Determination of nicotinamide in a multivitamin complex by Electrochemical-Surface Enhanced Raman Spectroscopy.

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Abstract

Time resolved Raman spectroelectrochemistry is a powerful technique to prepare useful Surface Enhanced Raman Scattering (SERS) substrates useful for quantitative analysis. Moreover, following the evolution of the Raman signal with potential the selection of the best conditions for the analysis is quite simple. In this work, gold SERS substrates are prepared on screen-printed electrodes for the determination of nicotinamide, vitamin B3, in a multivitamin complex. The formation of the substrate has been monitored using UV/Vis absorption spectroelectrochemistry and scanning electron microscopy, which confirmed the generation of gold nanoparticles on the electrode surface. The new

method has demonstrated to be suitable for the direct quantification of simple test samples using small sample volumes (50 µL). However, it failed when a complex sample, containing several interfering species, was analysed. A high interference on the preparation of the SERS substrate is observed during the analysis of a multivitamin complex. Despite that, nicotinamide was selectively determined in a multivitamin complex using the method of the standard addition, obtaining remarkable figures of merit (R^2=0.99, %RSD < 9 %).

Keywords

Spectroelectrochemistry; vitamin B3; EC-SERS; Raman spectroscopy; standard addition method;

1. Introduction

Nicotinamide is the amide form of vitamin B3, which belongs to vitamin B group or water-soluble vitamins. It has great importance in metabolic processes since it is the main precursor of the coenzymes NAD^+ and NADH, both regulators of the cellular energy metabolism. These compounds are also involved in different biologic reactions as reductive biosynthesis and antioxidation[1–3]. The deficit of this vitamin leads to pellagra, a disease characterized by the presence of diarrhoea, weakness, dementia and dermatitis[4]. During the past 50 years, many clinical reports have identified nicotinamide as a beneficial agent in the prevention and treatment of this disease, but also it is a candidate to be highly effective in the treatment of acne or in cancer chemoprevention[1,4–6]. Vitamin B3 can be found in several vitamin complexes. The importance of this vitamin makes quantification in a real sample interesting for different fields such as pharmacy, medicine, chemistry and biochemistry.

There are varieties of methods to quantify this vitamin, such as liquid chromatography (HPLC)[7–9] and UV/Vis spectrophotometry[10,11]. However, the sample preparation linked to those analysis is time-consuming and usually requires exhaustive pre-treatment of the sample. In the last years, surface-enhanced Raman spectroscopy has emerged as a good alternative to classical analytical methods due to its specific characterization and high sensitivity[12,13], which overcomes the classical drawbacks of Raman spectroscopy.

Surface-enhanced Raman spectroscopy is a surface-sensitive technique that enhances Raman scattering signal because of the adsorption of molecules on rough metal surfaces with plasmonic properties. Since its discovery in 1974[14], SERS has become in a highly studied phenomenon, due to the usefulness in trace and ultratrace analysis. Nowadays, there is an agreement on the mechanisms involved in this phenomenon[15–18], which depends on two phenomena: the electromagnetic mechanism (EM), related to the excitation of surface plasmon of nanoparticles or nanostructures, and the chemical mechanism (CM), associated to a charge transfer between the molecule and the substrate. Both mechanisms contribute to the SERS effect, being the most important the EM, which allows enhancements of the Raman scattering up to 10^6, meanwhile, the CM contributes with enhancements up to 10^3. This enhancement obtained by SERS has allowed to detect analytes even at a single molecule level[19–22]. Combination of SERS and electrochemistry is particularly interesting for analysis. Electrochemical processes can lead to the adsorption of the analytes, induced by the applied potential, and thus, improving the analytical signal[23]. Moreover, the first SERS spectra were obtained in an electrochemical cell and, since then, electrochemical-SERS (EC-SERS) has been frequently employed in the characterization and identification of a myriad of target molecules.

Due to the difficulty to obtain reproducible SERS substrates, initially surface enhanced Raman spectroscopy had been used almost exclusively in materials characterization[23]. However, the development of new protocols to prepare SERS substrates, such as arrays of nanoparticles, has promoted SERS as a good candidate not only for detection but also for quantification purposes[24,25]. Nevertheless, many protocols developed to obtain a good SERS substrate involve tedious preparation processes as well as a highly qualified operator. A good alternative to generate SERS-substrates with high reproducibility is the electrochemical roughening of a metal electrode (gold, silver or copper). Combining the latter with the use of screen printed electrodes (SPE), which have shown to be good candidates for substrate preparation, it is possible to obtain SERS substrates with high reproducibility in a very short time and applying a fairly easy protocol[26]. In this sense, Raman Spectroelectrochemistry (SEC) presents the advantage of generating the SERS substrate *in-situ* concomitantly with the detection of the target molecule in the same experiment. Moreover, this time-resolved multi-response technique allows us to validate the quality of the SERS substrate in a single measurement, providing useful information about the generation of the substrate as well as the adsorption process of the target molecule.

In this work, we demonstrate the capabilities of Raman SEC for the determination of nicotinamide, preparing the SERS substrates in a fast, simple and reproducible way and using a standard addition method for quantification of this molecule in a multivitamin complex.

2. Experimental

2.1. Chemicals and Materials

Nicotinamide ($C_6H_6N_2O$, 99 %, ACROS Organics) and potassium chloride (KCl, +99 %, ACROS Organics) were used as received. Multivitamin complex Jalea-Própolis® de Deliplus was used as test sample. All solutions were prepared using ultrapure water obtained from a Millipore DirectQ purification system provided by Millipore (18.2 MΩ·cm resistivity at 25 ºC).

2.2. Instrumentation

A customized SPELEC RAMAN instrument (Metrohm-DropSens) was used to perform the Time-Resolved-Raman-SEC (TR-Raman-SEC) experiments. This instrument has been developed by our group in collaboration with Metrohm-DropSens. It integrates a laser source of 638 nm in which the laser power was set at 61 mW in all experiments. The Raman SEC cell was used with SPEs. DropView SPELEC software (Metrohm-DropSens) was used to control simultaneously the potentiostat and the spectrometer, to obtain the time-resolved and synchronized spectroscopic and electrochemical responses and to perform a preliminary analysis of the results. Gold SPEs (DRP-220BT, Metrohm-DropSens) were used for the electrochemical generation of the EC-SERS substrate.

A customized UV/Vis SPELEC instrument (Metrohm-DropSens) was used to carry out the UV/Vis characterization of SERS substrate. This instrument has been also developed by our group in collaboration with Metrohm-DropSens. The instrument allows us to obtain simultaneously UV/Vis absorption and electrochemical during the experiments. Dropview SPELEC software (Metrohm-DropSens) was also employed to control this instrument. A DRP-REFLECELL cell (Metrohm-DropSens) was used in the UV/Vis-SEC experiments.

MATLAB® software was employed to analyze the SEC dataset.

2.3. Scanning electron microscope images

The morphology of the samples was examined by scanning electron microscopy using a field emission microscope, model JEOL JSM-7100, applying an electron beam of 5 kV and recording the response of the secondary electrons.

2.4. Spectroelectrochemistry measurements.

EC-SERS spectra were taken during a TR-Raman-SEC experiment and absorption spectra were taken during a TR-UV/Vis absorption SEC. Cyclic voltammetry (CV) was used as electrochemical technique, the vertex potentials were -0.70 V and +1.40 V, starting at +0.70 V in the anodic direction at 0.05 $V \cdot s^{-1}$. Spectra were collected simultaneously with electrochemical data. The integration time used in all the experiments was 1 s for TR-Raman-SEC and 0.1 s for TR-UV/Vis absorption SEC.

2.5. Sample preparation.

Nicotinamide solutions were prepared in 0.1 M KCl as supporting electrolyte, which is required both to generate the SERS substrate and to carry out the electrochemical experiment.

For the multivitamin complex a dilution 1:100 in water was required to adjust the concentration to the linear range, according to the nominal concentration in the prospect. To prepare the samples for the standard addition method, the diluted sample was used in all samples in a 0.1 M KCl solution, with a final nominal concentration of 6.45 μM of nicotinamide, according to the prospect, and concentrations of 0, 2, 4 and 6 μM of nicotinamide were spiked in the respective samples (s01, s02, s03 and s04).

It should be noted that just a volume of 50 µL is required to perform the SEC experiment.

3. Results and discussion

3.1. Nicotinamide EC-SERS spectra.

Before performing the determination of nicotinamide, the SERS spectrum is compared with the Raman spectrum of solid nicotinamide. SERS spectra were obtained using the electrochemical protocol described in section 2.4 while Raman spectra of nicotinamide solid were obtained placing the compound on a sample holder, without performing any electrochemical experiment. In order to have a better comparison, the main peak at 1025 cm^{-1} for the SERS spectrum at -0.40 V in the negative scan during the CV and at 1043 cm^{-1} for the Raman spectrum, both related to the breathing of the pyridine ring, were selected to normalize the spectra. Figure 1 shows the normalized Raman spectra of the solid (yellow line) and the normalized EC-SERS spectra of a 20 µM nicotinamide solution in 0.1 M KCl (blue line). This comparison allows us to confirm that nicotinamide has not suffered any chemical or electrochemical change during the potential scan that is used for its determination.

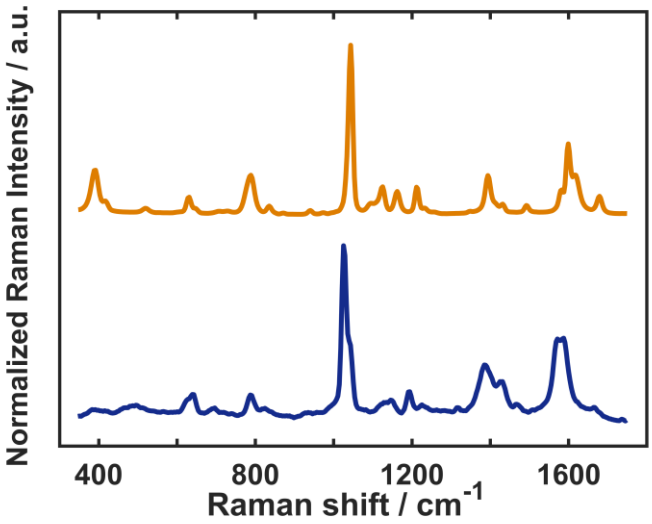

Figure 1. Normalized Raman spectrum of solid nicotinamide (yellow line) compare with normalized EC-SERS spectrum of 20 µM nicotinamide in 0.1 M KCl medium (blue line), registered at -0.40 V in the cathodic direction during a CV.

It can be observed that the two spectra are quite similar, and the apparent differences are mainly due to the interaction of the nicotinamide with the aqueous medium or with the SERS substrate. Band assignments are summarized in Table 1. The main peak, related to the pyridine ring breathing, at 1025 cm^{-1} was selected to perform quantitative measurements.

Table 1. EC-SERS band assignment for nicotinamide found in literature for SERS experiments.

Solid Raman bands / cm^{-1}	**EC-SERS bands / cm^{-1}**	**Band assignment**[27–29]
631	642	δ_{ring}
788	790	10b; γ (CH)
1043	1025	12; breathing pyridine ring
1125	1145 (broad band)	15; δ(CH)
1162		13; ν (C-X)
1211	1194	ν $(CC)_{ring}$, ρ$(CH)_{ring}$
1394	1385	ρ $(CH)_{ring}$, ν$(NC)_{ring}$, ν$(CC)_{ring}$
1432	1431	19b; ν_{ring} and

1492	1467	ν_{sym}(C=O)
1599	1571–1587	8a; ν_{ring}
1679	1668	Amide I; ν_{asym} (C=O)

δ, in-plane bending; γ, out-of plane bending; ν, stretching (symmetric or asymmetric); ρ, rocking; 10b, 12, 4, 2, 9b and 8a are referred to Wilson notation.

3.2. TR-Raman-SEC responses

As was mentioned above (Section 1), a SERS substrate is needed to obtain a good Raman spectrum of the analyte in a diluted solution. This SERS substrate can be easily generated by an *in-situ* oxidation and reduction cycles of a gold electrode in KCl medium. The presence of chloride is necessary to facilitate the dissolution of gold by forming the complex $[AuCl_4]^-$ and the later reduction to Au NPs. The SERS substrate is generated in 50 s, which is a very short time to generate a SERS substrate useful for quantitative analysis.

Figure 2 shows a comparison between the electrochemical (CV, garnet line) and the spectroscopic response (Raman intensity respect to the potential, denoted as VoltaRamangram, at 1025 cm^{-1}, blue line) of a 20 μM nicotinamide solution in 0.1 M KCl medium.

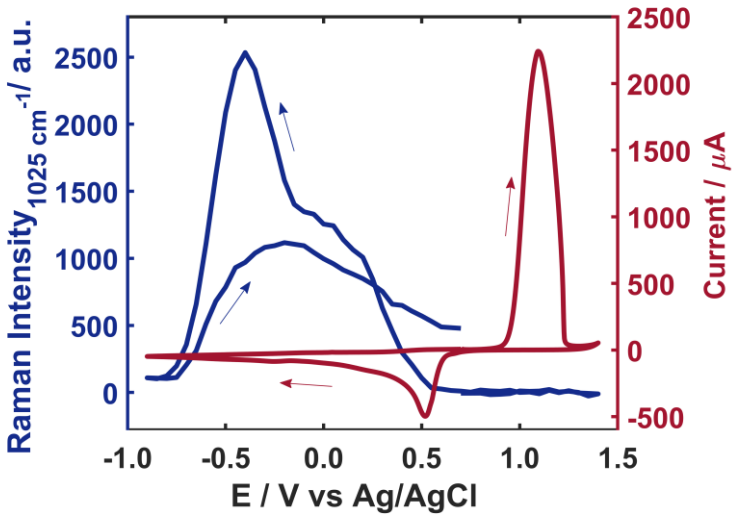

Figure 2. Cyclic voltammogram (garnet line) and voltaRamangram at 1025 cm^{-1} (blue line) of 20 µM nicotinamide in KCl medium. The CV experiment starts at +0.70 V, in anodic direction between vertex potentials (-0.70 V and +1.40 V). The voltaRamangram represents the evolution of the main peak at 1025 cm^{-1} of Raman spectrum as a function of the applied potential.

The voltammetric response (garnet line) shows one anodic and one cathodic peak. The anodic peak is related to the oxidation of Au from the electrode surface to form the $[AuCl_4]^-$ complex, reaching a maximum current at +1.20 V. Concomitantly, during this oxidation process, no Raman signal related to nicotinamide can be registered and only a broad band around 580 cm^{-1} due to the generation of different gold oxides can be observed (data not shown).

On the other hand, the cathodic peak, which starts at +0.60 V, and reaches its maximum at +0.50 V, is related to the reduction of $[AuCl_4]^-$ complex to form gold nanoparticles (AuNPs). These AuNPs are responsible for the SERS enhancement, making possible the detection of nicotinamide favored by the development of a well-defined SERS spectrum. The electrochemical response is only related to the oxidation/reduction of the gold substrate (Au-SPE), as can be deduced from Figure S1, where a blank experiment in absence of nicotinamide is performed under the same experimental conditions.

The voltaRamangram at 1025 cm^{-1} (blue line) shows the evolution of the main Raman band of nicotinamide with the applied potential. The Raman signal does not evolve until the reduction of gold complex takes place, yielding AuNPs (see section 3.3). When AuNPs are formed, the Raman signal starts to increase until reaching the maximum at -0.40 V in the cathodic direction. From this point downward, the Raman signal decreases, probably due to other associated electrochemical process such as chloride adsorption or oxygen reduction, which could interfere in the adsorption process of nicotinamide. As can be observed, there is a clear correlation between the AuNPs formation and the Raman response.

As can be seen, TR data allows us not only to follow the evolution of the Raman signal but also, and much more important for quantitative analysis, selecting the adequate applied potential. The best Raman response was easily obtained and used for the calibration of the method, by evaluation of the peak at 1025 cm^{-1}, as shown in the VoltaRamangram of Figure 2.

3.3. SERS substrate. Morphologic study.

The SERS substrate generated was studied by scanning electron microscopy (SEM). SEM images of the surface of an Au-SPE were taken after a SEC experiment, stopping the potential at -0.40 V in the cathodic scan, where the highest Raman signal was obtained. The electrochemical conditions and the electrolytic medium were the same that the one described previously. As can be seen in Figure 3A, small nanoparticles, more brilliant in the image, were generated during the SEC experiment on the surface of the Au microparticles that form the Au working electrode in the SPE. Therefore, AuNPs are covering with a high density the microparticles initially present in the Au-SPE surface, producing an aggregation of these NPs homogenously distributed on the surface.

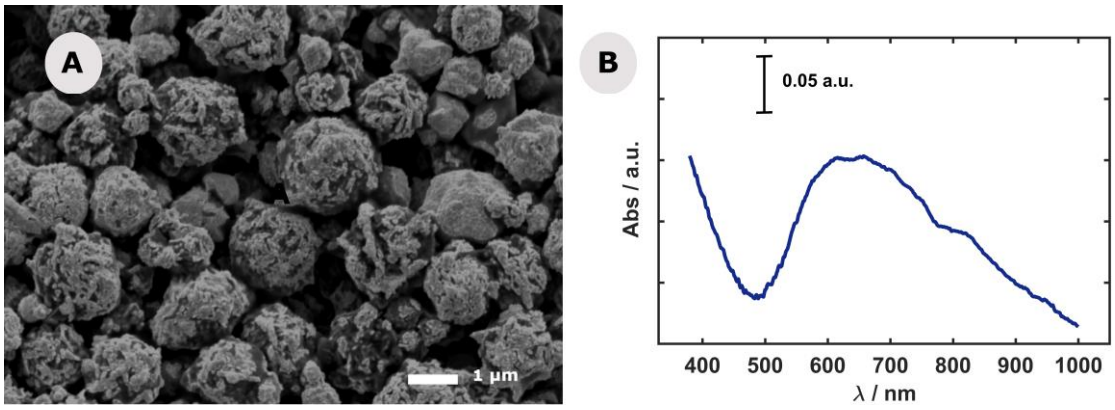

Figure 3. A) SEM image of Au NPs formed during a SEC experiment in which the potential was stopped at -0.40 V, using 20 µM nicotinamide in 0.1 M KCl medium and an Au-SPE. **B)** UV/Vis absorption spectrum for 20 µM nicotinamide in 0.1 M KCl solution recorded during

a SEC experiment (conditions described previously) at the potential of -0.40 V in the cathodic scan.

To further demonstrate the generation of AuNPs, a UV/Vis absorption SEC experiment was carried out, since the presence of a characteristic plasmon band can demonstrate the generation of AuNPs[30,31]. The experiment was carried out using the same experimental conditions than the Raman SEC experiment, in a 20 µM nicotinamide and 0.1 M KCl solution. The UV/Vis absorption spectrum, shown in Figure 3B, was obtained at -0.40 V, again, where the maximum of Raman signal is observed. Instead of the characteristic plasmonic band centered at around 520–550 nm, according with bibliography[30,31], the plasmonic band (Figure 3B) is centered at around 640 nm. This difference can be rationalized in terms of density of nanoparticles. A redshift of the absorption band is expected when a high number of NPs are present on the surface, mainly due to the interaction of metallic NPs in close mutual proximity[31,32]. Moreover, a broad plasmonic band is observed, which is indicative of both a high density of NPs and a high dispersion in the NPs size. UV/Vis absorption SEC experiment together with the SEM images confirm that the enhancement of the Raman signal is related to the generation of AuNPs during the cathodic scan.

TR-UV/Vis absorption SEC is very useful not only to obtain information about the deposited nanomaterial on the electrode but also to follow the evolution of plasmonic band with the applied potential. Figure 4 shows the voltabsorptogram at 640 nm (evolution of the maximum of plasmonic band with the applied potential) compared with the electrochemical response. Only the cathodic scan from +1.40 V to -0.90 V is plotted for a better understanding of the figure. During this scan, the reduction of the gold complex takes place to generate AuNPs, as is demonstrated by the growth of this plasmonic band. A small change in the slope of the absorbance increase with potential is

observed at -0.40 V, which could be related to the change of the response obtained in Raman SEC because from -0.40 V downward the Raman signal decreases.

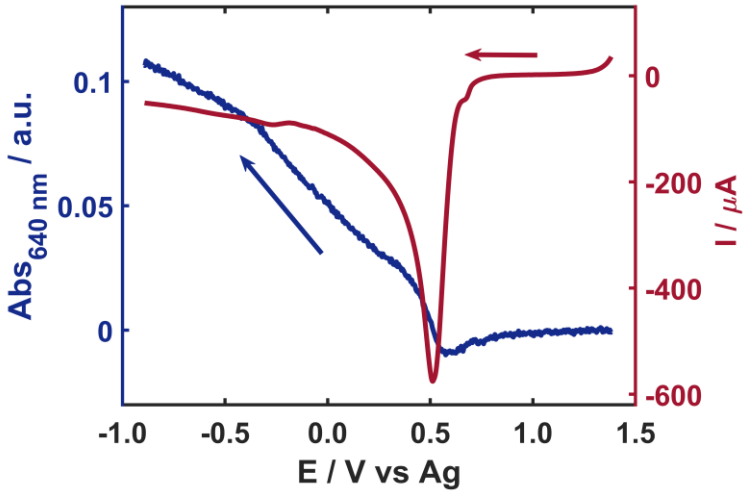

Figure 4. Comparison between the voltabsorptogram at 640 nm (blue line) and the voltammogram (garnet line) of 20 µM nicotinamide in 0.1 M KCl solution, during a SEC experiment. The experimental conditions are that of Figure 1, but only the negative scan from +1.40 V to -0.90 V is shown.

3.4. Determination of nicotinamide

3.4.1. Quantitative determination of nicotinamide in aqueous solutions

First, a calibration curve in 0.1 M KCl solution was performed to demonstrate the usefulness of this method for nicotinamide determination. As has been demonstrated, in Figure 5, the best spectroscopic response is obtained at -0.40 V in the cathodic direction. Therefore, this signal was selected to construct the calibration curve. A concentration range from 1 µM to 20 µM was selected and different measurements were made to study the capability of detection of the method. Figure 5 shows the voltaRamangrams corresponding to a set of calibrations samples, namely; 1, 3, 7, 16

and 20 µM. Inset shows the Raman band of the main peak at -0.40 V corresponding to the different samples. The peak height was used to construct the calibration curve.

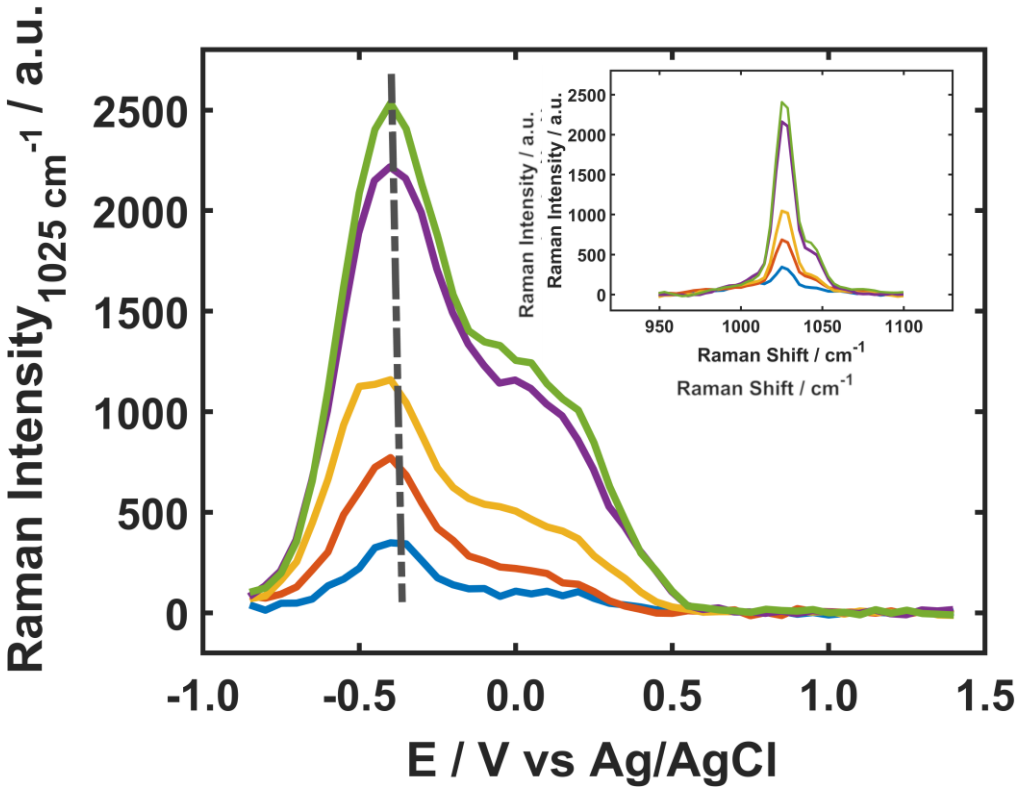

Figure 5. VoltaRamangram of different concentrations of nicotinamide in 0.1 M KCl solution between vertex potentials of -0.70 V and +1.40 V in the negative direction. The maximum of each signal is reached at -0.40 V. The Raman spectrum obtained at this potential (Inset) was taken to construct the calibration curve.

Figure 5 shows the calibration curve, with each sample being replicated three times. A test sample (10 µM nicotinamide in 0.1 M KCl) is included in the image to illustrate the high accuracy of the method. As can be observed, a good linear correlation between the peak height and the concentration was obtained (I_{Raman} = 119.9 $C_{Nicotinamide}$ + 264.7; $S_{yx=}$103.55), with a good R^2 value of 0.99. The data shows low dispersion with a limit of quantification (LOQ) of 1 µM and a limit of detection (LOD) below the lowest concentration measured, so both limits can be considered as 1 µM. The predicted concentration of the test sample was 9.48 µM, very close to the real one, obtaining a

recovery of 94.8 %. It is noteworthy that a relative standard deviation (%RSD) of 8.6 % was obtained in prediction, which is a very good value for SERS measurements, demonstrating the reproducibility of the measurements. It should be noticed that a very low integration time (1 s) is required to have a good sensibility but if it was necessary this integration time could be increased in order to have a higher signal and, which could improve the detection and quantification limits.

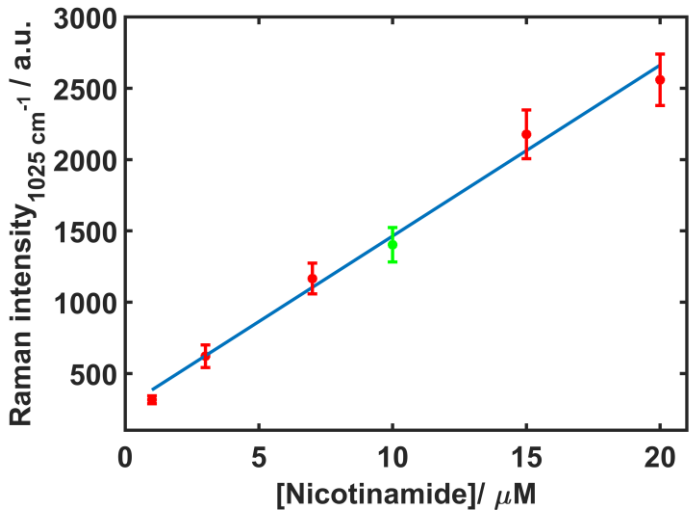

Figure 6. Regression curve of nicotinamide in 0.1 M KCl. The Raman response corresponds to the height of 1025 cm^{-1} peak in nicotinamide spectrum registered at -0.40 V during a SEC experiment. The calibration curve is plotted between 1 and 20 µM, adding a test sample of 10 µM nicotinamide.

3.4.2. Quantitative determination of nicotinamide in multivitamin complex.

Complex samples can be a challenge in quantitative analysis due to the matrix effect. For this reason, tedious pretreatments or separation processes need to be used to facilitate the analysis. We select a multivitamin complex to illustrate the capability of EC-SERS to determine nicotinamide in a system with a considerable matrix effect

An EC-SERS spectrum of the sample was registered and compared with pure nicotinamide (Figure S2). Both spectra were quite similar which indicates that, in this electrolytic medium and using the electrochemical conditions described in the previous section, nicotinamide is preferentially adsorbed onto the AuNPs and it seems that other compounds in the sample are not interferent. The electrochemical response does not present substantial differences. However, the matrix of the multivitamin complex probably affects to the generation of SERS substrate because a lower Raman intensity is obtained in the multivitamin sample compared with that obtained in aqueous medium (see Figure S2) for a similar concentration. Therefore, a simple calibration protocol cannot be directly used, selecting a method of standard addition to overcome the matrix effect.

The preparation of the samples is described in section 2.5 and the measurements were performed in fixed experimental conditions as the used for the calibration curve described above. According to the prospect, the test sample prepared for the analysis contains a nominal nicotinamide concentration of 6.45 μM. This test sample was determined by the standard addition method in 6.17 μM (I_{Raman} = 93.5 $C_{Nicotinamide}$ + 578.1, $S_{yx=}$19.9), with a recovery of 95.7 % (see Figure 7) and a good linear correlation (R^2=0.99). A %RSD of 8.60 % was obtained. These results demonstrate that EC-SERS can be used to quantify analytes in a very complex sample, containing different interfering compounds.

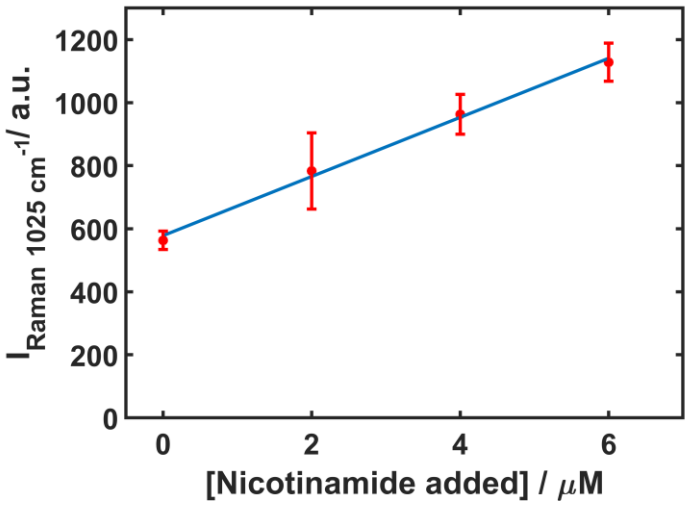

Figure 7. Calibration curve for the method of standard addition for nicotinamide in a multivitamin complex and 0.1 M KCl medium. The samples correspond to 0, 2, 4 and 6 µM nicotinamide spiked respectively.

4. Conclusions

This work presents a new method based on the *in-situ* preparation of reproducible EC-SERS substrates to determine nicotinamide in complex samples, with a sample volume of 50 µL. The new method is fast (~50 s) and feasible to use, demonstrating that EC-SERS can be a good alternative to other analytical techniques. TR-Raman-SEC exhibits the advantage of evaluating the SERS substrate during its formation, being easy to select the best conditions for the analysis of the target molecule and obtaining well-defined and reproducible Raman spectra. A high density of AuNPs were formed during the SEC experiment, confirmed by SEM images and UV/Vis absorption SEC. The low dispersion of the datademonstrates that the SERS substrate is highly reproducible. The good analytical figures of merit obtained with this methodology (R^2=0.99, %RSD < 9 %) demonstrate the capability of this technique for quantitative analysis.

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