (4), 890–897. <https://doi.org/10.1002/elan.201500576>.
- (283) Olmo, F.; Garoz-Ruiz, J.; Carazo, J.; Colina, A.; Heras, A. Spectroelectrochemical Determination of Isoprenaline in a Pharmaceutical Sample. *Sensors* **2020**, *20* (18), 1–13. <https://doi.org/10.3390/s20185179>.
- (284) Garoz-Ruiz, J.; Perales-Rondon, J. V.; Heras, A.; Colina, A.; Garoz-Ruiz, J.; Perales-Rondon, J. V.; Heras, A.; Colina, A. Spectroelectrochemistry of Quantum Dots. *Isr. J. Chem.* **2019**, *59* (8), 679–694. <https://doi.org/10.1002/ijch.201900028>.
- (285) Scherson, D. A.; Tolmachev, Y. V.; Stefan, I. C. Ultraviolet/Visible Spectroelectrochemistry. In *Encyclopedia of Analytical Chemistry*; Meyers, R. A., Ed.; John Wiley & Sons, Ltd: Chichester, UK, 2006; pp 1–54. <https://doi.org/10.1002/9780470027318.a5316>.
- (286) Keyes, T. E.; Forster, R. J. Spectroelectrochemistry. In *Handbook of Electrochemistry*; Zoski, C. G., Ed.; Elsevier B.V., 2007; pp 591–635. <https://doi.org/10.1016/B978-0-444-51958-0.50027-6>.
- (287) León, L.; Mozo, J. D. Designing Spectroelectrochemical Cells: A Review. *TrAC Trends Anal. Chem.* **2018**, *102*, 147–169. <https://doi.org/10.1016/j.trac.2018.02.002>.
- (288) Frantz, S.; Sieger, M.; Hartenbach, I.; Lissner, F.; Schleid, T.; Fiedler, J.; Duboc, C.; Kaim, W. Structure, Electrochemistry, Spectroscopy, and Magnetic Resonance, Including High-Field EPR, of $\{(\mu\text{-Abpy})[\text{Re}(\text{CO})_3\text{X}]_2\}^{0/+}$, Where Abpy=2,2'-Azobispyridine and X=F, Cl, Br. *J. Organomet. Chem.* **2009**, *694* (7–8), 1122–1133. <https://doi.org/10.1016/j.jorganchem.2008.09.034>.
- (289) Crayston, J. A. Spectroelectrochemistry. In *Comprehensive Coordination Chemistry II*; McCleverty, J. A., Meyer, T. J., Eds.; Elsevier, 2003; pp 775–789.

- <https://doi.org/10.1016/B0-08-043748-6/01240-8>.
- (290) Gonzalez-Baro, A. C.; Izquierdo, D.; Heras, A.; Colina, A. UV/Vis Spectroelectrochemistry of *o*-Vanillin: Study of the Antioxidant Properties. *J. Electroanal. Chem.* **2020**, 859, 113844. <https://doi.org/10.1016/j.jelechem.2020.113844>.
- (291) Ventosa, E.; Colina, A.; Heras, A.; Ruiz, V.; Garoz, J.; López-Palacios, J. One-Pot Synthesis of Gold/Poly(3,4-Ethylenedioxythiophene) Nanocomposite. *J. Nanoparticle Res.* **2012**, 14 (1), 661. <https://doi.org/10.1007/s11051-011-0661-z>.
- (292) Zanardi, C.; Terzi, F.; Pigani, L.; Heras, a.; Colina, a.; Lopez-Palacios, J.; Seeber, R. Development and Characterisation of a Novel Composite Electrode Material Consisting of Poly(3,4-Ethylenedioxythiophene) Including Au Nanoparticles. *Electrochim. Acta* **2008**, 53 (11), 3916–3923. <https://doi.org/10.1016/j.electacta.2007.07.057>.
- (293) Di Marino, M.; Marassi, R.; Santucci, R.; Brunori, M.; Ascoli, F. A Spectroelectrochemical Study of Carboxymethylated Cytochrome-C. *Bioelectrochemistry Bioenerg.* **1987**, 17 (1), 27–34. [https://doi.org/10.1016/0302-4598\(87\)80004-1](https://doi.org/10.1016/0302-4598(87)80004-1).
- (294) Kulesza, P. J.; Zamponi, S.; Malik, M. A.; Berrettoni, M.; Wolkiewicz, A.; Marassi, R. Spectroelectrochemical Characterization of Cobalt Hexacyanoferrate Films in Potassium Salt Electrolyte. *Electrochim. Acta* **1998**, 43 (8), 919–923. [https://doi.org/10.1016/S0013-4686\(97\)00212-0](https://doi.org/10.1016/S0013-4686(97)00212-0).
- (295) Kulesza, P. J.; Miecznikowski, K.; Chojak, M.; Malik, M. A.; Zamponi, S.; Marassi, R. Electrochromic Features of Hybrid Films Composed of Polyaniline and Metal Hexacyanoferrate. *Electrochim. Acta* **2001**, 46 (28), 4371–4378. [https://doi.org/10.1016/S0013-4686\(01\)00681-8](https://doi.org/10.1016/S0013-4686(01)00681-8).
- (296) Tesařová, E.; Heras, A.; Colina, Á.; Ruiz, V.; Švancara, I.; Vytrás, K.; López-Palacios, J. A Spectroelectrochemical Approach to the Electrodeposition of Bismuth Film Electrodes and Their Use in Stripping Analysis. *Anal. Chim. Acta* **2008**, 608 (2), 140–146. <https://doi.org/10.1016/j.aca.2007.12.023>.
- (297) Noyhouzer, T.; Snowden, M. E.; Tefashe, U. M.; Mauzeroll, J. Modular Flow-Through Platform for Spectroelectrochemical Analysis. *Anal. Chem.* **2017**, 89 (10), 5246–5253. <https://doi.org/10.1021/acs.analchem.6b04649>.
- (298) Ball, G. F. M. *Water-Soluble Vitamin Assays in Human Nutrition*; Springer US: Boston, MA, MA, 1994. <https://doi.org/10.1007/978-1-4615-2061-0>.
- (299) Reynolds, E. Vitamin B12, Folic Acid, and the Nervous System. *Lancet Neurol.* **2006**, 5 (11), 949–960. [https://doi.org/10.1016/S1474-4422\(06\)70598-1](https://doi.org/10.1016/S1474-4422(06)70598-1).

- (300) Donnelly, J. G. Folic Acid. *Crit. Rev. Clin. Lab. Sci.* **2001**, 38 (3), 183–223. <https://doi.org/10.1080/20014091084209>.
- (301) Reynolds, E. H. The Neurology of Folic Acid Deficiency. In *Handbook of Clinical Neurology*; Biller, J., Ferro, J. M., Eds.; Elsevier B.V., 2014; Vol. 120, pp 927–943. <https://doi.org/10.1016/B978-0-7020-4087-0.00061-9>.
- (302) Basu, P.; Burgmayer, S. J. N. Pterin Chemistry and Its Relationship to the Molybdenum Cofactor. *Coord. Chem. Rev.* **2011**, 255 (9–10), 1016–1038. <https://doi.org/10.1016/j.ccr.2011.02.010>.
- (303) O’Neil, M. J. *The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals*, 13th ed.; Whitehouse Station, Merck Manuals: New York, NY, 2001.
- (304) Baibarac, M.; Smaranda, I.; Nila, A.; Serbschi, C. Optical Properties of Folic Acid in Phosphate Buffer Solutions: The Influence of pH and UV Irradiation on the UV-VIS Absorption Spectra and Photoluminescence. *Sci. Rep.* **2019**, 9 (1), 14278. <https://doi.org/10.1038/s41598-019-50721-z>.
- (305) Gazzali, A. M.; Lobry, M.; Colombeau, L.; Acherar, S.; Azaïs, H.; Mordon, S.; Arnoux, P.; Baros, F.; Vanderesse, R.; Frochot, C. Stability of Folic Acid under Several Parameters. *Eur. J. Pharm. Sci.* **2016**, 93, 419–430. <https://doi.org/10.1016/j.ejps.2016.08.045>.
- (306) Kretzschmar, K.; Jaenicke, W. Der Redoxmechanismus Des Systems Folsäure—Dihydrofolsäure—Tetrahydrofolsäure II / The Redox Mechanism of the System Folic Acid — Dihydrofolic Acid—Tetrahydrofolic Acid II. *Zeitschrift für Naturforsch. B* **1971**, 26 (10), 999–1002. <https://doi.org/10.1515/znb-1971-1008>.
- (307) Ramírez-Herrera, D. E.; Reyes-Cruzaley, A. P.; Dominguez, G.; Paraguay-Delgado, F.; Tirado-Guizar, A.; Pina-Luis, G. CdTe Quantum Dots Modified with Cysteamine: A New Efficient Nanosensor for the Determination of Folic Acid. *Sensors* **2019**, 19 (20), 4548. <https://doi.org/10.3390/s19204548>.
- (308) Castro, R. C.; Ribeiro, D. S. M.; Páscoa, R. N. M. J.; Soares, J. X.; Mazivila, S. J.; Santos, J. L. M. Dual-Emission CdTe/AgInS₂ Photoluminescence Probe Coupled to Neural Network Data Processing for the Simultaneous Determination of Folic Acid and Iron (II). *Anal. Chim. Acta* **2020**, 1114, 29–41. <https://doi.org/10.1016/j.aca.2020.04.007>.
- (309) Azizi, S. N.; Shakeri, P.; Chaichi, M. J.; Bekhradnia, A.; Taghavi, M.; Ghaemy, M. The Use of Imidazolium Ionic Liquid/Copper Complex as Novel and Green Catalyst for Chemiluminescent Detection of Folic Acid by Mn-Doped ZnS Nanocrystals. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2014**, 122, 482–488.

- <https://doi.org/10.1016/j.saa.2013.11.036>.
- (310) Nagaraja, P.; Vasantha, R. A.; Yathirajan, H. S. Spectrophotometric Determination of Folic Acid in Pharmaceutical Preparations by Coupling Reactions with Iminodibenzyl or 3-Aminophenol or Sodium Molybdate-Pyrocatechol. *Anal. Biochem.* **2002**, *307* (2), 316–321. [https://doi.org/10.1016/S0003-2697\(02\)00038-6](https://doi.org/10.1016/S0003-2697(02)00038-6).
 - (311) Abo El-Maali, N. Carbon Paste Electrodes Modified with Palmitic Acid and Stearic Acid for the Determination of Folic Acid (Vitamin B9) in Both Aqueous and Biological Media. *J. Electroanal. Chem.* **1992**, *342* (3), 465–473. [https://doi.org/10.1016/0022-0728\(92\)85139-T](https://doi.org/10.1016/0022-0728(92)85139-T).
 - (312) Vaze, V. D.; Srivastava, A. K. Electrochemical Behavior of Folic Acid at Calixarene Based Chemically Modified Electrodes and Its Determination by Adsorptive Stripping Voltammetry. *Electrochim. Acta* **2007**, *53* (4), 1713–1721. <https://doi.org/10.1016/j.electacta.2007.08.017>.
 - (313) O'Shea, T. J.; Garcia, A. C.; Blanco, P. T.; Smyth, M. R. Electrochemical Pretreatment of Carbon Fibre Microelectrodes for the Determination of Folic Acid. *J. Electroanal. Chem. Interfacial Electrochem.* **1991**, *307* (1–2), 63–71. [https://doi.org/10.1016/0022-0728\(91\)85539-2](https://doi.org/10.1016/0022-0728(91)85539-2).
 - (314) Chen, Z.; Chen, B.; Yao, S. High-Performance Liquid Chromatography/Electrospray Ionization-Mass Spectrometry for Simultaneous Determination of Taurine and 10 Water-Soluble Vitamins in Multivitamin Tablets. *Anal. Chim. Acta* **2006**, *569* (1–2), 169–175. <https://doi.org/10.1016/j.aca.2006.03.099>.
 - (315) Brusač; Jeličić; Amidžić Klarić; Nigović; Turk; Klarić; Mornar. Pharmacokinetic Profiling and Simultaneous Determination of Thiopurine Immunosuppressants and Folic Acid by Chromatographic Methods. *Molecules* **2019**, *24* (19), 3469. <https://doi.org/10.3390/molecules24193469>.
 - (316) Gill, B. D.; Saldo, S.; Wood, J. E.; Indyk, H. E. A Rapid Method for the Determination of Biotin and Folic Acid in Liquid Milk, Milk Powders, Infant Formula, and Milk-Based Nutritional Products by Liquid Chromatography–Tandem Mass Spectrometry. *J. AOAC Int.* **2018**, *101* (5), 1578–1583. <https://doi.org/10.5740/jaoacint.18-0065>.
 - (317) Szakács, Z.; Noszál, B. Determination of Dissociation Constants of Folic Acid, Methotrexate, and Other Photolabile Pteridines by Pressure-Assisted Capillary Electrophoresis. *Electrophoresis* **2006**, *27* (17), 3399–3409. <https://doi.org/10.1002/elps.200600128>.

- (318) Thomas, A. H.; Suárez, G.; Cabrerizo, F. M.; García Einschlag, F. S.; Martino, R.; Baiocchi, C.; Pramauro, E.; Capparelli, A. L. Photochemical Behavior of Folic Acid in Alkaline Aqueous Solutions and Evolution of Its Photoproducts. *Helv. Chim. Acta* **2002**, *85* (8), 2300–2315. [https://doi.org/10.1002/1522-2675\(200208\)85:8<2300::AID-HLCA2300>3.0.CO;2-B](https://doi.org/10.1002/1522-2675(200208)85:8<2300::AID-HLCA2300>3.0.CO;2-B).
- (319) Chakraborty, P.; Bairi, P.; Roy, B.; Nandi, A. K. Improved Mechanical and Electronic Properties of Co-Assembled Folic Acid Gel with Aniline and Polyaniline. *ACS Appl. Mater. Interfaces* **2014**, *6* (5), 3615–3622. <https://doi.org/10.1021/am405868j>.
- (320) Thomas, A.; Einschlag, F. G.; Féliz, M. R.; Capparelli, A. L. First Steps in the Photochemistry of Folate in Alkaline Medium. *J. Photochem. Photobiol. A Chem.* **1998**, *116* (3), 187–190. [https://doi.org/10.1016/S1010-6030\(98\)00304-9](https://doi.org/10.1016/S1010-6030(98)00304-9).
- (321) Thomas, A. H.; Lorente, C.; Capparelli, A. L.; Pokhrel, M. R.; Braun, A. M.; Oliveros, E. Fluorescence of Pterin, 6-Formylpterin, 6-Carboxypterin and Folic Acid in Aqueous Solution: pH Effects. *Photochem. Photobiol. Sci.* **2002**, *1* (6), 421–426. <https://doi.org/10.1039/b202114e>.
- (322) Olmo, F.; Colina, A.; Heras, A. Determination of Ofloxacin in Urine Using UV/Vis Absorption Spectroelectrochemistry. *Microchem. J.* **2024**, *198*, 110186. <https://doi.org/10.1016/j.microc.2024.110186>.
- (323) Li, J.; Xue, Q.; Chen, T.; Liu, F.; Wang, Q.; Chang, C.; Lu, X.; Zhou, T.; Niwa, O. The Influence Mechanism of the Molecular Structure on the Peak Current and Peak Potential in Electrochemical Detection of Typical Quinolone Antibiotics. *Phys. Chem. Chem. Phys.* **2021**, *23* (25), 13873–13877. <https://doi.org/10.1039/D1CP01358K>.
- (324) Kloskowski, T.; Frąckowiak, S.; Adamowicz, J.; Szeliski, K.; Rasmus, M.; Drewa, T.; Pokrywczynska, M. Quinolones as a Potential Drug in Genitourinary Cancer Treatment—A Literature Review. *Front. Oncol.* **2022**, *12* (June), 1–14. <https://doi.org/10.3389/fonc.2022.890337>.
- (325) Bush, N. G.; Diez-Santos, I.; Abbott, L. R.; Maxwell, A. Contributions to Antibiotic Resistance. *Molecules* **2020**, *25* (23), 5662.
- (326) Appelbaum, P. C.; Hunter, P. A. The Fluoroquinolone Antibacterials: Past, Present and Future Perspectives. *Int. J. Antimicrob. Agents* **2000**, *16* (1), 5–15. [https://doi.org/10.1016/S0924-8579\(00\)00192-8](https://doi.org/10.1016/S0924-8579(00)00192-8).
- (327) Wong, A.; Silva, T. A.; Vicentini, F. C.; Fatibello-Filho, O. Electrochemical Sensor Based on Graphene Oxide and Ionic Liquid for Ofloxacin Determination at Nanomolar Levels.

- Talanta* **2016**, *161*, 333–341. <https://doi.org/10.1016/j.talanta.2016.08.035>.
- (328) Pi, Y.; Feng, J.; Song, M.; Sun, J. Degradation Potential of Ofloxacin and Its Resulting Transformation Products during Fenton Oxidation Process. *Chinese Sci. Bull.* **2014**, *59* (21), 2618–2624. <https://doi.org/10.1007/s11434-014-0293-7>.
- (329) Orimolade, B. O.; Oladipo, A. O.; Idris, A. O.; Usisipho, F.; Azizi, S.; Maaza, M.; Lebelo, S. L.; Mamba, B. B. Advancements in Electrochemical Technologies for the Removal of Fluoroquinolone Antibiotics in Wastewater: A Review. *Sci. Total Environ.* **2023**, *881*, 163522. <https://doi.org/10.1016/j.scitotenv.2023.163522>.
- (330) Redgrave, L. S.; Sutton, S. B.; Webber, M. A.; Piddock, L. J. V. Fluoroquinolone Resistance: Mechanisms, Impact on Bacteria, and Role in Evolutionary Success. *Trends Microbiol.* **2014**, *22* (8), 438–445. <https://doi.org/10.1016/j.tim.2014.04.007>.
- (331) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* **2012**, *9* (7), 671–675. <https://doi.org/10.1038/nmeth.2089>.
- (332) Abd El-Hamid, S. M.; Sadeek, S. A.; Zordok, W. A.; El-Shwiniy, W. H. Synthesis, Spectroscopic Studies, DFT Calculations, Cytotoxicity and Antimicrobial Activity of Some Metal Complexes with Ofloxacin and 2,2'-Bipyridine. *J. Mol. Struct.* **2019**, *1176*, 422–433. <https://doi.org/10.1016/j.molstruc.2018.08.082>.
- (333) Park, Hyeong Ryun ; Jeong, Gwang Yeong ; Lee, Hyeong Cheol ; Lee, Jin Gi ; Baek, G. M. Ionization and Divalent Cation Complexation of Quinolone Antibiotics in Aqueous Solution. *Ioniz. Complexation Quinolone* **2000**, *21* (9), 849–854. <https://doi.org/https://doi.org/10.5012/bkcs.2000.21.9.849>.
- (334) Baig, N.; Rana, A.; Kawde, A. N. Modified Electrodes for Selective Voltammetric Detection of Biomolecules. *Electroanalysis* **2018**, *30* (11), 2551–2574. <https://doi.org/10.1002/elan.201800468>.
- (335) Ping, J.; Wu, J.; Wang, Y.; Ying, Y. Simultaneous Determination of Ascorbic Acid, Dopamine and Uric Acid Using High-Performance Screen-Printed Graphene Electrode. *Biosens. Bioelectron.* **2012**, *34* (1), 70–76. <https://doi.org/10.1016/j.bios.2012.01.016>.
- (336) Tukimin, N.; Abdullah, J.; Sulaiman, Y. Electrodeposition of Poly(3,4-Ethylenedioxythiophene)/Reduced Graphene Oxide/Manganese Dioxide for Simultaneous Detection of Uric Acid, Dopamine and Ascorbic Acid. *J. Electroanal. Chem.* **2018**, *820*, 74–81. <https://doi.org/10.1016/j.jelechem.2018.04.065>.
- (337) Dinesh, B.; Vilian, A. T. E.; Kwak, C. H.; Huh, Y. S.; Saraswathi, R.; Han, Y. K. The Facile and Simple Synthesis of Poly(3,4-ethylenedioxythiophene) Anchored Reduced Graphene

- Oxide Nanocomposite for Biochemical Analysis. *Anal. Chim. Acta* **2019**, *1077*, 150–159. <https://doi.org/10.1016/j.aca.2019.05.053>.
- (338) Iranmanesh, T.; Foroughi, M. M.; Jahani, S.; Shahidi Zandi, M.; Hassani Nadiki, H. Green and Facile Microwave Solvent-Free Synthesis of CeO₂ Nanoparticle-Decorated CNTs as a Quadruplet Electrochemical Platform for Ultrasensitive and Simultaneous Detection of Ascorbic Acid, Dopamine, Uric Acid and Acetaminophen. *Talanta* **2020**, *207*, 120318. <https://doi.org/10.1016/j.talanta.2019.120318>.
- (339) Bagheri, H.; Pajooheshpour, N.; Jamali, B.; Amidi, S.; Hajian, A.; Khoshshafar, H. A Novel Electrochemical Platform for Sensitive and Simultaneous Determination of Dopamine, Uric Acid and Ascorbic Acid Based on Fe₃O₄[Sbnd]SnO₂[Sbnd]Gr Ternary Nanocomposite. *Microchem. J.* **2017**, *131*, 120–129. <https://doi.org/10.1016/j.microc.2016.12.006>.
- (340) Li, Q.; Huo, C.; Yi, K.; Zhou, L.; Su, L.; Hou, X. Preparation of Flake Hexagonal BN and Its Application in Electrochemical Detection of Ascorbic Acid, Dopamine and Uric Acid. *Sensors Actuators, B Chem.* **2018**, *260*, 346–356. <https://doi.org/10.1016/j.snb.2017.12.208>.
- (341) Wang, T.; Zhao, D.; Alvarez, N.; Shanov, V. N.; Heineman, W. R. Optically Transparent Carbon Nanotube Film Electrode for Thin Layer Spectroelectrochemistry. *Anal. Chem.* **2015**, *87* (19), 9687–9695. <https://doi.org/10.1021/acs.analchem.5b01784>.
- (342) Wang, Y.; Jiang, H.; Tian, J. J.; He, J. B. Spectroelectrochemistry of Salicylaldehyde Oxidation. *Electrochim. Acta* **2014**, *125*, 133–140. <https://doi.org/10.1016/j.electacta.2014.01.087>.
- (343) Murray, R. W.; Heineman, W. R.; O'Dom, G. W. An Optically Transparent Thin Layer Cell. *Anal. Chem.* **1967**, *39* (13), 1966–1968. <https://doi.org/10.1021/ac50156a052>.
- (344) Imai, K.; Okazaki, T.; Hata, N.; Taguchi, S.; Sugawara, K.; Kuramitz, H. Simultaneous Multiselective Spectroelectrochemical Fiber-Optic Sensor: Demonstration of the Concept Using Methylene Blue and Ferrocyanide. *Anal. Chem.* **2015**, *87* (4), 2375–2382. <https://doi.org/10.1021/ac504321u>.
- (345) Shtokyo, T.; Conklin, S.; Maghasi, A. T.; Richardson, J. N.; Piruska, A.; Seliskar, C. J.; Heineman, W. R. Spectroelectrochemical Sensing Based on Attenuated Total Internal Reflectance Stripping Voltammetry. 3. Determination of Cadmium and Copper. *Anal. Chem.* **2004**, *76* (5), 1466–1473. <https://doi.org/10.1021/ac034829i>.
- (346) Asadpour-Zeynali, K.; Maryam Sajjadi, S.; Taherzadeh, F. Second Order Advantage

- Obtained by Spectroelectrochemistry along with Novel Carbon Nanotube Modified Mesh Electrode: Application for Determination of Acetaminophen in Novafen Samples. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **2016**, *153*, 674–680. <https://doi.org/10.1016/j.saa.2015.09.035>.
- (347) Al Alamein, A. M. A.; Elwy, H. M.; El-Din, S. H. S. Univariate and Multivariate Spectrophotometric Methods for Simultaneous Determination of Avobenzone and Octinoxate in Pure Form and in Cosmetic Formulations: A Comparative Study. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **2019**, *206*, 37–47. <https://doi.org/10.1016/j.saa.2018.07.073>.
- (348) Moussa, B. A.; El-Zaher, A. A.; Mahrouse, M. A.; Ahmed, M. S. Resolution of Overlapped Spectra for the Determination of Ternary Mixture Using Different and Modified Spectrophotometric Methods. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **2016**, *165*, 127–137. <https://doi.org/10.1016/j.saa.2016.04.003>.
- (349) Gutiérrez, M. C. Derivative Spectroscopy Applied to the Determination of Alpha and Beta Acids in Hops. *J. Inst. Brew.* **1992**, *98* (4), 277–281. <https://doi.org/10.1002/j.2050-0416.1992.tb01108.x>.
- (350) Jin, B. K.; Li, L.; Huang, J. L.; Zhang, S. Y.; Tian, Y. P.; Yang, J. X. IR Spectroelectrochemical Cyclic Voltabsorptometry and Derivative Cyclic Voltabsorptometry. *Anal. Chem.* **2009**, *81* (11), 4476–4481. <https://doi.org/10.1021/ac9003634>.
- (351) Cheng, W. X.; Jin, B. K.; Huang, P.; Cheng, L. J.; Zhang, S. Y.; Tian, Y. P. Investigation on the π -Dimer/ σ -Dimer of 1,8-Dihydroxy-9,10- Anthracenedione in the Process of Electrochemical Reduction by Using IR Spectroelectrochemical Cyclic Voltabsorptometry and Derivative Cyclic Voltabsorptometry. *J. Phys. Chem. C* **2013**, *117* (8), 3940–3948. <https://doi.org/10.1021/jp3116947>.
- (352) Murphy, K. R.; Stedmon, C. A.; Graeber, D.; Bro, R. Fluorescence Spectroscopy and Multi-Way Techniques. PARAFAC. *Anal. Methods* **2013**, *5* (23), 6557–6566. <https://doi.org/10.1039/c3ay41160e>.
- (353) Bro, R. Review on Multiway Analysis in Chemistry - 2000-2005. *Crit. Rev. Anal. Chem.* **2006**, *36* (3–4), 279–293. <https://doi.org/10.1080/10408340600969965>.
- (354) Rueda, M.; Aldaz, A.; Sanchez-Burgos, F. Oxidation of L-Ascorbic Acid on a Gold Electrode. *Electrochim. Acta* **1978**, *23* (5), 419–424. [https://doi.org/10.1016/0013-4686\(78\)87040-6](https://doi.org/10.1016/0013-4686(78)87040-6).

- (355) Zhang, F.; Dryhurst, G. Oxidation Chemistry of Dopamine: Possible Insights into the Age-Dependent Loss of Dopaminergic Nigrostriatal Neurons. *Bioorg. Chem.* **1993**, *21* (4), 392–410. <https://doi.org/10.1006/bioo.1993.1033>.
- (356) Barreto, W. J.; Ponzoni, S.; Sassi, P. A Raman and UV-Vis Study of Catecholamines Oxidized with Mn(III). *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **1999**, *55* (1), 65–72. [https://doi.org/10.1016/S1386-1425\(98\)00164-4](https://doi.org/10.1016/S1386-1425(98)00164-4).
- (357) Martín-Yerga, D.; Pérez-Junquera, A.; González-García, M. B.; Perales-Rondon, J. V.; Heras, A.; Colina, A.; Hernández-Santos, D.; Fanjul-Bolado, P. Quantitative Raman Spectroelectrochemistry Using Silver Screen-Printed Electrodes. *Electrochim. Acta* **2018**, *264*, 183–190. <https://doi.org/10.1016/j.electacta.2018.01.060>.
- (358) Pinyayev, T. S.; Seliskar, C. J.; Heineman, W. R. Fluorescence Spectroelectrochemical Sensor for 1-Hydroxypyrene. *Anal. Chem.* **2010**, *82* (23), 9743–9748. <https://doi.org/10.1021/ac101883a>.
- (359) Verma, H. N.; Singh, P.; Chavan, R. M. Gold Nanoparticle: Synthesis and Characterization. *Vet. World* **2014**, *7* (2), 72–77. <https://doi.org/10.14202/vetworld.2014.72-77>.
- (360) Amir Tabrizi, Fatma Ayhan, H. A. Gold Nanoparticle Synthesis and Characterisation. *Hacettepe J. Biol. Chem.* **2009**, *37* (3), 217–226.
- (361) Shiraishi, Y.; Tanaka, H.; Sakamoto, H.; Ichikawa, S.; Hirai, T. Photoreductive Synthesis of Monodispersed Au Nanoparticles with Citric Acid as Reductant and Surface Stabilizing Reagent. *RSC Adv.* **2017**, *7* (11), 6187–6192. <https://doi.org/10.1039/C6RA27771C>.
- (362) Innocenzi, P.; Kozuka, H.; Yoko, T. Fluorescence Properties of the Ru(Bpy)₃²⁺ Complex Incorporated in Sol-Gel-Derived Silica Coating Films. *J. Phys. Chem. B* **1997**, *101* (13), 2285–2291. <https://doi.org/10.1021/jp970004z>.
- (363) Li, J.; Weng, Y.; Shen, C.; Luo, J.; Yu, D.; Cao, Z. Sensitive Fluorescence and Visual Detection of Organophosphorus Pesticides with a Ru(Bpy)₃²⁺-ZIF-90-MnO₂ sensing Platform. *Anal. Methods* **2021**, *13* (26), 2981–2988. <https://doi.org/10.1039/d1ay00841b>.
- (364) Kowacs, T.; Pan, Q.; Lang, P.; O'Reilly, L.; Rau, S.; Browne, W. R.; Pryce, M. T.; Huijser, A.; Vos, J. G. Supramolecular Bimetallic Assemblies for Photocatalytic Hydrogen Generation from Water. *Faraday Discuss.* **2015**, *185*, 143–170. <https://doi.org/10.1039/c5fd00068h>.
- (365) Smnová-Šloufová, I.; Vlčková, B.; Snoeck, T. L.; Stufkens, D. J.; Matějka, P. Surface-Enhanced Raman Scattering and Surface-Enhanced Resonance Raman Scattering

- Excitation Profiles of Ag-2,2'-Bipyridine Surface Complexes and of $[\text{Ru}(\text{Bpy})_3]^{2+}$ on Ag Colloidal Surfaces: Manifestations of the Charge-Transfer Resonance Contributions to Th. *Inorg. Chem.* **2000**, 39 (16), 3551–3559. <https://doi.org/10.1021/ic991372f>.
- (366) Silverstein, D. W.; Milojević, C. B.; Camden, J. P.; Jensen, L. Investigation of Linear and Nonlinear Raman Scattering for Isotopologues of $[\text{Ru}(\text{Bpy})_3]^{2+}$. *J. Phys. Chem. C* **2013**, 117 (40), 20855–20866. <https://doi.org/10.1021/jp4070505>.
- (367) Filevich, O.; Zayat, L.; Baraldo, L. M.; Etchenique, R. Long Wavelength Phototriggering: Ruthenium-Based Caged Compounds. *Struct. Bond.* **2014**, 165, 47–68. https://doi.org/10.1007/430_2014_169.
- (368) Leandri, V.; Liu, P.; Sadollahkhani, A.; Safdari, M.; Kloo, L.; Gardner, J. M. Excited-State Dynamics of $[\text{Ru}(\text{Bpy})_3]^{2+}$ Thin Films on Sensitized TiO_2 and ZrO_2 . *ChemPhysChem* **2019**, 20 (4), 618–626. <https://doi.org/10.1002/cphc.201801010>.
- (369) Pal, A. K.; Nag, S.; Ferreira, J. G.; Brochery, V.; La Ganga, G.; Santoro, A.; Serroni, S.; Campagna, S.; Hanan, G. S. Red-Emitting $[\text{Ru}(\text{Bpy})_2(\text{N-N})]^{2+}$ Photosensitizers: Emission from a Ruthenium(II) to 2,2'-Bipyridine 3 MLCT State in the Presence of Neutral Ancillary “Super Donor” Ligands. *Inorg. Chem.* **2014**, 53 (3), 1679–1689. <https://doi.org/10.1021/ic4028332>.
- (370) Luo, L.; Li, L.; Xu, X.; Liu, D.; Li, J.; Wang, K.; You, T. Determination of Pentachlorophenol by Anodic Electrochemiluminescence of $\text{Ru}(\text{Bpy})_3^{2+}$ Based on Nitrogen-Doped Graphene Quantum Dots as Co-Reactant. *RSC Adv.* **2017**, 7 (80), 50634–50642. <https://doi.org/10.1039/c7ra10247j>.
- (371) Ibáñez, D.; Begoña González-García, M.; Busto, J.; Pérez-Junquera, A.; Hernández-Santos, D.; Fanjul-Bolado, P. Development of a Novel Raman Cell for the Easy Handling of Spectroelectrochemical Measurements. *Microchem. J.* **2022**, 180 (April), 107614. <https://doi.org/10.1016/j.microc.2022.107614>.
- (372) Zheng, L.; Chi, Y.; Dong, Y.; Zhang, L.; Chen, G. Electrochemiluminescent Behavior of Tris(2,2-Bipyridine) Ruthenium(II)/Triethylamine in Ionic Liquid Solution. *J. Phys. Chem. C* **2008**, 112 (39), 15570–15575. <https://doi.org/10.1021/jp805609y>.
- (373) Norton, J. C. S.; Han, M. G.; Jiang, P.; Shim, G. H.; Ying, Y.; Creager, S.; Foulger, S. H. Poly(3,4-Ethylenedioxythiophene) (PEDOT)-Coated Silica Spheres: Electrochemical Modulation of the Optical Properties of a Hydrogel-Stabilized Core - Shell Particle Suspension. *Chem. Mater.* **2006**, 18 (19), 4570–4575. <https://doi.org/10.1021/cm060573w>.

- (374) Zheng, D.; Wang, N.; Wang, F. Q.; Dong, D.; Li, Y. G.; Yang, X. Q.; Guo, L. H.; Cheng, J. Sensitive Chemically Amplified Electrochemical Detection of Ruthenium Tris-(2,2'-Bipyridine) on Tin-Doped Indium Oxide Electrode. *Anal. Chim. Acta* **2004**, *508* (2), 225–231. <https://doi.org/10.1016/j.aca.2003.12.002>.
- (375) Venkateswara Raju, C.; Sornambigai, M.; Senthil Kumar, S. Ruthenium-Tris-Bipyridine Derivatives as a Divine Complex for Electrochemiluminescence Based Biosensor Applications. In *Ruthenium - An Element Loved by Researchers*; IntechOpen, 2022; pp 1–23. <https://doi.org/10.5772/intechopen.96819>.
- (376) Siddharthan, A.; Kumar, V.; Barooah, N.; Chatterjee, S.; Bhasikuttan, A. C.; Mohanty, J. Supramolecular Interaction of Ofloxacin Drug with P-Sulfonatocalix[6]Arene: Metal-Ion Responsive Fluorescence Behavior and Enhanced Antibacterial Activity. *J. Mol. Liq.* **2023**, *370*, 121047. <https://doi.org/10.1016/j.molliq.2022.121047>.
- (377) Jiang, C.; Wu, T.; Liu, J.; Wang, Y. Application of a Thermo-Sensitive Imprinted SERS Substrate to the Rapid Trace Detection of Ofloxacin. *Anal. Methods* **2020**, *12* (39), 4783–4788. <https://doi.org/10.1039/D0AY00616E>.
- (378) Teng, H.; Lv, M.; Liu, L.; Zhang, X.; Zhao, Y.; Wu, Z.; Xu, H. Quantitative Detection of NADH Using a Novel Enzyme-Assisted Method Based on Surface-Enhanced Raman Scattering. *Sensors* **2017**, *17* (4), 788. <https://doi.org/10.3390/s17040788>.
- (379) Perales-Rondon, J. V.; Hernandez, S.; Martin-Yerga, D.; Fanjul-Bolado, P.; Heras, A.; Colina, A. Electrochemical Surface Oxidation Enhanced Raman Scattering. *Electrochim. Acta* **2018**, *282*, 377–383. <https://doi.org/10.1016/j.electacta.2018.06.079>.

10. ABBREVIATIONS

5-MTHF	5-methyltetrahydrofolate
A	Absorbance
AA	Ascorbic Acid
BSEC	Bidimensional Spectroelectrochemistry
CA	Chronoamperogram
CAbs	Chronoabsorptograms
CE	Counter electrode
CM	Chemical mechanism
CNTs	Carbon nanotubes
CORCONDIA	Core consistency diagnostic
CT	Charge transfer
CV	Cyclic voltammogram
CVA	Cyclic voltabsorptogram
CVF	Cyclic voltaFluorogram
CVR	Cyclic voltaRamangram
DA	Dopamine
DAQ	Dopaminequinone
DHA	Dehydro-L-ascorbic acid
DHF	7,8-dihydrofolate
DNA	Deoxyribonucleic Acid
ECDs	Electrochromic devices
EM	Electromagnetic mechanism
EP	Epinephrine
EPQ	Epinephrinequinone
EPR	Electron paramagnetic resonance
FA	Folic acid
FQ	Fluoroquinolone
Glu	L-glutamic acid

I	Intensity
I₀	Initial intensity
IR	Infrared
ITO	Indium tin oxide
LED	Light emitting diode
MAD	Multipulsed amperometric detection
MBTBT	4,4'-bis(2-methylbutylthio)-2,2'-bithiophene
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
NTDs	Neuronal tube defects
OFL	Ofloxacin
ORC	Oxidation-reduction cycles
OTE's	Optically transparent electrodes
PABA	<i>p</i> -aminobenzoic acid
PARAFAC	Parallel Factor Analysis
PGA	<i>p</i> -aminobenzoyl-L-glutamic acid
PL	Photoluminescence
PMMA	Poly(methyl methacrylate)
POMs	Polyoxometalates
RE	Pseudo-reference electrode
RHE	Reversible hydrogen electrode
RGO	Reduced graphene oxide
RNA	Ribonucleic Acid
SEC	Spectroelectrochemistry
SECM	Scanning electrochemical microscopy
SEM	Scanning Electron Microscopy
SERS	Surface Enhanced Raman Scattering
SOERS	Surface Oxidation Enhanced Raman Scattering

SPE	Screen-printed electrode
T	Transmittance
THF	5,6,7,8-tetrahydrofolate
TIRF	Total internal reflection fluorescence
UA	Uric acid
UV/Vis	Ultraviolet/Visible
WE	working electrode
WHO	World Health Organization

