

Determination of 8-Hydroxy-2 Deoxyguanosine in *Pseudomonas Fluorescens* Freeze-Dried Exposed to the ROS Using HPLC with Electrochemical Detection Method

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Abstract— Oxidative DNA damage is involved in the cell death induced by freeze-dried powder during storage. Cell 8-hydroxy-2'-deoxyguanosine (8-oxodG) is widely accepted as a biomarker of the "freeze-dried bacteria" oxidative DNA damage. The aim of this study was to introduce a method for determination 8-oxodG in cell freeze-dried samples using high-performance liquid chromatography with electrochemical detection. In the tested range of 0.5 $\mu\text{mol L}^{-1}$ to 1.0 nmol L^{-1} , the calibration curve was linear ($r^2=0.9995$) and the limit of detection was 0.05 $\mu\text{mol L}^{-1}$. The used method did not allow highlighting the presence in the samples of the 8OH within the limits of detection. A more successful method (more sensitive) would be needed to detect possibly the 8OH.

Keywords— *Reactive Oxygen Species (ROS), DNA, 8-Hydroxy-2 deoxyguanosine.*

I. INTRODUCTION

The drying by lyophilisation of microorganisms has some consequence on the viability of the latter. The powder of lyophilized bacteria remains vulnerable on the environmental conditions such as temperature, ultraviolet, reactive species of oxygens. The latter have mainly four consequences: the loss of the cellular viability caused by an oxidation of the biological materials like lipids, proteins and DNA [1,2,3]. The drying method cause severe damage in the cellular membranes such as the lipid peroxidation, denaturation of proteins and DNA leading to a loss of viability [4].

Freeze-drying and the storage of cells lead damage in proteins, fatty acids and DNA [5, 6]. Although the DNA damage undergone by the cellular membrane during the freeze-drying. It still plays an essential role in the loss of viability. The damage of the cellular components DNA and ARN affects considerably the viability of the freeze-dried bacteria during storage. The DNA is very sensitive to the drying, as demonstrated on *Echericha coli* [7, 8].

There are numerous modifications observed after the oxidation of the DNA, the major product of DNA oxidative damage is 8-hydroxyguanine (8-oxoG) and its nucleoside 8-hydroxy-2'-deoxyguanosine (8-oxodG). 8-oxodG has widely been accepted as a biomarker of DNA damage and cellular oxidative stress [9].

The present study will be focus on the development of novel method for the detection and quantification of the 2-déoxyguanosine 2dG and the 8-hydroxy-2-déoxyguanosine 8OH in extracts of DNA bacterial of *Pseudomonas fluorescens* freeze-dried. Because the 8-hydroxy-2-déoxyguanosine is the main oxidized by-product of the DNA used as a biomarker of the oxidative stress.

II. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria strain.

The *Pseudomonas fluorescens* BTP1 of Wallon Center of Industrial Biology laboratory (CWBI) was used as the strain in this study [2].

2.2 Methods

2.2.1 Photosensibilisation

The samples (bacterial powder) were exposed to methylene blue (MB) at 40 μM of in Light presence. The methylene blue and an incandescent bulb produce singlet oxygen [6,10].

2.2.2 Method of extraction of the genomic DNA of bacteria

Take a sample of cells (1,5 ml of night culture) and spin-dry it 5 minutes at 13000 rpm. Rinsing of the nerve with a PCR, then the sample were taking back the cap with 480 μL of EDTA (50 mm, pH 8). The addition of 30 μL of a solution of lysozyme to 20 mg/ml and let incubate 1 hour in 37°C by shaking. Add 30 μL of a solution of proteinase K to 20 mg/mL and let incubate 30 minutes in 37°C under agitation. Spin-dry 3 minutes at 13000 rpm, taken back by the cap in 600 μL of solution Nuclei Lysis by pipetting and incubate 10 minutes in -80°C. Incubate then 10 minutes in 80°C (dry bath); let it cool at room temperature [11].

Add 200 μL of solution protein precipitation shake 20 seconds and incubate 10 minutes in the ice. Spin-dry the solution during 5 minutes to 13000 rpm. Transfer the floats in a containing sterile tube 600 μL of isopropanol, mix delicately by inversion and spin-dry 5 minutes at 13000 rpm. Wash the cap with 600 μL of ethanol 70 %, spin-dry it 5 minutes in 13000 rpm and dry it under the basket (10-15 minutes). Rehydrate the cap in 50 μL of rehydration solution, incubate 60 minutes at 50°C and store the DNA during 1 month.

2.2.3 Extracts of bacterial DNA

The DNA was extracted according to the process described by P.Duez (Chromatographic determination of 8-Oxo-7,8-dihydro-2'-deoxyguanosine in cellular DNA).

2.2.4 Chemicals preparation

8-oxodG standard potassium chloride, acetonitrile, and methanol were purchased from Sigma Chemicals (St. Louis, MO, USA) and potassium dihydrogenphosphate and water were from Merck (Darmstadt, Germany). All chemicals were of *pro analysis* grade. Water, acetonitrile, KOH and methanol used for mobile phase were of HPLC grade. Cartridges (Bond Elut C18, Bond Elut C18 OH and Bond Elut Certify) for solid phase extraction (SPE).

2.2.5 Standard solution preparation

Stock solution of 8-oxodG (8-OH) was prepared dissolving the 8-oxodG standard in purified water to give a final concentration of 1,5 mmol L^{-1} and 2 mmol L^{-1} for 2-déoxyguanosine (2dG). The stock solution was aliquoted and stored at -18 °C.

2.2.6 Electrochemical conditions

HPLC-EC (High-Performance Liquid Chromatography with an Amperometric Detection).

HPLC (stocking) EC " Pump: Gilson 307, system of injection: Rheodyne 7125 (with two positions: LOAD / INJECTIONS), column Atlantis C18 (internal diameter: 3,0 μm , size: 4,6 mm x 100 mm) curls, locks up 20 μL ; detector amperometric in thin layer BASi (working electrode in glassy carbon), a reference electrode (electrode Ag / AgCl in a gel of NaCl 3 M), a stainless steel auxiliary electrode, a potentiostat LC-4B (BASi) and a recorder: PowerChrom 280.

2.2.7 Chromatography's conditions

Different mobile phases were studied. That used consists of: 50 mM KH_2PO_4 , KOH, pH 5.5, 13% Methanol, Water MilliQ 2L with a potential of 1.1 V. If we need to detect the two molecules simultaneously the potential required is 1.1 V; but for one molecule such as 8OH, the potential required is 700 mV. A scale of 1 to 5 nA, depending on the concentration with a flow rate of 0.8 mL / min.

2.3 Statistical analysis

Data from three replications were analyzed by using analysis of variance to determine if significant difference ($P \leq 0.05$) existed between mean values.

III. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Curves of calibration

Different curved of calibrations were realized with solutions between 0.5µM and 1nM. The dilutions were prepared in the mobile phase **figure** and **table 1**.

TABLE 1
OXIDATION POTENTIAL OF VARIOUS ELECTRODES GCE AND BDD

	GCE	BDD
Potentiel oxydation : (mV)	2.50	0.56

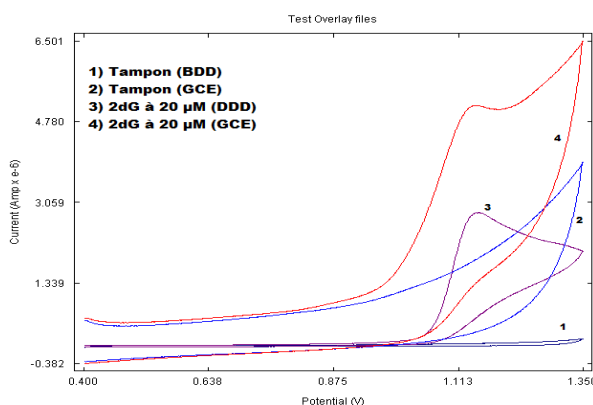


FIGURE 1: STUDY BY CYCLIC VOLTAMPEROMETRY OF THE 2-DEOXYGUANOSINE. REFERENCE ELECTRODE: Ag / AgCl, INDICATOR ELECTRODE: BDD (ELECTRODE OF DIAMOND DOPED IN THE BORON) / GCE (ELECTRODE OF CARBON GLASSY), AUXILIARY ELECTRODE: PLATINUM, BUFFER: ACETIC ACID, ACETATE OF SODIUM 0,1 M-pH 4,3, POTENTIAL OF 400 mV in 1350 mV, SPEED OF SCANNING OF 10mV.

According two days experiment, we observed a variation of the sensibility, to the high potential of oxidation and to the state of the surface of the working electrode according table 2 and figure 2.

TABLE 2
CALIBRATION OF THE METHOD BY THE DILUTION OF STANDARDS

Conc.	2dG (day 1)	2dG (day 2)	8OH (day 1)	8OH (day 2)
0.50 µM	2.50	2	1.50	0.80
0.10 µM	0.56	0.52	0.30	0.22
0.05 µM	0.28	0.22	0.14	0.10
1 nM	Nd	0.05	Nd	nd

Nd : not detected

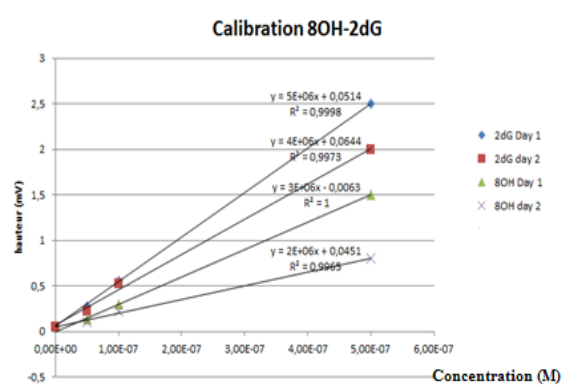


FIGURE 2: THE SENSIBILITY VARIATION OF THE METHOD

3.1.2 Chromatograph before and after the addition of the sample

We performed analysis of a standard 8OH and 2 dG from Sigma Aldrich, the unique indicators of the oxidation of DNA and injected samples. The results are shown in Figure 3.

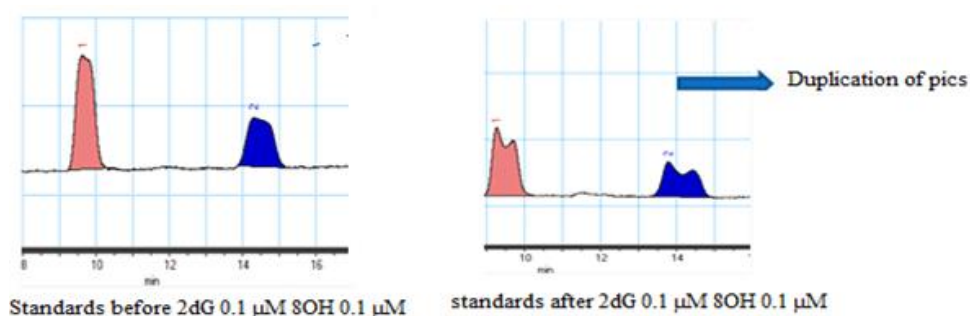


FIGURE 3: STANDARDS BEFORE AND AFTER INJECTION OF A SAMPLE

3.1.3 Dosage of the 8OH and 2dG

The DNA sample was extracted using the method described above on the lyophilized powder of *Pseudomonas fluorescens*. The results are illustrated in the chromatogram as shown in the figures 4.

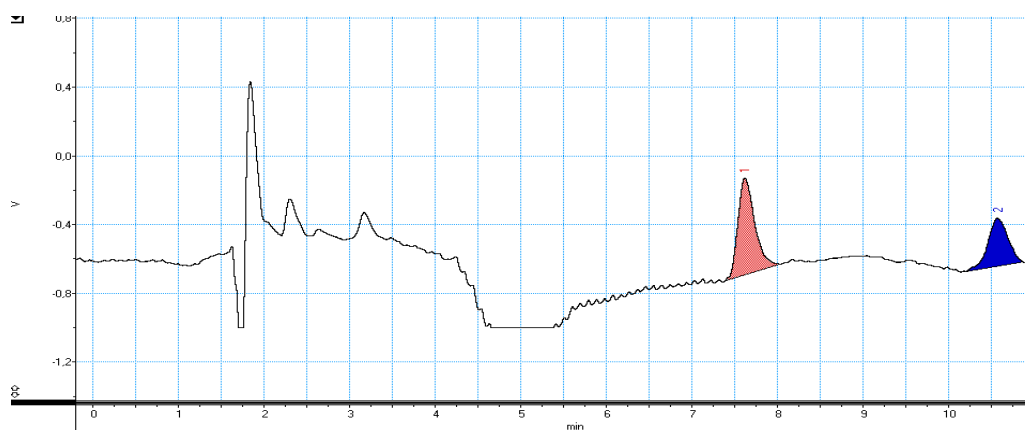


FIGURE 4: SPECTRE HPLC OF THE 8OH AND 2dG OF THE FREEZE-DRIED *PSEUDOMONAS FLUORESCENS* BTP1 STRAIN.

3.2 Discussions

3.2.1 Curves of calibration

The mobile phase was not stamped. We need to notice that other buffers were tested but none of them were well satisfactory due to the loss of sensibility, the important background noise detected during the experiment. The potential was observed brought up. It is essential to work with a potential of 1.1V to put in evidence the same time both molecules (the 8OH and 2dG) simultaneously figure 1. Indeed, the 2dG in a high potential of oxidation observed at 900 mV on electrode of glassy carbon as shown in figure 2. The 8OH has a potential of oxidation of 500mV on glassy carbon.

During the analysis of our sample, we observed a doubling of peaks probably due to degradation of the column and more particularly C18 groups [Figure 3].

The guard column was placed in order to avoid damaging the column. In the present experiment, we also precipitate residual proteins by adding methanol. It is important to notice that it is preferable to work in an acid medium for a better precipitation, which is not possible in our case because of the non-buffered mobile phase.

Then, the analysis of the samples was preceded by using the appropriate dilution for an efficient detection with the HPLC. The results are shown in the chromatogram in the figures 4.

3.2.2 Dosage of the 8OH and 2dG

The curve of calibration was realized every day for the dosage of the 2dG. In the present study, we proceeded that determination by using the method of standard additions due to the lower amounts of 8OH. The sample preparation was performed as follow: Collect 75 HL then add 25 µl of methanol (total 100 µL), mixer 30 seconds and centrifuge 5 min at 13,000 rpm. It is difficult to detect at the same time 8OH and 2dG in our conditions. Nevertheless, our results support the hypothesis that the DNA of the lyophilized the powder freeze-dried of bacteria mainly *Pseudomonas fluorescens* undergoes a damage during storage.

IV. CONCLUSION

The main purpose of this study was to confirm the hypothesis which supported that DNA also is subject of the oxidation in the same manner as lipids and proteins. The sensitivity of the 2dG was better measured in our operating conditions compared to the 8OH one. The current method was not able to highlight within the limits of detection the presence of the 8OH samples. An additional method would be needed for such to detection. Some scientists describe the tests on the oxidation of the ARN instead of the DNA, which represent an interesting way to explore [8].

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