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Characterisation of Electrochemical Parameters for the Stabilisation of Anthocyanins from Hibiscus Sabdarrifa L

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Abstract The high nutritional value of *Hibiscus sabdariffa L* calyxes is now known almost worldwide. The multiple benefits of Hibiscus juice on human health explain the new consumer demands on the preservation techniques of the nutritional qualities of Hibiscus juice. The instability of anthocyanins, the molecules responsible for the red colouring of *Hibiscus sabdariffa* calyxes, remains a problem despite the numerous stabilisation techniques on the subject. Platinum electrode oxygen reduction is a new athermal technique using a two-compartment electrolysis cell separated by a cationic membrane. The fruit juice is stabilised by the passage of the reduction current for a specified time. Cyclic voltammetry was used to characterise the electroactive element, the reduction peak, which is dissolved oxygen. The electroreduced Hibiscus juice was then stored at 37°C for 30 days with an untreated control. After one month of storage, determination of the anthocyanin concentration of the electroreduced extracts and the control made it possible to retain the 30mn/-6mA couple presenting a significant difference compared to the control for the stabilisation of the juices. Bubbling with nitrogen gas not only justified the negative impact of dissolved oxygen in the *Hibiscus sabdariffa* juice but also confirmed that oxygen is the electroactive element in the extract. The result obtained on bubbling also reveals that it is necessary to bubble for 2 hours to obtain an anthocyanin concentration of 279.21 mg/l after storage at 37°C for 30 days, whereas 282.66 mg/l is obtained for the same extract treated electrochemically (30mn/-6mA) and stored under the same conditions; both concentrations are significantly different from the control 263.37 mg/l. The electrochemical treatment with the time/intensity couple of 30min/-6mA for 500ml of Hibiscus sabdariffa juice on platinum/ECS electrode allowed to keep more than 10% of anthocyanins at 25°C and 37°C after 30 days of storage, significant compared to the control.

Keywords: *Hibiscus sabdariffa, Anthocyanin, Stabilisation, Oxygen, Storage, Electrochemistry*

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1. Introduction

The multiple benefits of anthocyanins are due to their antioxidant properties [1,2,3], a chemical substance found in several foods, notably those of *Hibiscus sabdariffa L.*, commonly known as bissap in Senegal, which have the power to neutralise the free radicals that damage cells, thus causing several diseases [4,5].

The particularity of the calyxes of *Hibiscus sabdariffa L.* also lies in their mineral composition, especially vitamin C. The anthocyanins in Hibiscus sabdariffa L. can prevent cancer, reduce the risk of cardiovascular disease, reduce the risk of high blood pressure, strengthen bones and teeth, strengthen the immune system, improve intestinal regularity, promote healthy weight, etc. [8-13].

However, the instability of anthocyanins due to its component flavylium cation, which is highly reactive to factors such as temperature, light, pH, enzymes and oxygen, among others, causes Hibiscus sabdariffa-based juices to discolour during storage [14-20].

The impacts of oxygen on the sensory quality of food products and the underlying reactions have been reviewed by several authors [20,21,22].

As a result, much work has been done on the stabilisation of these pigments.

Heat treatment has long been used by researchers for the stabilisation of food products, notably the work of Mady Cissé [23,24].

Pasteurisation and hot extraction have often been used to preserve anthocyanins from *Hibiscus sabdariffa*, as in the work of Alé Kane at 70°C/30 minutes [25,26].

Thermal treatment may allow a certain microbial stability but the nutritional qualities of the product remain

to be desired with a notable loss of anthocyanin concentration during preservation in addition to the relatively high energy cost [27,28].

Thermal processes remain the most widely adopted technology for the preservation of fruit juices. However, the strong consumer demand for nutritious food over time has pushed researchers towards new non-thermal techniques for better stability while maintaining the organoleptic qualities of fruit juices [23,27,29].

In addition, co-pigmentation, which consists of adding tannins such as gum arabic to obtain a good colour, is also a recent technique, as shown by the work of Papa Guedel Faye [30,31], but the change in the basic components of the juice remains problematic because the starting product is modified.

Non-thermal technologies are effective preservation treatments at room temperature minimising the thermal effects that degrade anthocyanins but also the organoleptic qualities of foods [29,32].

New athermal food preservation techniques considerably reduce microorganisms in fruit juices, thus reducing spoilage of food products during storage as shown by B K Tiwari in his paper [27,29].

Several of these techniques have been reviewed in the literature namely: high pressure treatment, pulsed electric field, irradiation, ultrasound, electrodialysis, tangential microfiltration, osmotic distillation, ultrafiltration among others [23,27,29,32,33,34,35].

The non-thermal technologies reviewed by these authors have the potential to eliminate almost all microorganisms but the potential development of the latter during storage favoured by the presence of oxygen is problematic in addition to the opening of the flavylium cation cycle under the action of oxygen leading to colourless products subsequently transformed into brown or black products, thus limiting these new techniques as a real alternative to thermal treatment. Moreover, their use in developing countries remains relatively expensive.

Oxygen reduction is attracting considerable attention due to the many applications that require electrode materials with simultaneously high catalytic activity, good electrical conduction, large specific surface area and above all improved stability [36,37,38,39].

The reduction of oxygen in solutions is not widely used. It was studied on iron and stainless steels in natural seawater by Nathalie LE BOZEC in her thesis in 2000 [40]. Platinum is a reference element in terms of catalytic efficiency [41]. However, its application on food products is rare or even non-existent to our knowledge.

Oxygen management in food products is one of the keys to ensuring better quality juices for different types of markets and consumers [21].

Oxygen reduction has been used as a process to prevent the auto-oxidation of food substances that may degrade by oxidation with oxygen. This involves adding an enzymatic compound comprising an oxidase and its substrate to these food substances and then studying the impact of oxygen [42].

Most of the work on oxygen reduction has been found in patents for dry food preservation.

In a Japanese patent, an invention was made with the aim of obtaining a milk that has good taste, flavour and texture by improving the physicochemical properties [43].

It was a combination of some processes for the removal of water and monovalent cations but also the penetration of divalent cations. In addition, the product is contacted with an inert gas (nitrogen) to reduce dissolved oxygen and then a heat treatment to sterilise the milk [44].

In the case of this study, the use of the electrode for juice preservation was used for the first time.

In contrast to conventional thermal techniques and athermal techniques used, oxygen reduction in juices is used for the first time to our knowledge to extend the shelf life of the product. In order to ensure that the treated juice keeps its organoleptic properties, several physico-chemical analyses will be carried out. This new approach would allow the reduction of dissolved oxygen; this would allow the extension of the shelf life of the juice without adding any stabiliser while keeping the organoleptic qualities of the product.

2. Materials and Methods

2.1. Material

2.1.1. Plant Material

The test was carried out using whole calyxes of *Hibiscus sabdariffa L.* We made a mixture of calyxes/water with the ratio 1/20 (kg/kg), the maceration was carried out at room temperature with distilled water for about 4h and the mixture was filtered.

2.1.2. Electrochemical Equipment

The electrochemical measurements carried out in this work used a three-electrode potentiostatic set-up. It comprises a platinum working electrode, the site of the electrochemical reactions studied, a stainless steel auxiliary electrode which closes the electrical circuit and a saturated calomel reference electrode, which allows the potential of the working electrode to be monitored and measured at any time. The system used is a potentiostat connected to a computer equipped with cyclic voltammetry software.

The electrochemical cell is a Plexiglas electrolysis cell with two compartments separated by a cationic membrane of the nafion type. On one side of the membrane, *Hibiscus sabdariffa* juice is in contact with a platinum cathode and on the other side, 0.1M hydrochloric acid (HCl) is in contact with a stainless steel anode.

2.1.3. Experimental Set-up

Figure 1. Electrochemical experimental setup

2.2. Methods

2.2.1. Voltammetric Study

One of the objectives of this study is to determine the oxygen reduction potential on a platinum electrode. In addition, it is also a question of determining the oxidation-reduction activity of bissap, i.e. whether or not there is the presence of electroactive compounds, as shown by the appearance of oxidation or reduction peaks on the curves obtained. The applied potential difference was set between a stainless steel reference electrode and the platinum plate working electrode, the stainless acid counter electrode. Both electrodes were immersed in one of the compartments of the electrolysis cell filled with Hibiscus sabdariffa juice in the presence of a bar magnet to agitate the solution and the counter electrode in the compartment containing the 0.1N HCl solution.

The potential sweep was performed between -0.5V and 1.5V/ECS at a rate of 100mV/s.

At the cathode, oxygen is reduced on the platinum electrode according to the reaction in acid medium:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \quad (1)$$

Platinum is satin-like, malleable and ductile. It is not affected by oxygen or water and is only soluble in aqua regia or molten alkali. In particular, platinum catalyses the direct reduction of oxygen by the 4-electron process [40].

2.2.2. Electroreduction of Bissap

The potential difference applied between the two electrodes, anode and cathode, is produced by an electric current generator, in potentiostatic mode, connected to a potentiostat which fixes the potential between the cathode and the reference electrode. The applied electrolysis potentials are fixed with respect to this reference.

The treatment was carried out on 500ml of bissap placed in the cathode compartment using an equivalent volume of hydrochloric acid in the anode compartment. The current intensities applied and the duration of the treatment were determined following the voltammetric study (see results and discussion).

At the end of each electrolysis, the treated product was placed in small vials wrapped in aluminium foil for storage at 37°C. After 4 weeks of storage, the anthocyanin content will be measured.

2.3. Determination of Anthocyanins

The determination was carried out on *Hibiscus sabdariffa* extract.

The principle is based on the modification of the anthocyanin coloration according to the pH (pH-differential method).

After dilution of the calyx extract in two buffer solutions at pH = 1.0 and pH = 4.5, the absorbance is measured at 510 and 700 nm, the values read at the two wavelengths for each solution are used to calculate the anthocyanin concentration by the following formula:

$$Ca = \frac{P_m * F_d * A * 1000}{\varepsilon} \quad (2)$$

Ca: anthocyanin concentration in mg/L.

Pm: molecular weight of the anthocyanin. In this case, Ca is expressed in relation to Delphinidin Sambubioside, which is the majority anthocyanin in the calyxes of *Hibiscus sabdariffa L*. Its molecular weight is 597 g/mol.
ε: molecular extinction coefficient and is equal to 26 000 L. mol^{-1}. Cm^{-1}.
Fd: dilution factor
A: absorbance, calculated using the formula:

$$A = (A1 - A2) - (A3 - A4)$$

A1 = absorbance measured at pH 1 at 510 nm
A2= absorbance measured at pH 1 at 700 nm
A3 = absorbance measured at pH 4.5 at 510 nm
A4 = absorbance measured at pH 4.5 at 700 nm.

3. Results and Discussion

In this section, we present the results of the cyclic voltammetric characterisation of dissolved oxygen, the treatment time/reduction current intensity and the electrode surface in solution. The working electrode is a platinum plate, the counter electrode is stainless steel and the reference electrode is saturated calomel.

The results obtained after electrochemical characterisation were applied to the Hibiscus sabdariffa extract which will be subsequently stored at 4°C, 25°C and 37°C with the untreated control.

Analyses of variance (ANOVA) were performed with STATISTICA 7.1 software and significance is represented as superscript and lower case letters. Identical superscripts on a result mean that there is no significant difference and when the superscripts are different then the difference is significant.

3.1. Cyclic Voltammetry of Hibiscus Sabdariffa Extract

In order to characterise the presence of electroactive elements in *Hibiscus sabdariffa* extract, we applied cyclic voltammetry to it. The first result obtained is presented in the following figure.

The curve I = f(E) in Figure 2 shows a voltammogram with a peak (arrow) corresponding to an intensity of -5mA for the *Hibiscus sabdariffa* extract (A) and next to it (B) a voltammogram (the blank: distilled water + salt) with a peak at -13mA. This result shows the presence of an electroactive element in the solution. Indeed, the presence of a peak (Figure 2) on the voltammogram attests that there is an electroactive element (which exchanges electrons) in solution, the nature of which will be confirmed by other experiments (Table 1 and Figure 3).

The difference noted in the intensity of the peak is due to the nature of the solution. Indeed, dissolved oxygen decreases with concentration. The effect of oxygen in bissap juice is limited by the vitamin C [45].

At first sight, it seems that oxygen is the electroactive element in the solution. In order to justify this, we found it necessary to bubble the extract with nitrogen while measuring the concentration of dissolved oxygen with the oximeter but also and especially to proceed to the backflush during the electroreduction of some samples by observing the cathodic reduction peak to corroborate our hypothesis.

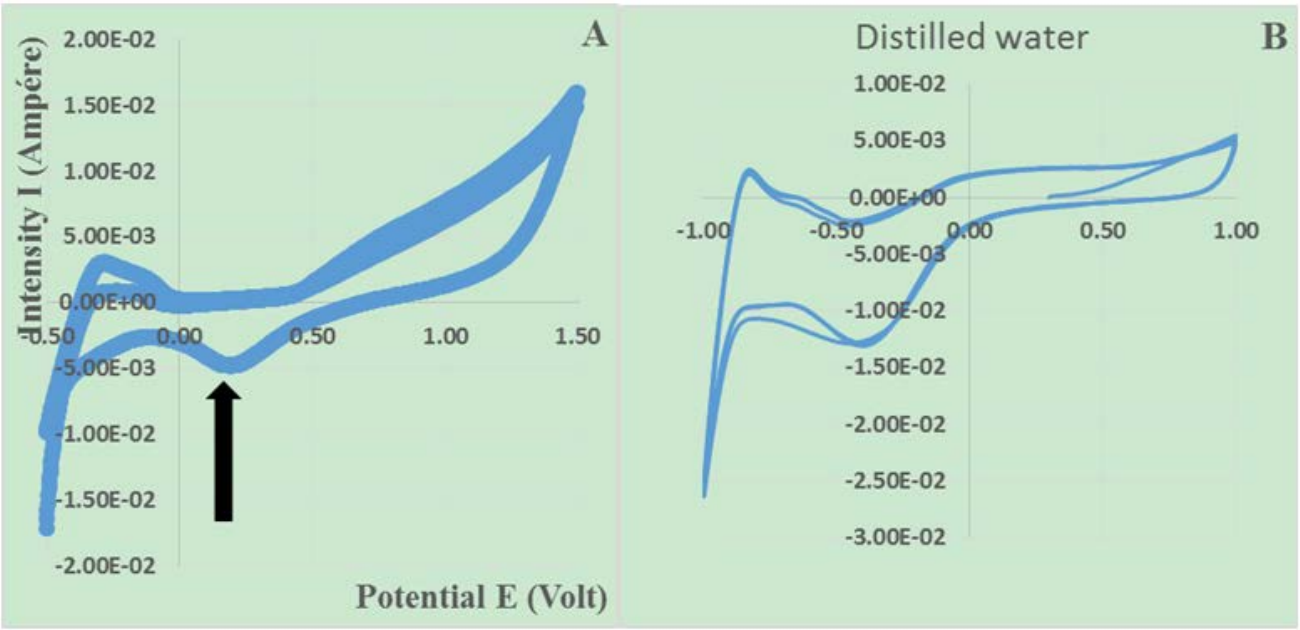

Figure 2. A-Voltammogram of *Hibiscus sabdariffa* extract; B-Voltammogram of the blank

Table 1. Dissolved oxygen and anthocyanin concentration (after one month's storage at 37°C) of Hibiscus sabdariffa extract after bubbling with nitrogen gas

Paddling time	Initial	30minutes	1hour	1h30min	2heures	2h30min
$[O_2]$ en mg/l	8	5,62	3,15	2,37	2,11	1,53
Anthocyanin Concentration in mg/l	393,33$^{(a)}$	250,30$^{(b)}$	256,02$^{(bc)}$	264,29$^{(c)}$	268,88$^{(dc)}$	279,21$^{(d)}$

The following Table 1 presents the anthocyanin and dissolved oxygen concentration as a function of the bubbling time of Hibiscus sabdariffa extracts. After bubbling with nitrogen gas for a given time for each 500 ml extract, the samples are then stored at 37°C for one month before determining the anthocyanin concentration.

The results presented in Table 1 show the degrading effect of oxygen on anthocyanin concentration. The bubbling of Hibiscus extracts with nitrogen gas in one-hour increments shows a significant difference in anthocyanin concentration after one month of storage at 37C. However, there was no significant difference in the concentration of anthocyanins bubbled in 30-minute steps.

The initial anthocyanin concentration (393.93 mg/l) has a significant difference compared to the anthocyanin concentration of all other extracts bubbled and stored at 37°C for one month : the temperature factor predominates over the other factors. Nevertheless, dissolved oxygen significantly degrades anthocyanins during storage as shown in the results in Table 1.

The reduction of dissolved oxygen in the Hibiscus juice can be justified by a return scan on the already electroreduced extract with the intensity of the peak characterised by cyclic voltammetry (Figure 2).

The results on the second scan at a speed of 100mv/s are presented in the following Figure 3.

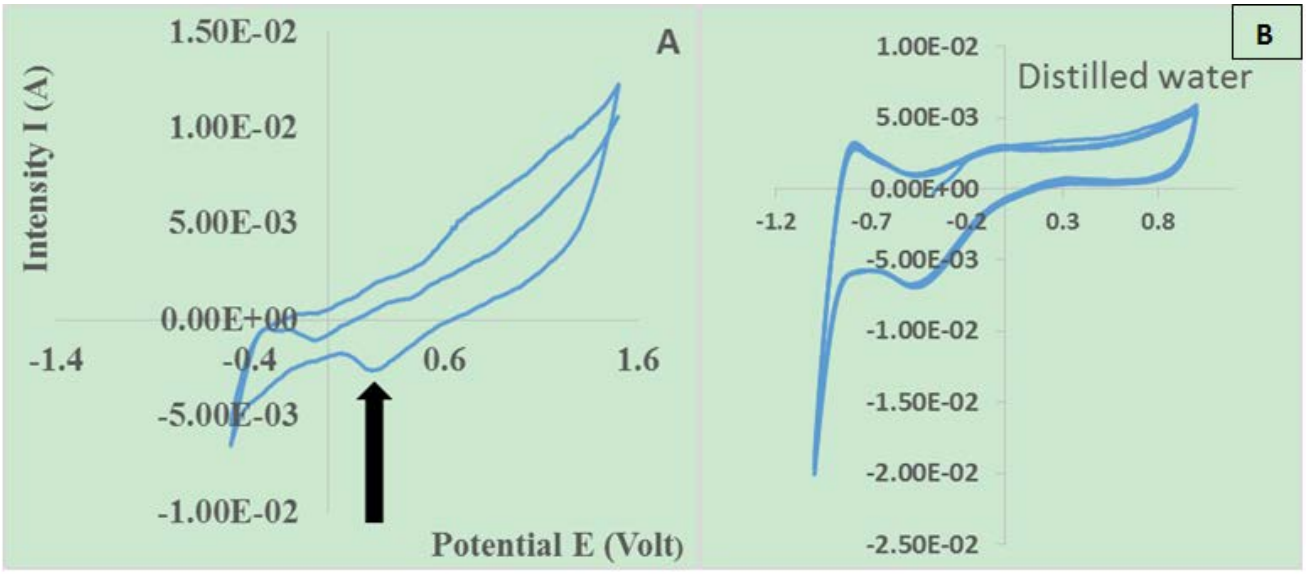

Figure 3. A-Voltammogram of electroreduced Hibiscus sabdariffa extract B-Voltammogram of electroreduced blank

Figure 3 shows the cyclic voltammetry curves (feedback) of the Hibiscus extract and the blank (distilled water) after electroreduction at the respective intensities (-5mA and -13mA) obtained in Figure 2.

It is clearly seen in Figure 3 that the intensity of the reduction peak decreased (-2.5mA) for the extract and (-6.5mA) for the blank after electrochemical treatment compared to the curve in Figure 2 where there was no electroreduction yet. Indeed, the chronopotentiometric treatment consisting in applying the peak current intensity (during a well-defined duration) obtained after cyclic voltammetry of the extract as a function of time I=f(t) made it possible to show that the electroactive element (oxygen) had been reduced.

3.2. Optimisation of the Time/Intensity Pair

The characterisation of the electrochemical parameters is fundamental for the treatment of juices. The reduction of juices on a platinum electrode makes it possible to electrochemically transform one oxygen molecule into two water molecules. The aim is to carry out chronopotentiometry by applying the intensity of the reduction peak (-5mA) obtained by cyclic voltammetry (Figure 2) for a specific period of time.

Use of experimental designs for the determination of the treatment time

In order to reduce the number of tests and still get good results, we opted for experimental designs to determine the treatment time.

In our case, we used the quadratic model with the following input parameters: treatment time and intensity of the reduction peak while choosing the minimum and maximum values carefully. The output parameters are the concentration and colour of anthocyanins. The evaluation of the method by the STATGRAPHICS software gave 9 trials.

It should be noted that in this optimisation, the counter electrode is made of stainless steel. As for the reference electrode, it is made of saturated calomel.

Table 2 below shows the anthocyanin concentrations, standard deviations and percentages of anthocyanin loss after 30 days of storage at 37°C.

We note that, after 30 days of storage, there is a significant degradation of anthocyanins for all samples compared to the initial concentration due to the temperature factor. However, we observe a significant difference between the anthocyanin concentration of extracts treated with the following couples : 5mn/-10mA ; 23mn/-2mA ; 23mn/-6mA; 23mn/-10 and 40mn/-6mA and that of the control kept under the same conditions.

For a good choice of the treatment time/current intensity couple, another experiment is necessary but first, let us optimise the electrode surface.

Table 2. Anthocyanin concentration of the 9 samples after one month of storage at 37°C

Couple time/Intensity (min/mA)	Anthocyanin Concentration (mg/l)	Standard deviation	Percentage loss in anthocyanin
INITIAL	384,48$^{(a)}$	1,94	0,00
5min/-2mA	263,66$^{(cd)}$	11,07	33,16
5min/-6mA	276,30$^{(bd)}$	6,32	29,96
5min/-10mA	283,96$^{(b)}$	21,24	28,02
23min/-2mA	283,58$^{(b)}$	1,15	28,11
23min/-6mA	277,83$^{(b)}$	9,94	29,57
23min/-10mA	284,51$^{(b)}$	5,84	27,88
40min/-2mA	269,42$^{(bd)}$	13,79	31,70
40min/-6mA	278,98$^{(b)}$	16,68	29,28
40min/-10mA	267,88$^{(bd)}$	6,73	32,09
Control	263,37$^{(c)}$	11,64	33,24

3.3. Optimisation of the Current Density

In order to know the appropriate surface to immerse the electrode, we have optimised the surface of the electrode.

To do so, we chose the 30min/6mA couple and the platinum plate working electrode by varying the immersed part of the electrode by steps of 1cm to the bottom.

The results obtained are presented in the following Table 3.

Table 3. Anthocyanin concentration of the electroreduced extracts with variation of the surface of the working electrode by 1cm steps after 4 weeks of storage at 37°C

Time/Intensity	anthocyanin Concentration (mg/l)	% loss	Standard deviation
Initial	393,33$^{(a)}$	0	18,42
30-6_1cm	250,05$^{(b)}$	36,43	6,51
30-6_2cm	271,18$^{(c)}$	31,06	11,35
30 -6_3cm	277,38$^{(c)}$	29,48	15,69
30-6_4cm	282,66$^{(d)}$	28,14	11,38
30-6_El fond	266,58$^{(bc)}$	32,22	8,33
CONTROL	245,00$^{(b)}$	37,71	12,00

According to the experiment, the immersed part of the electrode should be longer than 1cm and should not touch the bottom either. Lengths of 2, 3 and 4cm are acceptable (significant difference with the control) but 4cm is the best option as it has a significant difference with all other samples. Thus, a 4cm immersion will be adopted for the electrodes.

This result is also understandable as it is an electron transfer in aqueous solution that takes place between the working and reference electrodes. The migration concerns all charged species between these two electrodes, hence the importance of the electrode surface for a good movement of ions. This justifies the value obtained for an immersion of 4cm.

In reality, it is the experiment that will decide the best couple among those selected.

3.4. Optimisation of the Pre-selected Torques

The study of a new technology always requires prior definition of all the parameters involved. Thus, in order to carry out the study of this new electrochemical technique applied to food products, we must experimentally choose between these pre-selected time/intensity couples while respecting an immersion of 4cm of all the electrodes (Table 4).

Table 4. Anthocyanin concentration of electroreduced extracts with the platinum plate as working electrode

Time/Intensity	Anthocyanin Concentration (mg/l)	% loss	Standard deviation
30-10_4cm	260,38$^{(ab)}$	33,80	5,53
40-6_4cm	272,09$^{(a)}$	30,82	9,36
30-6_4cm	282,66$^{(a)}$	28,14	11,38
CONTROL	245,00$^{(b)}$	37,71	12,00

The results in Table 4 show a non-significant difference in the concentration of anthocyanins in the electroreduced samples with the 30mn/-10mA_4cm pair and the control. On the other hand, there is a significant difference between the concentration of the pairs: 40mn/ 6mA_4cm; 30mn/-6mA_4cm and the control.

The juice stabilisation treatment can be carried out for 30 minutes at a current intensity of -6 mA. This result on the anthocyanin concentration (282.66 mg/l) corroborates those in Table 3 (30mn/-6mA/4cm), showing at the same time the reproducibility of the method.

Comparing this result with those obtained by bubbling with nitrogen gas (Table 1), we can see that bubbling for 2 hours is necessary to obtain approximately the same anthocyanin concentration (279.21 mg/l) obtained after 30 minutes of electroreduction at an intensity of -6 mA. This allows us to conclude that this new electrochemical technique could also be an alternative to bubbling.

3.5. Monitoring of Electroreduced Extracts and the Control at 3 Temperatures after One Month of Storage

One of the aims of our laboratory study is to evaluate the effect of the reduction of dissolved oxygen in the juices by quantifying it in a reproducible way. Therefore, we monitored electroreduced extracts on platinum/ECS electrodes stored at 4°C, 25°C and 37°C compared to an untreated control stored under the same conditions as the samples.

The concentration of anthocyanins after 4 weeks of storage at 4°C, 25°C and 37°C of the sample and the control are presented in the following Figure 4.

Figure 4 shows the results obtained after 4 weeks of storage at three different temperatures. We note a degradation at all temperatures, especially at 37°C where the temperature factor predominates over the others. However, the electroreduced extract (Sample) degrades less at all temperatures compared to the untreated extract (Control). At 4°C, the difference between the sample and the control is not significant compared to the other temperatures (25 and 37°C) because the effect of oxygen on anthocyanins would be catalysed by a certain temperature during the first days of storage [20].

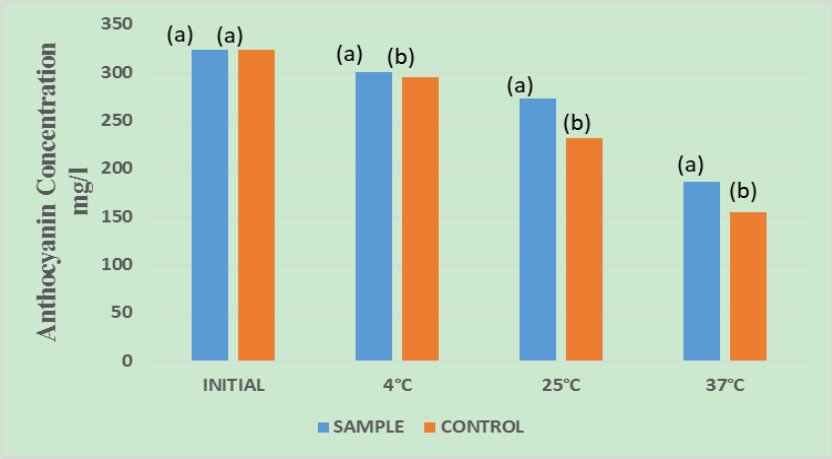

Figure 4. Monitoring of Hibiscus sabdariffa juice and the control at 4°C, 25°C, 37°C after 30 days of storage

In addition, stabilisation studies carried out by Mady CISSE [17] have shown considerable losses caused by heat treatment in relation to the initial concentration, thus showing the limits of heat treatment. In the same vein, M. CISSE [46] followed up with tangential microfiltration on a ceramic membrane with an increasing volume reduction factor. After 2 months of conservation, no microbiological development was noted in the extracts conserved at temperatures of 4, 20 and 37°C. However, losses in anthocyanins and vitamin C were observed. Still referring to the work of Mr CISSE, concentration by vacuum evaporation leads to concentrates with final characteristics far removed from those of fresh products (losses in vitamin C, anthocyanin, total sugars and aroma compounds of between 50 and 100%).

In the same logic of anthocyanin stabilisation, Pape Guedel FAYE [30] tested the effect of gum arabic on Hibiscus sabdariffa extract. The 10g/L gum arabic ratio reduced anthocyanin losses by 6% compared to the control after only 120 days of storage at 4°C. The treatment had no effect on the extract at higher temperatures and sometimes degraded the syrups compared to the control.

Compared to our results, we were able to preserve 12% at 25°C and 10% at 37°C and almost 3% at 4°C after 30 days of storage without altering the juice.

Innovation and quality are essential concepts for industrial success and market conquest, so this new electrochemical technique could be a real alternative for the stabilisation of food products.

3.6. Colour Monitoring

In order to confirm the results obtained on the monitoring of anthocyanins, the red colour was also characterised using a colorimeter. The coordinates (L, a*, b*) according to the CieLab system are read directly on the screen. The coordinate a* corresponding to the red proportion is presented in the following Table 5.

Table 5. Monitoring of the red colour of the electroreduced extract and the control at 4°C, 25°C and 37°C

4°C		25°C		37°C	
Sample[(a)]	Control[(a)]	Sample[(a)]	Control[(b)]	Sample[(a)]	Control[(b)]
56,14	56,06	56,05	55,99	55,75	52,52
56,13	56,11	56,09	55,97	55,73	52,62
56,16	56,16	56,06	55,95	55,7	52,66

The results in Table 5 corroborate those already obtained on the concentration of anthocyanins (Figure 4). Indeed, there is a significant difference in the red colour of the treated extract (Sample) and the untreated Hibiscus extract (Control) preserved at 25 and 37°C. At 4°C, there was no significant difference between the sample and the control.

The consistency of the results on the monitoring of anthocyanins and red colour can be explained by the fact that the molecules responsible for the red colouring of Hibiscus sabdariffa juice are anthocyanins.

4. Conclusion

In this study it was clearly demonstrated that the reduction of oxygen on a platinum electrode in Hibiscus sabdariffa L juice considerably reduces the degradation of anthocyanins at room temperature during the first month of storage.

Nitrogen bubbling tests followed by storage under the same conditions as the electroreduced extract gave similar results. However, the implementation of bubbling in industries is costly and impractical.

By optimising different electrochemical parameters using the experimental designs, we know that for a stabilisation of 500 ml of fruit juice, with a ratio of 1/20, on a platinum/ECS plate (L=8.5cm ; W=4cm), a treatment time and reduction current of 30 minutes/-6mA with an electrode immersion of 4cm is required.

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