Optimization of Cyclic Voltammetric Curve Parameters to Measure Lactate Concentration in Urine Samples

Giulio Rosati[1(✉)], Matteo Scaramuzza[2], Elisabetta Pasqualotto[1,2], Alessandro De Toni[2], and Alessandro Paccagnella[1]

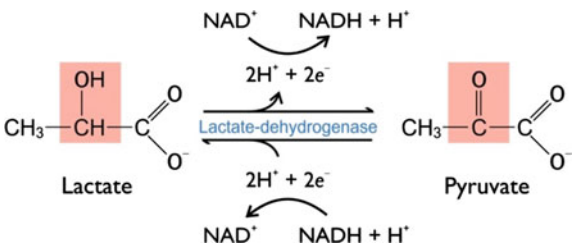
[1] Department of Information Engineering, University of Padova, via G. Gradenigo 6/b, 35131 Padua, Italy
rosatigiulio@gmail.com
[2] ARC - Centro Ricerche Applicate s.r.l, via J. da Montagnana, 49, 35132 Padua, Italy

Abstract. In this work, veal urine dilutions in Hepes-buffered Ringer's solution (HBRS) are tested by both UV-visible absorption spectroscopy and Cyclic Voltammetry (CV) to assess their viability as mediums for the detection of lactate, through the Lactate Dehydrogenase enzyme (LDH) reaction which involves the formation of NADH. Several data analysis algorithms for the recorded CV data are proposed and compared, in order to optimize the NADH detection in the urine samples dilutions. UV-visible spectroscopy was adopted as reference for NADH quantification.

Keywords: Biosensors, Cyclic Voltammetry, Lactate, SPCE, NADH, Urine

1 Introduction

Lactate detection in cattle urine samples is a non-invasive method which could help to prevent syndromes, e.g., sub-acute ruminal acidosis (SARA), that alter the quality of dairy products and seriously injure the animals in the herds [1, 2]. Electrochemical biosensors employing the Lactate Dehydrogenase enzyme (LDH) for the Lactate detection are a well-known technology [3, 4]. These sensors are based on the LDH catalysis, which reduces NAD^+ to NADH in presence of Lactate, and on the NADH electrochemical quantification (Scheme 1).



Scheme 1 Lactate Dehydrogenase (LDH) enzyme catalysis (elte.prompt.hu paragraph 7.6)

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The NADH detection can be performed both optically (absorbance at 340 nm or fluorescence at 460 nm) and electrochemically, e.g. by Cyclic Voltammetries (CVs) which oxidize NADH generating an electrical current proportional to its concentration [5, 6]. However, since the NADH oxidation is an irreversible reaction which usually requires a high overpotential, two approaches are currently adopted: using a mediator to perform an electrocatalytic oxidation requiring lower overpotentials [7, 8], or developing new materials for the mediatorless oxidation at lower potentials [9, 10].

With the latter approach, no standard data analysis methods are reported for the construction of the NADH calibration curve [11]. Therefore, we introduce and compare six parameters extracted from the cyclic voltammograms, to quantify the NADH concentration produced by the LDH enzyme in presence of the different Lactate dilutions in Hepes Buffered Ringer's Solution (HBRS). Lastly, we validate these parameters by performing the same measurements with Lactate in veal urine diluted in HBRS at 1, 10 and 100%.

2 Materials and Methods

All the chemicals and biochemicals used in this work were purchased from Sigma Aldrich. The Nicotinamide Adenine Dinucleotide reduced (NADH) and oxidized (NAD^+) coenzymes were purchased in powder form, with a purity ≥ 97 and 96.5% (HPLC), respectively, and dissolved in the mediums prior to use. Lactic acid was purchased with a purity $\geq 98\%$ in powder form, dissolved in the mediums with a 100 mM concentration and conserved at 4 °C for maximum 5 days. The rabbit muscle Lactate dehydrogenase enzyme was purchased dehydrated, dissolved in PBS to a concentration of 100 U/ml, and aliquoted in 1 ml tubes stored at −20 °C.

The veal urine samples were provided by the Venetian Zooprophylactic Experimental Institute of Legnaro (Padova), from a 6 months old veal, and stored at −80 °C.

The sensors were purchased from Dropsens Inc. (Dropsens 110). They consist of screen-printed electrodes on ceramic substrate composed by carbon (working and counter electrodes) and silver (pseudo-reference electrode and contacts), respectively. The sensors were used with a Dropsens adapter both in the three and two electrodes configurations, for CV and EIS measurements, respectively.

The measurement techniques involved in this work are Cyclic Voltammetry (CV), and UV-Visible spectrometry (UV-Vis). The CVs were recorded using a CH Instruments Inc. 440a potentiostat with 100 µl of solution, while the UV-Vis measurements were performed using a Mapada UV 1600PC spectrophotometer with 4 ml quartz cuvettes (1 cm path length) filled by 2 ml of solution, i.e., a sufficient volume to completely cover the incident light spot.

The data analysis was performed by Matlab 2012a, using its native curve fitting tool for the linear fits.

3 Results and Discussion

The veal urine sample was diluted in HBRS to several concentrations ranging from 1 to 100% (pure sample).

The dilutions were characterized by UV-Visible spectroscopy and CV in order to define the level of interference which the sample exhibits at 340 nm, and at 456 mV, respectively. The former value is usually associated to the NADH absorption peak, while the latter to oxidation on the carbon electrodes. The results are depicted in Fig. 1.

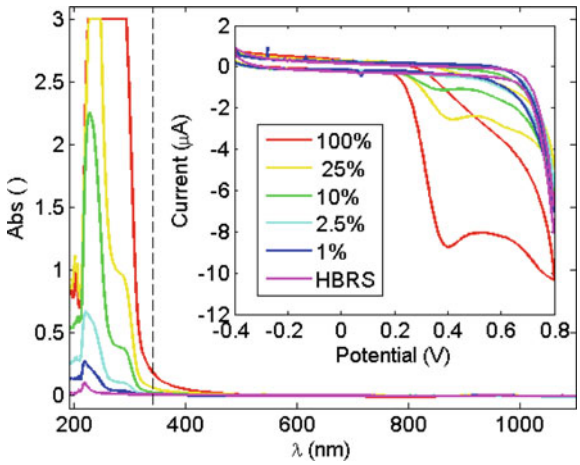

Fig. 1. UV-Visible absorption spectra of veal urine dilutions in HBRS. The *inset* shows the CV data of the same solutions

The adsorption at 340 nm is slightly disturbed by the urine sample. Conversely, the oxidation of one or more interfering species appears over 300 mV in the CVs, and the associated current is relevant at high sample concentrations.

Four urine sample dilutions in HBRS were selected for the next experiments, i.e. 100% (pure sample), 10, 1, 0% (HBRS). Each urine sample solution was spiked by 10 Lactate dilutions, ranging from 1 μM to 50 mM.

The prepared dilutions were tested by UV-Vis kinetic measurements at 340 nm for 300 s by mixing in the quartz cuvette 625 μM NAD^+ in the respective sample dilution (1.6 ml), 100 U/ml LDH in PBS (0.2 ml), and each Lactate dilution (0.2 ml). Then, at the end of the NADH formation kinetics, 100 μl of the cuvette solution were drop-casted on 3 electrochemical sensors for the CV NADH measurement.

Figures 2 and 3 show the UV-Vis kinetics and CVs for the Lactate dilutions in pure HBRS. The 300 s absorbance values, obtained from the UV-Vis kinetics allow the calculation of the final NADH concentration through the Lambert-Beer equation and the millimolar absorption coefficient 6.22 Abs mM^{-1} cm^{-1}.

Depending on which parameter of the CV curves is considered, different NADH calibration curves can be obtained, with respect to the UV-Vis determined NADH

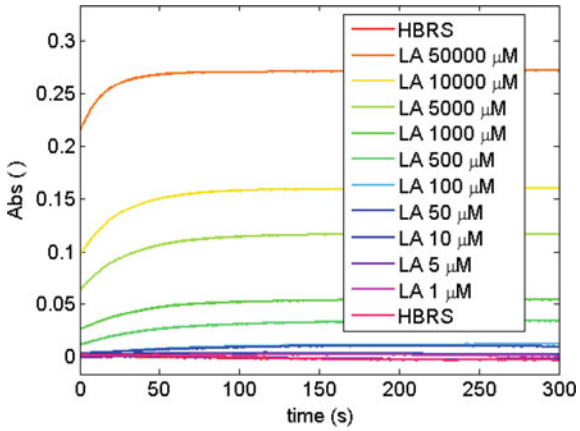

Fig. 2. UV-Vis kinetics at 340 nm of the Lactate dilutions in pure HBRS

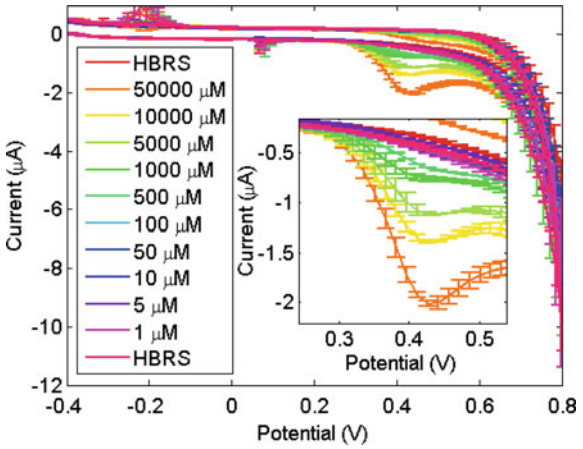

Fig. 3. CVs on the sensors of 100 µl of the Lactate testing solutions in the quartz cuvette at the end of the UV-Vis kinetic measurements

concentration. Therefore, we propose six CVs parameters for the NADH quantification in each urine sample dilution, defined as:

- P1: current value at 456 mV;
- P2: area under the CV curve;
- P3: area under the CV curves between 0.2 and 0.8 V;
- P4: area under the CV curves between 0.2 and 0.55 V;
- P5: current recorded at the potential which give the maximum R^2 value of the linear fit of the currents against the NADH concentrations;
- P6: current at the potential which give the absolute maximum slope of the linear fit of the currents against the NADH concentrations.

The definitions of these parameters reflect all the possible interpretations of the CV data. For example, P1 represent the current values at a fixed potential (obtained as the average of the NADH dilutions oxidation peak potentials). Conversely, P2, P3 and P4 are related to the charge accumulation, i.e., to the capacitance, developed by the NADH oxidation. They become more specific for this reaction as the boundaries of the area calculation becomes closer to the NADH oxidation potential. Finally, P5 and P6 are focused on the NADH calibration curve instead on the single CV.

Figures 4 and 5 show the absolute values of the slopes and the R^2 values of the linear fits of the NADH calibration plots, obtained by the six parameters considered from the UV-Vis and the CV measurements in the Lactate dilutions. The slopes represent the NADH detection sensitivity by the sensors while the R^2 values are a good indicator of the linearity of the detection in the considered range.

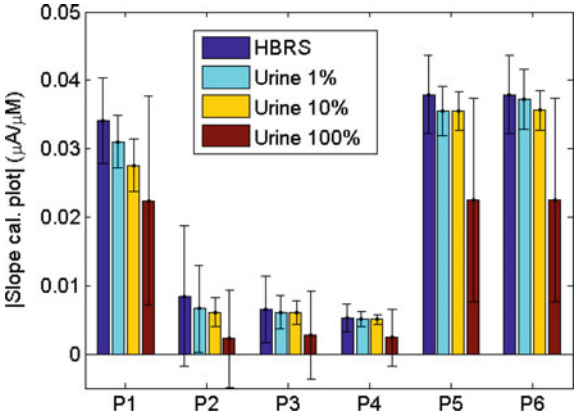

Fig. 4. Average modules of the NADH calibration plot linear fits' slopes and respective 95% confidence intervals, obtained by the CV parameters with respect to the 300 s UV-Vis kinetics determined NADH concentrations

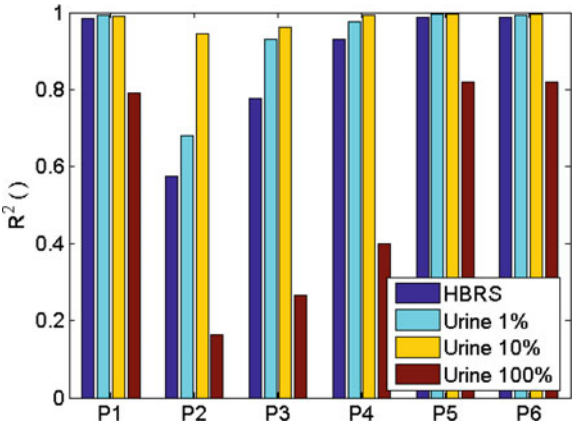

Fig. 5. R^2 values obtained from the linear fits of the CV parameters with respect to the 300 s UV-VIS kinetics determined NADH concentrations

From Fig. 4 it's evident that the P5 and P6 parameters allow a higher sensitivity, but the Lactate measurements in the pure urine sample shows a drastic sensitivity reduction and a standard deviation boost, most probably caused by the interferences observed in Fig. 1 inset.

Since the R^2 values represent the linear fit quality, it is interesting to notice that the P1 and P6 show a fit quality very similar to P5, which has the highest R^2 as it is formulated to maximize it. Moreover, as expected, the R^2 values of the area related parameters tend to those of P1, P5 and P6 as the portion of area is limited around the 456 mV current peak.

Another way to define the quality of the linear fits is to consider the fitted slope 95% confidence intervals. The linear fits which maximize the slope and minimize the interval is the best candidate as CV-related parameter for the NADH sensitive quantification. Therefore, the maximization of the ratio between the slope and its confidence interval becomes an important quality factor (QF), as described in Eq. 1, and shown in the inset of Fig. 6.

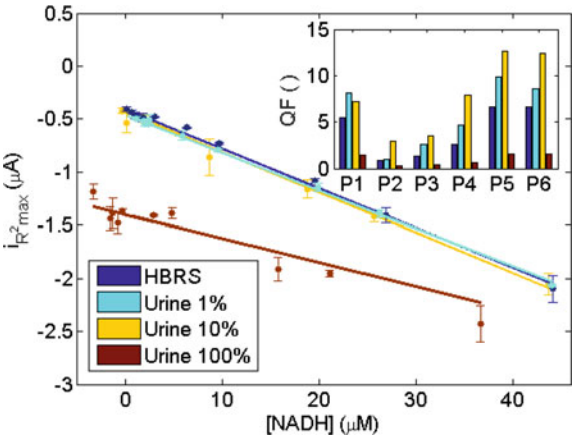

Fig. 6. NADH calibration plots and linear fits obtained by the P5 parameter for the Lactate concentrations in 100, 10, 1, and 0% sample urine. The *inset* shows the quality factor defined by the ratio between the linear fit slope and its 95% confidence interval for the NADH calibration plots of the CV parameters, with respect to the 300 s UV-Vis kinetics determined NADH concentrations

$$QF = |m| / cf_m^{95\%} \tag{1}$$

Where m is the considered parameter linear fit slope, and $cf_m^{95\%}$ is its 95% confidence interval obtained by the fitting procedure.

Again P5 and P6 result the best parameters and the measurements in the pure urine sample show very low values. However, the solutions which show the best ratios are 10 and 1% sample urine instead of HBRS, as could be expected.

Figure 6 shows the calibration plots and the respective linear fits obtained from the P5 parameter for the CV analysis, which proved to be the best in terms of quality of the linear fit (by definition), slope of the fit (and thus NADH sensitivity), and ratio between the slope and its 95% confidence interval.

4 Conclusions

In this work we have proved that the Lactate detection and quantification in veal urine is possible both optically by standard techniques such as UV-Visible spectroscopy, and electrochemically by Cyclic Voltammetries on low-cost disposable carbon screen-printed electrodes.

Furthermore, we have proposed several methods to analyze the CV data obtained after the stabilization of the LDH enzyme Lactate catalysis, and thus the produced NADH concentration. We compared these methods by evaluating their linearity, their sensitivity, and their linear fitting errors, with respect to the NADH concentration produced by the LDH catalysis.

We found that the best CV parameters are the currents recorded at the potentials that maximize the slope, i.e., the sensitivity (P6) of the linear fit of the calibration curve, and that maximize its R^2 value (P5). Therefore, the best approach to the data analysis in this case is to define in this way the potential by the calibration curve and then to use this parameter for the quantification of unknown Lactate concentrations.

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