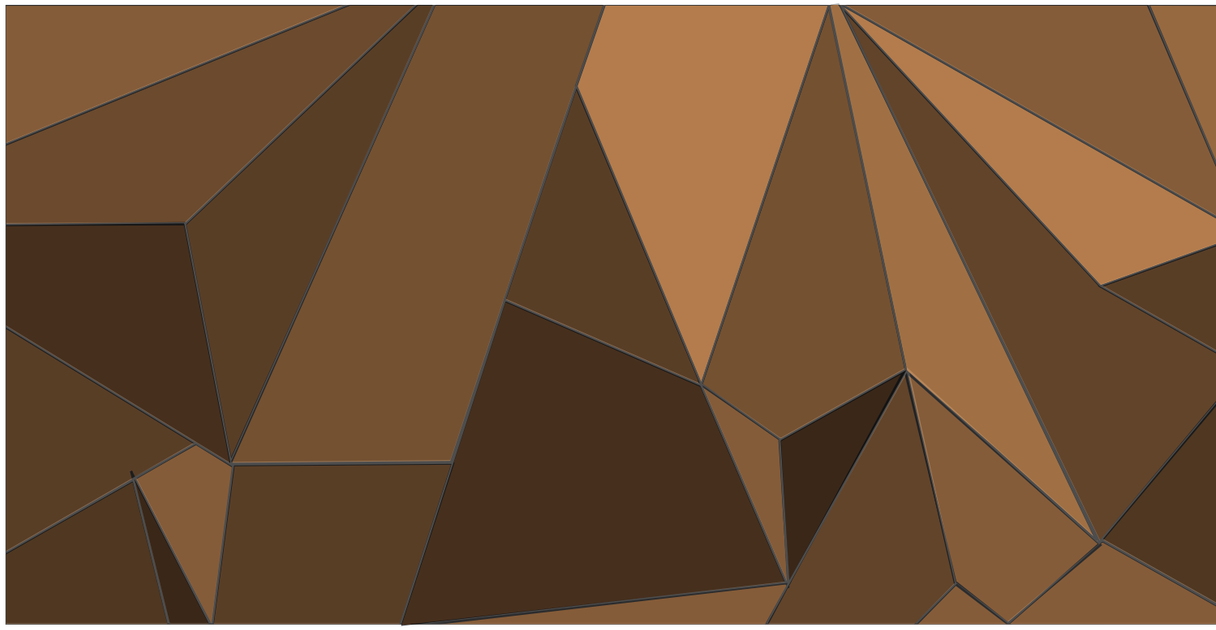

ATP-BIOLUMINESCENCE TECHNIQUE FOR FRUIT QUALITY ASSESSMENTS: A DETAILED METHODOLOGY FOR ANALYSIS OF ADENYLATE NUCLEOTIDES

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ABSTRACT

The bioluminescence technique has been used during a long time for measurement of adenylate nucleotides (ATP, ADP, and AMP) in several investigation fields. Until the end of the seventies, the assay was complicated and the results uncertain because of the low purity reagents, which were initially, extracted directly from desiccated lanterns of firefly (*Photinus pyralis*), and poorly controlled reaction conditions at the laboratory. With the introduction of a new type of luciferin-luciferase reagent, the ATP Monitoring Reagent (AMR), these disadvantages have been overcome, and therefore, the application of the assay has been extended. The new reagent results in the emission of light of an almost constant intensity, proportional to the ATP concentration. This is in contrast to the flash obtained with conventional reagents. This new technique facilitates the measurements and improves the sensitivity of the assay. The reagent may also be used to monitor changes in ATP concentration because the ratio between the ATP concentration and light intensity remains constant. This work describes the principle and a detailed methodology for determination of ATP, ADP, and AMP by the luciferin-luciferase reaction in fruits such as apples, pears and sweet cherries and some applications.

Keywords: Adenylate nucleotides, fruit quality monitoring, fruit biochemistry, high precision

1 INTRODUCTION

Bioluminescence is a widespread phenomenon characterized by light emission produced by enzyme-catalyzed reactions. Most systems consist of an enzyme, luciferase, catalyzing the oxidation of the O_2 or H_2O_2 of a substrate, luciferin: The light emission is of high quantum yield, and the color varies with the species, from blue to red, depending on the type of luciferin-luciferase system involved (Harvey, 1952).

Bioluminescence methods using the firefly luciferase enzyme finds its applications in various areas such as clinical testing, drug screening, development of biosensors for environmental pollutants, detection of microbial contamination (Lundin, 2000), genetic reporter assays in molecular biology (Gould and Subramani, 1988), detection of phosphatase activity (Miska and Gaiger, 1988), DNA sequencing (Ronaghi et al., 1996) and assessments of fruit quality after harvest, either during normal ripening studying the fruit physiology and biochemistry or during storage under regular air and controlled atmosphere systems (Saquet et al., 2000; Tan and Bangerth, 2001; Saquet et al., 2001; Harb et al., 2003; Saquet et al., 2003a; Saquet et al., 2003b; Streif and Saquet, 2003; Saquet and Almeida, 2015; Almeida and Saquet, 2016).

The aim of this report is, therefore, to present a detailed laboratory protocol in order to allow researchers to use this technique for assessments not only fruits and vegetables but as a tool for various other research investigation fields.

2 METHODOLOGY FOR FRESH FRUITS AND VEGETABLES

2.1 Equipment

The procedure described here is designed for two kinds of luminometer:

a) Luminometer model 1251 (LKB-Wallac, Turku, Finland). The instrument is fitted out with an auto-sampler and a water-through system in order to keep the temperature in the range of 25 °C. Other very important factor is the control of the environmental room temperature, which should kept in the range of 25 °C.

b) Synergy 2 Multi-Mode Reader (BioTek, Winooski, USA) adjusted with the sensitivity 200 and wavelength of 590/35 nm. This instrument does not have an internal system to control the temperature therefore, it is very important that the laboratory has an efficient air conditioning system in order to keep the sample incubation and further assay in the range of 25 °C.

2.2 Reagents

Purity of the reagents and solution preparation: luciferase and luciferin must be of the highest purity available. All other chemicals must be of reagent grade. For the preparation of solutions, it is very important that they be prepared in re-purified water and the containers must be sterilized to prevent microbial contamination.

2.3 Sources of error

The light emission can be considerably inhibited by the following effects:

a) Physically, by light absorption, if there is a substance present in the sample whose absorption spectrum overlaps the emission spectrum of the luciferase reaction.

b) By inhibition of the luciferase activity:

- By neutral salts which act on the one hand, by ionic concentration alone, and on the other hand, by a specific effect of the anions exerted in accordance with the chaotropic series.
- By specific inhibitors of the ATP or luciferin binding sites of luciferase.

All these interfering factors can be accounted for by carrying out control measurements with an internal ATP standard. The inhibitory effect can be corrected by the difference in signal emitted, therefore the crucial importance of the use of an internal standard during measurements.

2.4 Extraction and assay procedures

2.4.1 Sample preparation and adenylates extraction

Samples of fresh or freeze-dried fruits can be used. In the first case, reduced losses are achieved because of the direct use of fresh fruit material without the drying process. In the case of freeze-dried samples, they should be obtained from a low temperature vacuum system in order to avoid losses of nucleotides. It is recommended to use a vaccum system at -50 °C temperature and 1 bar negative pressure to dry simples. The advantage of this method is that it can be used for a great number of samples, keeping them at -30°C or below until analyzed. Samples can be taken from fruit flesh or the skin, depending of the aim of the research. Seeds and other plant materials can be used. In this report, results from the flesh of pears, apples and sweet cherries are described.

For the extraction of ATP, ADP and AMP one gram of dried and powdered sample is used. The samples were then placed in centrifuge tubes, on ice, containing a solution of 5% trichloracetic acid (2 mM) and homogenized. Samples were kept for 30 min for extraction and immediately centrifuged at least at 18,000 *g* during 15

min at 4 ºC. It is very important, before centrifugation to cool down the centrifuge at 4 °C. In order to minimize the inhibition effect of anions of the trichloracetic acid, samples were diluted 40 times with 0.1 M Tris EDTA buffer (2 mM, pH 7.75) before assessment. Here is also very important the exact adjustment of the pH of the Tris EDTA buffer.

2.4.2 Assay

For the assessment of ATP the following were mixed in a cuvette: 10 µL extract, 50 µL AMR (ATP monitoring reagent) and 440 µL of 0.1 M tris-EDTA buffer (2 mM, pH 7.75). The luminescence of this reaction was measured with a 1251 Luminometer at 25 °C (LKB Wallac, Turku, Finland). After measurement of each sample, an internal standard was fed and the luminescence recorded again. The ATP concentration was calculated using both values.

If used the Synergy 2 Multi-Mode Reader with the microplate, each microplate well contains a final volume of 200 µL. In this case it is necessary to adjust the volumes of each reagent and keeping the sample volume of 10 µL.

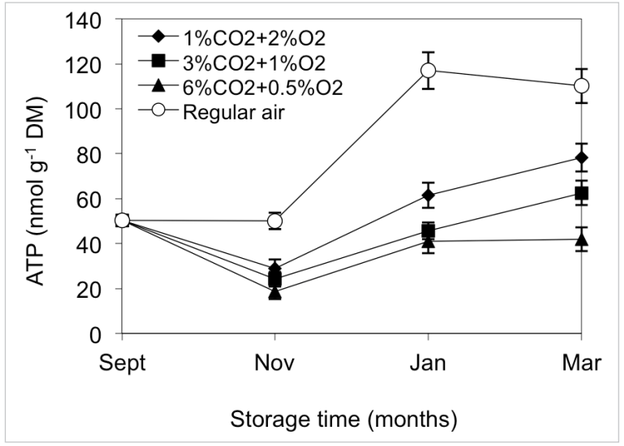
To measure ADP, it was first necessary to convert it to ATP. Samples were then incubated with pyruvate kinase (120 units/mL PEP buffer) at 25 °C for 30 min. The total ATP concentration was assayed as described above and ADP calculated by difference.

Before the AMP assessment, it needs to be converted to ADP and this converted to ATP by incubation of the samples with a mixture of myokinase (180 units/ml PEP buffer) and pyruvate kinase (120 units/mL PEP buffer) for 30 min at 25°C. Myokinase converts AMP to ADP and pyruvate kinase converts ADP to ATP. For these incubations and for measurements it is very important to keep the room temperature stable in the range of 25 °C.

3 SOME RESULTS IN FRUITS

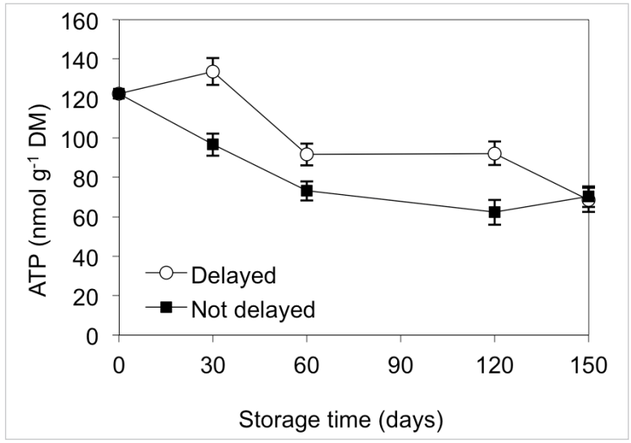
The following figures and table show examples of data with measurements of adenylate nucleotides in apples, pears, and sweet cherry.

Figure 1 - ATP levels in 'Jonagold' apple during storage period (DM = dry mass)



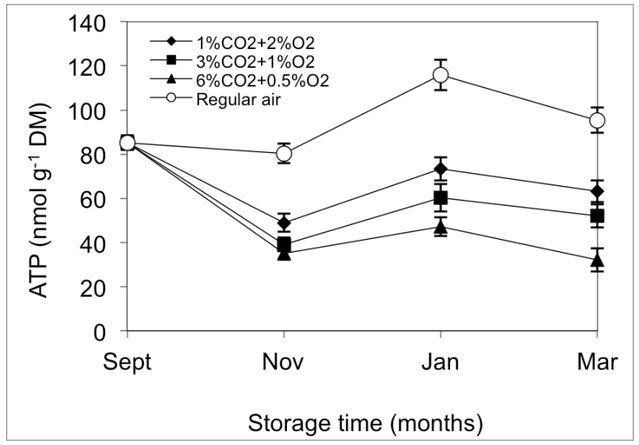
Fonte: Saquet et al., 2000

Figure 2 - ATP levels in 'Braeburn' apple during the storage period. Fruits were stored under 4% CO_2 plus 1% O2 (DM = dry mass)



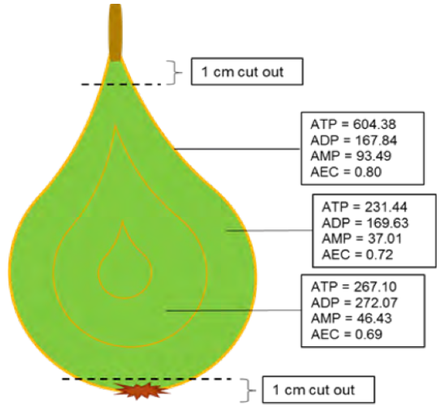
Fonte: Saquet et al., 2003b.

Figure 3 - ATP levels in 'Conference' pear during storage period (DM = dry mass)



Fonte: Saquet et al., 2000.

Figure 4 - Gradients of adenylate nucleotides within 'Rocha' pear fruit (values in nmol g^{-1} FM) (FM = fresh mass)



Fonte: Saquet and Almeida, 2015.

4 DISCUSSION

Assays using ATP bioluminescence are faster and more accurate than spectrophotometric or fluorometric procedures. While the spectrophotometric assay is limited to concentrations higher than 1.1 mol, the determination of adenylates by bioluminescence offers considerable advantages regarding the sensitivity in the range of nmols as shown in various results in the figures presented in this text. Furthermore, the technique allows a large number of samples to be assayed in a reasonable time, and the ATP is measured directly rather than after phosphorylation of a substrate participating in a NAD(P)NAD(P)H converting reaction. Thus, the risk of analytical interference by auxiliary reactions is lower in the bioluminescence assay. This risk of error is further reduced by the use of an internal ATP standard.

The firefly assay of ATP has found many applications in the study of cellular systems. The central role of ATP in intracellular metabolism makes ATP an interesting parameter in various physiological, biochemical and signaling studies. ATP has been used as an index of biomass in oceanography, limnology, clinical bacteriology, hygiene monitoring, food chemistry, and biochemistry, signaling in plants, and in connection with sewage treatment plants (Geigenberger et al., 2009).

In the field of plant biochemistry, ATP bioluminescence has been extensively applied. The roles of ATP, ADP, and AMP in cell metabolism are very important and many studies have been developed. Saquet et al. (2000, 2001, 2003a, 2003b), Saquet and Almeida (2015), and Almeida and Saquet (2016) used the technique to measure the energy charge related to the incidence of physiological disorders in apples and pears during controlled atmosphere storage as well as to study the physiology and biochemistry of pear fruit ripening. The role of ATP in the biosynthesis of fatty acids in fruits was also investigated as related to the integrity of the membranes and the production of aroma compounds in apples (Saquet et al., 2003a) and sweet cherries, related to the aroma production during storage (Harb et al., 2003).

In fruit metabolism, ATP plays an essential role because it is the unique source of energy for reactions of synthesis, transport and degradation processes. It is necessary for the maintenance of cell function, integrity and repair mechanisms of cell membranes (Tan, 1999). The involvement and importance of ATP in the synthesis and desaturation of fatty acids are well known (Mazliak, 1994; Ohlrogge and Browse, 1995). However, its possible involvement in the development of physiological disorders in fruits and the aroma production during CA-storage has not been enough studied. In all kinds of investigated fruits, a decrease in the concentrations of ATP during storage under low oxygen and/or combined with high CO_2 partial pressures was observed. Fruits stored under regular air showed the highest concentrations of ATP as well as the highest tissue energy charge.

The relationship between the energy charge of tissues and fatty acid contents with aroma production during the storage time was also observed. Especially in the studied apple cultivars, in which lipids are the precursors of esters, which are the impact aroma compounds, a negative effect of CA-storage on aroma production during CA-storage was found. The lower the O_2 and/or the higher the CO_2 partial pressures during CA-storage, the lower was the ATP and fatty acid contents, and

consequently lower production of aroma volatiles was measured (Saquet et al., 2003a).

The energy charge in pear fruits under CA-storage was more important in relation to the incidence of physiological disorders, because of its involvement in the metabolism of membrane fatty acids, cell integrity, and maintenance. Pear aroma production also depends on ester production, but pears also produce large amounts of alcohols during ripening minimizing the negative effect of CA-storage in reducing aroma production during long-term storage.

In sweet cherry, a non-climacteric fruit, ATP also seems to play an important role in aroma production during ripening and storage. The storage of cherry fruits under low oxygen and/or combined with high CO_2 partial pressures resulted in a strong decrease in the ATP levels of fruit tissues and aroma production.

5 CONCLUSIONS

The ATP bioluminescence technique is a very sensitive and reliable method for investigating plant metabolism.

The methodology described is detailed enough to allow those other researchers use this technique in their laboratories in many investigation fields.

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