New Voltammetric Sensor Based on LDH and $H_2O_2$ for L-Proline Determination in Red and White Wines

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Abstract: Taking inspiration from our recent work in which a new sensor for hydrogen peroxide was proposed, our research group has now developed a simple, fast, and inexpensive voltammetric system for determining proline concentration both in standard solutions and in real samples (red and white wines). This system uses a non-enzymatic sensor based on a working electrode of glassy carbon (GC) modified with a layered double hydroxide (LDH) compound, of the type GC-Ag(Pasteur)-LDH-$H_2O_2$, with hydrogen peroxide in solution at fixed concentration, in a three electrode cyclic voltammetry setup. Using an increasing concentration of standard solutions of L-proline, the method shows a linearity range, in semilogarithmic coordinates, between $125 \mu mol L^{-1}$ and $3200 \mu mol L^{-1}$ of proline, with a limit of detection (LOD) value of $85.0 \mu mol L^{-1}$ and a limit of quantitation (LOQ) value of $95.0 \mu mol L^{-1}$. The developed method is applied to the determination of proline in several samples of commercial Italian wines. The results are compared with those obtained by applying the classic spectrophotometric method of ninhydrin, obtaining a good correlation of the results.

Keywords: cyclic voltammetry method; GC sensor; layered double hydroxides; catalytic sensor; proline sensor; proline determination in wines

1. Introduction

Proline is a very important amino acid, as it is present in numerous foods, such as wines, musts and honey [1]. In some of them, such as grape juice and wines, proline is often the predominant amino acid [2], therefore its analytical determination is frequently required. For this reason, different methods for its determination have been proposed and developed over the years, including chromatography [3,4], gas chromatography [5,6], HPLC analysis [7–9], ion exchange chromatography [10,11], spectrophotometric [12–15] and chemiluminescence methods [16]. Some electrochemical methods have also been proposed for the determination of amino acids [17,18]. The amperometric sensor reported in Ref. [17], which is based on a cheap, simple, and rapid assay, responds to many common L- and D-amino acids, except proline. The method described in Ref. [18] for non-enzymatic determination of L-proline is based on an innovative electrochemical sensing platform, showing excellent sensitivity as well as long-term stability. However, the proposed sensor is notably more complex than that fabricated in our present study; furthermore, the possible interference with other amino acids is not discussed in that study [18]. Perhaps, among electrochemical methods, those most interesting are the electro-enzymatic ones, although those based on the amino acid-oxidase enzyme are not at all specific towards the different amino acids [19], whereas highly specific methods based on proline dehydrogenase [20–22] are not commercially viable due to the enormous cost of proline dehydrogenase (or oxidase).
The same is true when very sophisticated techniques are used for the determination, simultaneously based on antibody processes and electrochemical impedance spectroscopy [23].

Currently, one of the most interesting fields, where many quantitative analysis techniques are nowadays proposed, is that concerning wines, since the level of L-proline in these foods is often evaluated as an index of the genuineness of the wines themselves [2]. For this purpose, the most popular and used method is certainly the spectrophotometric one based on ninhydrin [11–15,24,25], of which there are several versions, precisely because it is not without drawbacks [25]. In fact, it is necessary to pay close attention, in addition to the purity of the necessary reactants, also to the time and temperature of heating and cooling, as well as to the time elapsed between the execution of the analytical procedure and the spectrophotometric measurement; all parameters that need to be well standardized. Finally, the analysis time is also not entirely negligible. For these reasons, faster and cheaper analytical methods are always much appreciated by analysts involved in the determination of L-proline in real samples, particularly in wines.

Recently, our research group developed different types of biosensors and electrochemical sensors for the rapid analysis of hydrogen peroxide, mainly present at low concentrations and in real samples [26–28], all based on a type of lamellar compound, namely layered double hydroxide (LDH) [26]. In our last work [28] we fabricated non-enzymatic electrocatalytic sensors for $\text{H}_2\text{O}_2$ (both voltammetric and amperometric), of considerable interest, which were able to reach very low values of limit of detection (LOD), of about 0.15 mmol L$^{-1}$ in static air and 0.05 µmol L$^{-1}$ under nitrogen atmosphere. This is thanks to the simultaneous presence both of a LDH mixture, glued, using Ag paste, to a glassy carbon (GC) electrode (which in itself exerts a discrete catalytic power on the oxidative decomposition of $\text{H}_2\text{O}_2$ [27,28]), and of L-proline amino acid free in solution. Indeed, some other authors had already observed that sensors for the determination of $\text{H}_2\text{O}_2$ with very low LOD values could be obtained using LDH over the surface of iron oxide nanospheres [29], or by immobilizing proline on silver microspheres [30]. As mentioned above, our research group obtained excellent sensors for hydrogen peroxide using LDH compounds, in solutions containing L-proline at a fixed and optimized concentration. Although the role of proline in these sensors has not yet been fully clarified, we believe, on the basis of some recent experiments [28], that proline acts positively on the electron transfer mechanism [28,29,31], while other authors [30] believe that proline essentially exerts a cleaning mechanism, specifically on the surface of silver beads deposited on the GC.

While investigating the determination of hydrogen peroxide, we saw the possibility of analyzing with the same voltammetric sensor as described above, also the unknown concentration of proline possibly present in solution once the concentration of hydrogen peroxide was fixed and kept constant. Then, by suitably varying the concentration of proline and keeping hydrogen peroxide constant, a specific voltammetric calibration curve could be constructed. In this regard, in the present research a voltammetric sensor for the analysis of proline was developed, which, after being validated from an analytical point of view, was used for the determination of proline in different samples of red and white wines. The results were then compared with those obtained by applying, on the same wine samples, the classic spectrophotometric method using ninhydrin [25], obtaining sufficiently correlated results.

2. Materials and Methods
2.1. Materials

Zinc nitrate hexahydrate (Zn(NO$_3$)$_2$ $\cdot$ 6 H$_2$O) and aluminium nitrate nonahydrate (Al(NO$_3$)$_3$ $\cdot$ 9 H$_2$O), L-proline (BioUltra, 99.5%), valine, glycine, lysine, methionine, phenylalanine, tryptophan, arginine hydrochloride and glutamine, were supplied by Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide and 0.1 mol L$^{-1}$ pH 7 phosphate buffer solution (PBS) from Fisher Scientific (UK). Potassium chloride was from Fluka BioChemika (Buchs, Switzerland), ammonium nitrate, formic acid, and isopropanol were from Carlo Erba (Milan, Italy), and ninhydrin was from Sigma-Aldrich.
Five samples of commercial wine were analysed, two whites and three reds, all purchased in local stores. All the samples were sold in dark glass containers (bottles), or in special plasticized cardboard packages. Each sample was tested immediately after opening.

2.2. LDH Preparation and Characterization

Several methods for LDH preparation are reported in the literature: hydrothermal growth [32,33], which was also used to fabricate our previous enzymatic sensors [26,27], coprecipitation [34], sol-gel techniques [35], exchange reaction methods [36], or else calcination-reconstruction routes [36,37]. In this work, the coprecipitation method was used for the synthesis of LDH of the type [Zn$^{ll}$Al$^{ill}$ (OH)$_2$]$^+$ (NO$_3$)$^- \cdot H_2O$, hereinafter referred to as (Zn-Al-NO$_3$), see Figure 1, was employed to build our catalytic sensors. This choice in turn was the most convenient, in order to have greater quantities of raw LDH available; indeed, the coprecipitation method, as explained in a previous paper [28], does have a very high synthesis yield, compared to other methods, for instance the in situ hydrothermal growth [32].

![Schematic representation of the (Zn-Al-NO$_3$) LDH structure.](image)

In this regard, LDH of type (Zn-Al-NO$_3$) was synthesized by dissolving in 200 mL of distilled and deionized water 50 mmol L$^{-1}$ of aluminium nitrate and 150 mmol L$^{-1}$ of zinc nitrate, bringing the solution to pH 10 with NaOH and keeping the solution in a closed container, in the oven at 90 °C for 12 h. Afterwards, the precipitated LDH slurry, after several centrifugations and washes with ethanol and deionized water, was dried at 45 °C, sealed in closed small containers, and stored at room temperature.

The structural characterization of the LDH synthesized by coprecipitation was carried out using X-ray diffraction (XRD) (see Figure S1). See also the comparison with that of LDH previously obtained by means of the in situ hydrothermal growth process reported in previous work (ref. [28]), while the same has been already characterized by using several other instrumental techniques as summarized in a previous report [27]. A perfect superimposition of the two XRD spectra can be clearly observed, indicating the same layered structure of the two raw materials [28]. In addition, a morphology comparison by means of scanning electron microscope (SEM) images of the two types of LDH is reported in Figure S2, revealing a randomly oriented aggregation of crystallite clusters for coprecipitated LDH and a closely interconnected hierarchical network of nanoplatelets for hydrothermal grown LDH.

2.3. Sensor Preparation

The GC electrodes were modified according to the same route used in our previous work [28]. A schematic of the modified electrode is shown in Figure 2. First, the surface head of the GC cylindrical rod (0.5 cm in diameter) was polished by sandpaper, rinsed with deionized water and ethanol, and finally dried. Then, 15 mg of (Zn-Al-NO$_3$) LDH, gently homogenized, were glued on the GC electrode surface, which was previously smeared with
a very thin coat of silver paste glue (Agar Scientific, Stansted Essex, UK, 60% solid silver in 4-methylpentan-2-one). Lastly, the cylindrical head of the LDH modified GC electrode was gently screwed to the end of the electrode stem. Finally, the electrode was immersed in a PBS and KCl solution containing an optimized concentration of hydrogen peroxide.

![Schematic diagram of the GC-Ag\(\text{paste}\)-LDH-\(\text{H}_2\text{O}_2\) catalytic sensor used in this work.](image)

**Figure 2.** Schematic diagram of the GC-Ag\(\text{paste}\)-LDH-\(\text{H}_2\text{O}_2\) catalytic sensor used in this work.

### 2.4. Electrochemical Apparatus and Experimental Cyclic Voltammetry Measurements

#### 2.4.1. Cyclic Voltammetries and L-Proline Determination in Standard Solution

All cyclic voltammetries (CVs) were measured from −1.5 V to +1.5 V, with a scan rate of 40 mV s\(^{-1}\), using a VersaSTAT3 Potentiostat (AMETEK Scientific Instruments, Princeton USA), a glass cell (thermostated at 25 °C) and three electrodes, namely, the working electrode (i.e., the modified GC electrode), a platinum counter electrode and a reference electrode of Ag/AgCl/Cl\(^{-}\) (see Figure 3). The three electrodes were dipped in 40 mL of 0.1 mol L\(^{-1}\) pH 7, phosphate buffer, 50 mmol L\(^{-1}\) in KCl and containing also an optimized fixed addition of hydrogen peroxide (200 µL 1%), then making additions of the standard solution L-proline, so increasing its concentration in solution, to obtain calibration curves. Trends in CV curves with increasing proline concentration and related blank curves were recorded. The voltammetric calibration curves were obtained by reading the current value at each peak, i.e., reduction and oxidation peaks, by adding different volumes of proline solution added. The changes in the current values at the oxidation or reduction voltammetric peaks after the additions of the respective interfering amino acids were then compared with those recorded after the addition of the proline solution alone.

#### 2.4.2. Optimization of Hydrogen Peroxide in Solution

In order to optimize the \(\text{H}_2\text{O}_2\) concentration in solution analogous experiments were carried out by the same way as described in the previous section, but with addition of different hydrogen peroxide concentrations in solution, equal to 200 µL 1%, 150 µL 10%, and 225 µL 10%, so that the final concentrations of hydrogen peroxide in 40 mL were 1.5 mmol L\(^{-1}\), 11.1 mmol L\(^{-1}\), and 16.7 mmol L\(^{-1}\), respectively. The best concentration of hydrogen peroxide in solution turned out to be equal to 1.5 mmol L\(^{-1}\).

#### 2.4.3. Possible Interferences from Other Different Amino Acids

The possibility that other amino acids present in the analysed real samples can interfere was studied using the same procedure employed to obtain calibration curves, but by adding, after an addition of 25 µL of proline standard solution (0.1 mol L\(^{-1}\)) to 40 mL of 0.1 mol L\(^{-1}\), pH 7, phosphate buffer, containing 50 mmol L\(^{-1}\) of KCl, and 200 µL of 1% \(\text{H}_2\text{O}_2\), subsequent additions of 25 µL of each of the other amino acids considered, all at the same concentration (0.1 mol L\(^{-1}\)) of the proline solution added. The changes in the current values at the oxidation or reduction voltammetric peaks after the additions of the respective interfering amino acids were then compared with those recorded after the addition of the proline solution alone.
Figure 3. Schematic of the three-electrodes voltammetric system used in the present research for proline measurement: (a) counter electrode, (b) reference electrode, (c) working electrode, (d) hydrogen peroxide in PBS containing KCl, and (e) thermostated electrochemical cell. In the inset a schematic representation of the catalytic sensor GC-Ag(paste)-LDH-H$_2$O$_2$, is shown.

2.4.4. Voltammetric Measurements in Real Samples (Wines)

First, each wine sample was suitably diluted (white wines 1:2, red wines 1:3) before being subjected to analysis. Then, a first cyclic voltammetry was recorded in 37.5 mL of phosphate buffer solution alone, containing KCl. A second cyclic voltammetry was carried out in the same solution after adding 200 μL of 1% H$_2$O$_2$ solution. After, 2.5 mL of the diluted wine sample was added and a new cyclic voltammetry was performed. Finally, subsequent additions were made, i.e., volumes between 50 μL and 600 μL of standard solution of L-proline (0.1 mol L$^{-1}$), recording the corresponding CV curve each time, as in the case of the construction of calibration curves, and reading the current intensities of the oxidation and reduction peaks thus obtained.

In this way, matrix calibration curves were constructed [28], while the calculation of the proline contained in the wine sample was carried out by applying the Gran’s Plot format [38,39]. Also in this case, the modest dilution of the solution due to subsequent additions was considered.

2.5. Spectrophotometric Method

2.5.1. Reagents and Apparatus

- L-Proline aqueous solution (100 mg L$^{-1}$, i.e., 8.68 mol L$^{-1}$)
- Ninhydrin aqueous solution (2%)
- Formic acid
- 1:1 isopropanol-deionized water solution
- Single beam spectrophotometer (Onda Spectrophotometer, UV-21) and 1.00 cm optical path glass cuvettes were used for all the measurements
2.5.2. Spectrophotometric Format

Five different volumes of standard proline solution (namely, 1.0, 3.0, 5.0, 7.0, and 9.0 mL of 100 mg L\(^{-1}\) of L-proline) were added in 5 respective flasks of 10.0 mL. Then, 0.5 mL of each sample was picked up and added to 0.5 mL of formic acid and 2 mL of 2% ninhydrin aqueous solution, contained in a special glass test tube. Another sample was prepared by adding the same reagents to 0.5 mL of deionized water to also obtain a reagents blank. Each of these samples was hermetically sealed and placed into boiling water for 20 min. After that, samples were cooled at room temperature for 5 min and then transferred to corresponding flasks of 10.0 mL which were then filled to the maximum volume, i.e., 10.0 mL, with 1:1 isopropanol-deionized water solution. A part of each solution was then transferred into glass cuvettes for UV-Vis analysis and the absorbance peak was read at 517 nm. The absorbance by the reagents blank was measured and subtracted to the absorbance measured from each sample.

Finally, a spectrophotometric calibration curve was constructed, as shown in Figure 4.

![Figure 4](image_url)

**Figure 4.** Straight-line spectrophotometric calibration curve built for proline measurements in real samples (wines), using ninhydrin spectrophotometric format.

2.5.3. Spectrophotometric Measurements of Wine Samples

All the wine samples for the spectrophotometric analysis were initially diluted 1:20 in distilled water, to obtain absorbance values, due to the reaction of contained proline with the added ninhydrin, that fall within the range of the calibration curve.

One-half of each diluted wine was picked up and added to 0.5 mL of formic acid and 2 mL of 2% ninhydrin aqueous solution. Another sample was prepared by adding the same reagents to 0.5 mL of deionized water to obtain also a reagents blank. Finally, another sample was prepared by adding the same volume of 1:20 diluted wine to 2.5 mL of deionized water to obtain also a “blank of wine” without reagents.

Each of the wine samples prepared were then analyzed using the same protocol described in the previous section, i.e., reading the absorbance at 517 nm and then subtracting blanks’ absorbance. The unknown concentrations of proline in wine were finally determined from the calibration curve previously obtained (see Figure 4).
3. Results

As mentioned in the introduction section, the operation of our sensor is based on the oxidation process of hydrogen peroxide, catalyzed by the presence of the (Zn-Al-NO₃)₃ LDH and assisted by proline, according to the reaction

$$2\text{H}_2\text{O}_2 \xrightarrow{\text{Proline-LDH Catalyst}} \text{O}_2 + 2\text{H}_2\text{O} \quad (1)$$

which, in the recorded CV curves, corresponds to the anodic oxidation peak of H₂O₂, between 0.65 V and 0.72 V. The oxygen reduction process, produced in the following reaction

$$\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O} \quad (2)$$

results in the cathodic reduction peak at about −0.58 V. Noteworthy, by fixing the concentration of H₂O₂ in solution, the intensity of these peaks increases with increasing proline concentration in solution, at least within certain limits, making it possible an indirect determination of proline concentration through the intensity variation of these oxidation and reduction peaks.

For this purpose, the first step was to optimize the concentration of hydrogen peroxide to be kept fixed and constant in solution. Three series of cyclic voltammetries were recorded, using the system represented in Figure 3, with increasing proline concentration between 63 μmol L⁻¹ and 10.7 mmol L⁻¹, carried out with different (but constant) concentrations of H₂O₂ in solution (see Section 2.4.2). These three series of cyclic voltammetry are shown in Figure 5.

![Figure 5. Three series of CV obtained, with increasing proline concentration between 63 μmol L⁻¹ and 10.7 mmol L⁻¹, using three different fixed concentrations of hydrogen peroxide in PBS and KCl solution: (a) 1.5 mmol L⁻¹, (b) 11.1 mmol L⁻¹, (c) 16.7 mmol L⁻¹ of H₂O₂, respectively.](image)

From Figure 5a, it can be clearly observed that the greatest increases in the intensity of the oxidation or reduction voltammetric peaks, upon the subsequent additions of proline in solution between 63 μmol L⁻¹ and 10.7 mmol L⁻¹, are obtained for a fixed concentration of hydrogen peroxide in solution equal to 1.5 mmol L⁻¹. Subsequently, using these values of the intensity of the oxidation or reduction peaks, the calibration curves were constructed, both relating to the oxidation process and to the reduction process, as shown in Figure 6a,d, respectively.

The obtained calibration curves show a logarithmic trend, which of course can be made approximately linear on a semilogarithmic plot (Figure 6b,e). The respective straight-lines and their confidence intervals are shown in Figure 6c,f. The analytical data are listed in Table 1. The repeatability of new straight-lines, fabricated in different days, has been resulted ≤5.50%.
Figure 6. Calibration curves obtained, at first day of sensor fabrication, by increasing proline concentration between 63 μmol L⁻¹ and 10.7 mmol L⁻¹ (H₂O₂ concentration in solution equal to 1.5 mmol L⁻¹) and reading the current values at the oxidation (a–c) and reduction (d–f) peaks of CV curves. Curves (a,d), plotted using linear scales, show logarithmic trends; curves (b,e), plotted on semilogarithmic scales, show about linear trends; (c,f) straight-line intervals of curves (b,e). Each point represents the mean of three determinations.

Table 1. Analytical method validation: main analytical data of the straight-line curves reported in Figure 6c,f, using oxidation peak and reduction peak, respectively.

| Main Analytical Data | Oxidation Peak | Reduction Peak |
|----------------------|---------------|---------------|
| Linearity range (μM) | 125.0–3200    | 125.0–3200    |
| Equation *           | y = a·log(x) + b | y = a·log(x) + b |
| Intercept (mA)       | 2.420 ± 0.1033 | 3.972 ± 0.1667 |
| Slope value (mA)     | 0.902 ± 0.0360 | 1.330 ± 0.0574 |
| R²                   | 0.99629        | 0.99577       |
| Residual Sum of Squares | 0.00958     | 0.02493       |
| RSD%                 | 5.50          | 7.00          |
| LOD (μM)             | 85.0          | 85.0          |
| LOQ (μM)             | 95.0          | 95.0          |

* y current in mA, x is the ratio between the concentration value and 1 μM.

In Table 1, it can be observed that in the same linearity range, between 125 μmol L⁻¹ and 3200 μmol L⁻¹ of proline, the straight-line constructed with the values by the oxidation peak shows a correlation coefficient (R²) comparable to that of the straight-line by the reduction peak, as well as lower values of residual sum of squares and RSD% and a slightly lower slope value. A better precision in proline determination is therefore expected using the oxidation peak. Finally, in Figure 7a,b,d,e the calibration curves, built by oxidation (upper panels) and reduction (lower panels) peaks recorded in the third and fifth days from the preparation of same the sensor, are shown in linear and in semilogarithmic scales, respectively (the calibration curves of the first day of sensor operation are reported for comparison). In Figure 7c,f the corresponding straight-lines are displayed.
In Table 1, it can be observed that in the same linearity range, between 125 μM and 1000 μM, the current values at the maximum of oxidation (a–c) and reduction (d–f) peaks of CV curves. Curves (a,d), plotted using linear axes, show logarithmic trends; curves (b,e), plotted on semilogarithmic scales, show about linear trends; (c,f) straight-line intervals of curves (b,e). Each point represents the mean three determinations.

The main analytical data of the method are listed in Table 2, where the straight-line equations and relevant parameters for the curves constructed by the oxidation and the reduction peaks, obtained as the days pass, are summarized.

Table 2. Main analytical data of the straight-line curves displayed in Figure 7c,f, recorded in the first, third and fifth day from sensor fabrication.

| CV Peaks   | Day    | Found Equation                                      | R²      | RSD% |
|------------|--------|-----------------------------------------------------|---------|------|
| Oxidation  | 1st day| \(y = (0.902 \pm 0.0360) \cdot \log(x) + (2.420 \pm 0.1033)\) | 0.99629 | 5.5  |
|            | 3rd day| \(y = (0.810 \pm 0.0330) \cdot \log(x) + (3.868 \pm 0.09640)\) | 0.99499 | 9.8  |
|            | 5th day| \(y = (0.313 \pm 0.0140) \cdot \log(x) + (5.970 \pm 0.03910)\) | 0.99063 | 11.5 |
| Reduction  | 1st day| \(y = (1.330 \pm 0.05740) \cdot \log(x) + (3.972 \pm 0.1667)\) | 0.99577 | 7.0  |
|            | 3rd day| \(y = (0.786 \pm 0.0470) \cdot \log(x) + (7.226 \pm 0.1407)\) | 0.98607 | 13.0 |
|            | 5th day| \(y = (0.303 \pm 0.0110) \cdot \log(x) + (9.262 \pm 0.03130)\) | 0.99061 | 11.5 |

In Table 2, it can be observed that the slopes of the straight-lines decrease as the days pass from the construction of the sensor, but that this can be used for about a week, albeit with decreasing sensitivity values. It is therefore advisable, especially for measurements on real samples, to always use the sensor on the first day of its preparation, when it provides the best analytical performance. Indeed, it can be observed that the values of the slopes, as well as those of R² and of RSD% are generally better for data obtained from the oxidation peaks than for the ones obtained from the reduction peaks. Moreover, as noticed in Table 1 for the first day of use, by comparing the analytical data obtained from the oxidation and reduction peaks over the whole operation period from sensor fabrication, it still results that a better precision in proline determination is expected using the oxidation peak.
Furthermore, the possible interferences caused by traces of the other main amino acids, which, according to the literature, can be contained in wines, were evaluated with the procedure described in Section 2.4.3. The possible interference produced by the ammonium ion was investigated as well. The obtained results are summarized in Table 3 and compared with those reported in other papers [25,40,41], found using the spectrophotometric method of ninhydrin.

Table 3. Interferences in proline determination, expressed as the percentage response variation to proline, using the catalytic GC-Ag(paste)-LDH-H₂O₂ voltammetric sensor, to several possible amino acids contained in wines and to ammonium ion. Each added interfering species had a final concentration in solution equal to that of proline, i.e., 0.1 mol L⁻¹ (the reported values are the mean of three determinations). Results from this work are compared with those reported in the literature for the same interfering species.

| Interferents   | GC-LDH Sensor Interference (%) (RSD% ≤ 5.80) | Interference (%) from Literature [41] | Interference (%) from Literature [25] | Interference (%) from Literature [40] |
|----------------|---------------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| Tryptophan     | +12.5                                       | +4.8                                  | +1.4                                  | -                                     |
| Lysine         | +14.0                                       | +4.4                                  | +4.4                                  | -                                     |
| Phenylalanine  | −10.0                                       | −6.1                                  | -                                     | -                                     |
| Glycine        | +6.00                                       | +1.8                                  | 0.0                                   | -                                     |
| Valine         | +7.00                                       | +2.1                                  | -                                     | -                                     |
| Methionine     | +8.00                                       | +3.5                                  | -                                     | -                                     |
| Arginine       | −2.00                                       | -                                     | -                                     | ≤3.4                                  |
| Glutamine      | −7.50                                       | −3.2                                  | -                                     | -                                     |
| Ammonium       | −12.5                                       | -                                     | -                                     | −12.6                                 |

Once the method was validated, the fabricated catalytic–voltammetric sensor was used to perform the analysis of five samples of Italian commercial wines, two whites and three reds, using the procedure described in Section 2.4.4. An experimental example of the analysis for the determination of proline content is shown in Figure 8 for two different samples, one of red wine and one of white wine.

![Figure 8](image_url)

**Figure 8.** CV curves for the determination of proline content in two different wine samples, (a) of red wine and (b) of white wine, using the catalytic sensor described in this paper. “Blank” is the CV of the buffer-KCl solution and fixed oxygen peroxide concentration; “Matrix” is the CV after diluted wine sample addition; while other CV curves are those recorded after proline addition, whose final concentration is reported as μM in the respective legends.

The proline concentration for all five examined samples, using both the oxidation and reduction peaks, are shown in Table 4. These values are compared with those found by the spectrophotometric method of ninhydrin [25], using the spectrophotometric calibration curve shown in Figure 4, constructed with the procedure reported in Section 2.5. It is worth...
to point out that each experimental value found by CV analysis using both oxidation and reduction peaks is the mean of three determinations. The application of the t-test to each triplet of experimental values, found by CV, the means of which, obtained for each wine, are reported in the Table 4, in columns 3 and 4, derived respectively by using the oxidation peak (Ox.) and the reduction peak (Red.), for \( p = 95\% \), \( \nu = 2 \), always gives as a result: N.S. (not significant).

**Table 4.** Experimental values of proline concentration found in five different wine samples using the catalytic voltammetric sensor developed in this work, and comparison with the same data obtained using the ninhydrin spectrophotometric method. All values of proline concentrations are the real found values of not diluted wine samples. The percentage differences \( \Delta\% \) between the values of proline concentration obtained by the CV method and by the spectrophotometric method are also reported.

| Samples (Wines) | UV-Vis Spectrophotometric Values (mg L\(^{-1}\)) (a) | Cyclic Voltammetry Values (mg L\(^{-1}\)) (b) | Proline Concentration | \( \Delta\% \) | \( \Delta\% \) |
|-----------------|-----------------------------------------------|-----------------------------------------------|-----------------------|----------------|----------------|
|                 | Ox. (RSD\% \( \leq 8.50\))                   | Red. (RSD\% \( \leq 7.20\))                   | Ox.                   | Red.          |
| Red W. 1        | 610.0                                        | 561.6                                        | 642.8                 | −7.90         | +5.40         |
| Red W. 2        | 368.4                                        | 410.0                                        | 326.4                 | +11.5         | −11.4         |
| Red W. 3        | 980.0                                        | 939.9                                        | 936.1                 | −4.10         | −4.50         |
| White W. 1      | 343.7                                        | 312.6                                        | 301.1                 | −9.00         | −12.4         |
| White W. 2      | 419.5                                        | 442.6                                        | 428.4                 | +5.50         | +2.10         |

Interestingly, the percentage differences \( \Delta\% \) between the values of proline concentration obtained by the CV method, using the reduction peak, and the ones by the spectrophotometric method are not very different from those deriving from the oxidation peak, although the latter show a slightly better agreement with the spectrophotometric method, at least in two of the five examined wines. This confirms what has been previously observed, when working on standard solutions rather than real samples, i.e., that generally better results are expected if the analysis is carried out based on the oxidation peak, rather than on the reduction one.

Finally, to further evaluate the accuracy of the proposed method, the “standard addition method” was used to calculate, by means of voltammetric analysis, the percentage recoveries for proline addition on some of the wine samples tested in the present study. The obtained results are summarized in Table 5. It can be observed that the recovery values (%), calculated on two of the wine samples considered, are between 93.2% and 99.2%, and therefore, the recovery values are also generally acceptable.

**Table 5.** Experimental percentage recovery for proline addition (\( \mu\)M) in red and white wines, using the standard addition method. The reported found values are the mean of three determinations. The concentrations reported in the second column provide the concentrations of samples after dilutions introduced by applying the format described in Section 2.4.4, therefore they do not represent the real found values of not diluted wine samples, such as those listed in Table 4, where concentrations are expressed in mg L\(^{-1}\).

| Sample          | Found Concentration in Wine Sample (\( \mu\)M) | Proline Additions (\( \mu\)M) | Found + Added Nominal Value (\( \mu\)M) | Found Experimental Value (\( \mu\)M) | \( \Delta\% \) (%) (RSD\% \( = 5.80\)) | Percentage Recovery |
|-----------------|------------------------------------------------|------------------------------|----------------------------------------|-------------------------------------|----------------------------------------|---------------------|
| White 1 red     | 54.50                                         | 690.0                        | 744.5                                  | 729.4                               | −2.0                                   | 98.0                |
| White 1 ox      | 56.60                                         | 688.0                        | 744.6                                  | 714.8                               | −4.0                                   | 96.0                |
| Red 3 red       | 101.6                                         | 642.8                        | 744.4                                  | 738.5                               | −0.80                                  | 99.2                |
| Red 3 ox        | 102.1                                         | 640.4                        | 742.5                                  | 691.7                               | −6.8                                   | 93.2                |
4. Discussion

A voltammetric system, which uses a non-enzymatic sensor of the type GC-Ag\(^{\text{(paste)}}\)LDH-H\(_2\)O\(_2\), the latter in solution at fixed concentration, has been developed. This platform allows, by means of cyclic voltammetry with three electrodes, to determine the concentration of proline both in standard and in real samples. When operating with standard solutions of L-proline, the method shows a linearity range, in semilogarithmic coordinates, between 125 µmol L\(^{-1}\) and 3200 µmol L\(^{-1}\) of proline, with an LOD value equal to 85.0 µmol L\(^{-1}\). With this sensor it is possible to operate for about a week, albeit with a decreasing sensitivity. To obtain the best results it is advisable to operate with a sensor immediately after it has been assembled, which is still very simple, fast, and cheap.

It has also been experimentally demonstrated that this sensor allows to determine, in a rapid and inexpensive way, the proline concentration contained in white and red wines, with sufficient precision (RSD\% ≤ 5.80%) and an accuracy, with an average percentage recovery of about 97%.

Finally, the comparison of determinations of proline concentrations contained in real samples found with the voltammetric method (using the oxidation peak) and with the spectrophotometric method of ninhydrin, has shown in two cases a percentage deviation less than about 5%, in other two cases a Δ\% < of 10%, while only in one case the Δ\% was found to be about 11%. Based on these data, it is therefore possible to affirm that the two methods are sufficiently correlated, especially if one takes into account the considerable difference between the two analytical techniques used. Moreover, as regards the possibility of interferences, with the main other amino acids that could be contained in wines, they appear to be of the same level as those reported in the literature, when using the classic ninhydrin method, even if the level of interference is generally different, in the two methods, for the single amino acids. However, this can be an advantage if the two methods are applied simultaneously.

5. Conclusions

In the present research, we have demonstrated the feasibility of determining the concentration of L-proline in solution by means of a non-enzymatic voltammetric sensor of the type GC-Ag\(^{\text{(paste)}}\)LDH-H\(_2\)O\(_2\), the latter in solution at a fixed concentration determined by an optimization procedure. This sensor showed an LOD of 85.0 µmol L\(^{-1}\) and a linearity range between 125–3200 µmol L\(^{-1}\), both operating on the oxygen reduction peak and on the hydrogen peroxide oxidation peak.

In conclusion, we have proposed a new electrochemical method, completely new, based on a voltammetric sensor of simple and rapid construction. The method is sufficiently correlated with the classic determination method based on spectrophotometric analysis with the aid of ninhydrin. However, our novel, fast, and inexpensive approach is unaffected by the known issues of the spectrophotometric method, such as complexity, cost and time-consuming; therefore, it is advantageous to be applied for the determination of proline in real samples, such as wines.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/10.3390/cryst12101474/s1. Figure S1, Title: X-ray diffraction patterns of LDH obtained by the coprecipitation method. The main basal reflections of (Zn-Al-NO\(_3\)) LDH phase are labelled by diamonds (♦). Figure S2, Title: SEM images of (Zn-Al-NO\(_3\)) LDH obtained by: (a) coprecipitation method; (b) grown in situ by hydrothermal method.

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