Enzymatic time-temperature integrator device for chromatic quality check of Cova da Beira's cherry (Portugal)

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Abstract: *Cherry is a fruit widely appreciated by consumers in general. In Portugal, within the Beira Interior region, and particularly, Cova da Beira, is the most representative area with a production of more than 50% of the national production. The peculiar characteristics of cherries, such as color, firmness, palate, among others, increase the demand for this fruit, which can only be commercialized during a short period. Its high perishability interferes with the shelf life and consequently generates undesirable changes in the cherry flow chain. In order to ensure food quality and safety and prevent food waste, a time-temperature integrator (TTI) device to monitor the quality of the cherry in real time is proposed. This device suffers a chromatic change with the temperature variation over time. For the specific case of the cherry, the kinetic parameters for thermal inactivation are determined which leads to the proposal of an enzymatic-type TTI, where the degradation of the phenolic compounds occurs, which are substrates of the polyphenol oxidase enzyme, whose hydroxylation reaction of a monophenol in o-diphenol leads to the oxidation in o-Quinone. This device aims to help retailers to decide when and where to sell the food items taking into account the remaining shelf life, as well as support the decision of purchase by the consumer predicting through a chromatic and expedite stamp the food quality and safety*

Keywords: Cherry; TTI (Time-Temperature Integrators); enzymatic reaction; Temperature; Quality.

1. INTRODUCTION

Nowadays, the progressive population increase, as well as a situation of economic crisis and production deficit in several developed countries, heightens the need to reduce losses and wasting food in order to increase the world food intake. This is considered an ethical commitment to be assumed by us all, thus improving the nutritional level of the population and, consequently, the fight hunger [1]. In order to achieve this goal, it is needed to create an alliance between the countries policy and their populations, as well as motivate all the elements belonging to the food chain, towards a modification of attitudes, applied processes and effective management systems. In addition, the food safety and quality that the products marketed reach the end consumer is an increased concern [2]. As temperature is one of the factors that most influences the state of food preservation, there is a need to develop and test new appropriate sensors. Time Temperature Integrators (TTI), based on the introduction of two proteins and/or chemical systems, to quantify the impact of quality or safety of thermal processes are one of and more promising options [2]. These sensors are one of the main missing links to improve the introduction of new monitoring technologies, which reduce both production costs, predict food quality and safety, and may have a positive effect in mitigating food waste. This need for monitoring is increased when the food products are highly perishable, which require a stricter, qualified and rigorous control [3]. The purpose of this paper is to contribute to the implementation of TTI in commercialization of cherries, in order to promote food quality and safety, reducing the food waste, providing a way to predict the best time to sell the food products, or alternatively, the remaining shelf life, and thus fostering the commercialization gains by reducing losses.

2. GENERAL SPECIFICATIONS

The fruits and vegetables are integrated in the agricultural products but they stand out essentially for their perishability and the impact that the visual appearance has in the choice of the consumer at the moment of the purchase. The peculiar characteristics of horticultural products (morphological, anatomical, physiological and chemical) are responsible for the precocity of their degradation compared to other food products [4].
Besides being used for immediate consumption, fresh fruits and vegetables are an important raw material for many food industries, from which wine, olive oil, juices and frozen products can be named. In most situations, in a commercial establishment, the quality and freshness of horticultural products can stand out in a positive way [4].

2.1. Case study: Cova da Beira's Cherry

The cherry tree cultivation occupies about 14% of the area of fresh fruits in Portugal and reaches 2191 ha in the Beira Interior region, constituting more than 50% of the national production of the cherry tree [5].
According to the systematic classification, the cherry tree belongs to the family Rosaceae, subfamily Prunoideae, genus Pronus L., and species Prunus Avium L. Sweet cherries, which we refer to throughout this document, come from regions with climate appearances in the Mediterranean and Central Europe, North Africa, Near and Far East, South Australia and New Zealand, the United States of America and Canada, Argentina and Chile, having a harvest period between May and July [6]. See Figure 1 for an example of usual cultivar in the Beira Interior region, the cultivar Santina.

Figure 1. Example of Cherry cultivar Santina.

Cherries are very susceptible to mechanical damages, besides being highly perishable fruits presenting a very limited harvest period. This process requires a large amount of tests in order to enable its storage time and to preserve all its components. Unavoidably, the control and monitoring of this product is central and essential for optimal consumption in its highest state of quality, as well as for human health. Economically, it is also an advantageous process, which translates in the reduction of food waste [6], [7].
The characteristics most valued by the consumer are the dark red color, a sweetened flavor, a high diameter (about 28-30 mm), no cracks and a perfect rounded shape [7]. The quality of the fruit is often evaluated through firmness, since this directly affects the shelf life, consumer acceptance and the susceptibility, not only to mechanical damages, but also to infection by microorganisms [8], [9].
Unsurprisingly, the climatic conditions, cultivation, maturation time and subsequent harvesting and storage directly influence all these characteristics, that defines which of the cherries falls to the consumer's choice [10].

2.2. Compounds in Cherries and its relevance

Prunus avium contains numerous chemical elements such as calcium, iron, proteins, fibers, magnesium, minerals, potassium, phosphorus, and vitamins A, B and C. With regard to the benefits of cherries consumption, it provides to the medical field the ability to soothing, disinfectant, detoxifying, diuretic, regulating intestinal, antibacterial, and non-inflammatory, among others. These properties will only be recognized as beneficial if the cherry is in perfect conditions of consumption, otherwise it will not be able to exert the same effects [11]. Polyphenols directly influence color, sensory properties and nutritional properties. In order to improve marketing, cherries as well as other food products are previously processed in several ways. Before consumption, the functional and nutritional characteristics as well as their attributes and preservation capacity are improved [12].

In horticultural products, there is a vast diversity of phenolic compounds, the result of innumerable combinations of nature. Chemically, the phenolic compounds have a varied nomenclature due to the significant structural differences. They are classified according to the number of phenol rings as well as their binding. Of course, polyphenols have a common structure, which integrates an aromatic ring surrounded by one or more hydroxyl groups, passing through simple phenolic molecules to molecules with a high degree of polymerization [11], [13]. Phenolic compounds play an important role in the growth and reproduction of plants, which gives them not only metabolic elasticity (alteration of the environments to which they are exposed) but also pigmentation. The bioavailability of polyphenols and their concentration in tissues varies between them, depending essentially on the structure [11].

2.3. Methods of conservation and marketing parameters

Horticultural products are living tissues, which undergo constant transformations during the period of harvest. About these transformations, some are desirable for the consumer, but many of them are considered as problematic and damaging to their quality, culminating even in the loss [14].

The conservation process must be aimed at inhibiting microbial propagation, while preserving the quality of the product [15]. Generally, it is common to resort to low temperatures to retard this growth as well as at high temperatures to delay growth. During dehydration, pH decreases and several chemical additives with preservative functions are other maintenance techniques, which allow controlling the surrounding environment to which the product is submitted [16]. In addition to temperature, the ideal conservation environment can also be achieved by handling the concentration of oxygen and carbon dioxide and by manipulating the relative humidity (in order to reduce water losses) through the air circulation, since the temperature of the air goes up as it removes heat from the product [14]. In the cherry preservation process, which is susceptible to damage due to cold temperature below 0 °C, the optimum storage temperature is -1 to -0.5 °C. The temperature for transport should be between 0 and 3 ºC. The relative humidity should be between 90 to 95% and the concentrations of oxygen and carbon dioxide should be 0.5 to 2% and 20 to 25%, respectively [17]. The cherry, because of the yield of the harvest, has a relatively low impact with respect to the total production of fresh fruits in Portugal. However, their yield is not affected due to its market value, being a fruit that usually generates a good revenue to the producers [5].

The export process should take into account the fragility of the cherry, carefully adjusting the handling of this fruit. The success of this process is thus dependent on obtaining cherries with the highest possible quality, a good dimension, and adequacy of modern postharvest strategies and optimization of distribution and marketing circuits [18], [19].

3. STATE OF ART

3.1. Introduction

Quality models have always been developed in order to predict the quality of different perishable products, being subject to different environments both in terms of the temperature to which they are subject as well as the atmospheric composition. These factors are peculiarly important not only when the products are arranged in large bulk storage rooms but also because horticultural products generate heat throughout the storage process [20].

The main objective of the manufacturers is mainly to offer safe products with the maximum possible nutritional value. The two techniques most used in the evaluation of thermal processes are the *in-situ* approach and the physical-mathematical method. At in situ method, changes in the product are monitored before and after processing to provide a direct and accurate information about the food status. However, in practice, the microbial count, measurement of texture, vitamin content and organoleptic quality is laborious, time-consuming, expensive and, in some cases, impossible due to the value detection limit available techniques and/or sampling requirements [21].

3.2. Temperature integrating devices

In order to overcome the limitations and disadvantages inherent in these two methods, TTIs were developed as an alternative for the monitoring of these processes, especially perishable products [22]. A TTI state can be defined as "*a small measuring device which shows a measurable, temperature-dependent irreversible change, which easily and accurately illustrates the changes of a target attribute, being subject to temperature variation*" [23]. The attribute of destination may be any related to safety issues or for example inactivation of microorganisms (spores), loss of a particular vitamin, texture or color. The change during the thermal process must inevitably be irreversible in order to be able to quantify the impact of the process after processing. Exceptionally, after reading the monitoring system, there is the possibility of reversing the change to reuse the indicator [21]. The main advantage of TTIs is the ability to quantify the integrated time-temperature impact of the target attribute without any information about the real-time history and temperature of the product. The TTIs can be used as an alternative tool in the design of the process, in the evaluation of processes as well as in their optimization when the approach of conventional methods is not viable [24].

3.3. Enzymatic TTI

Temperature variation over time can be easily monitored. When a reference is made to enzymatic systems, there are certain properties that determine the kinetic parameters for thermal inactivation. The temperature sensitivity of the rate constant, E_a, and the rate constant, k_{TTI}, can be reported. Equation 1 gives the Arrhenius expression that illustrates the temperature dependence of the product quality [22],

$$\propto k_{A_{Ref}} \ \exp\left(\frac{-E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right) \quad (1)$$

Where k_{ref} is the kinetic constant of the reaction at a reference temperature T_{ref}, E_a is the activation energy (temperature sensitivity) of the reaction that controls the loss of quality and R is the universal gas constant. Most of the enzymes used in the development of TTIs reveal relatively natural thermal stability. Temperature variation over time can be easily monitored [25]. There are several types of TTIs that can be highlighted, such as: CheckPoint® that is an adhesive label, which reacts to time and temperature in a similar way to the food product. This process allows an evaluation of the qualitative parameters of the product under study, since it reacts to the existing enzymatic activity [26]. This type of TTI is based on a physiological, visible principle, that is to say, it is translated in a change of color, which is due to a decrease of the pH. This pH variation is a consequence of the controlled enzymatic hydrolysis of a given lipid substrate. The hydrolysis process will cause a release of an acid, which allows verifying by the progressive alteration of the color, starting from a deep green and culminating in an orange-red, as shown in Figure 2 [26].

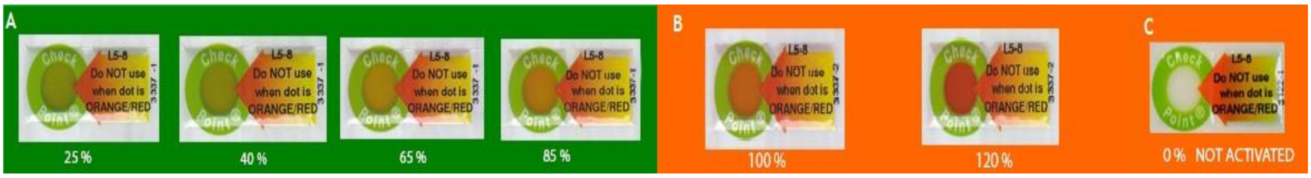


Figure 2. Illustration of the hydrolysis process of CheckPoint®. a) Primary TTI color state (green); b) Red-orange states, which indicates that the product is not fit to be consumed; c) TTI has a white coloration, which means that it has not been activated and cannot monitor the product's conservation status [26].

Another commercial TTI is Fresh-Check®, which is based on a chemical polymerization reaction, where the colorless acetylene monomer polymerizes and becomes opaque. The temperature-dependent reaction occurs in the solid state, with the reaction product having a colored polymer as the monomer polymerizes [21]. This formation of the polymer, which is accompanied by the change in color, is explained by the decrease in reflectance. Since the reflectance is the ratio between the flux of electromagnetic radiation incident on a surface and the reflected flux, there is a clear decrease of the latter [21]. The shape of the TTI is circular, whereby the color of the "active" center is evaluated and compared to the reference color of the surrounding ring, the color change extending from the transparent to a black threshold. At a stage prior to the use of this TTI, care must be taken to store them (at around -24 °C) still frozen, since they are active during manufacture in order to improve the yield of the chemical reaction [20]. The Fresh Check® indicator can be applied to a package in a self-adhesive format, such as a circle surrounded by a dark colored ring, as shown in Figure 3 [21].

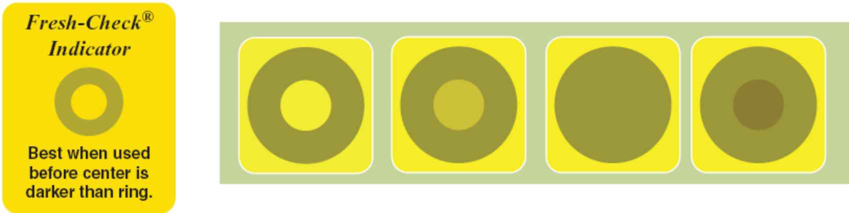


Figure 3. Illustration of a TTI based on a Fresh-Check® polymer [21].

Finally, OnVu™ can be considered an indicator of freshness, not replacing the expiration date, but providing a complement of information. The dynamics of operation is based on a solid-state photochromic reaction, which occurs due to the presence of organic crystals that alter the color according to the temperature variation registered, as shown in Figure 4 [21]. The organic crystals present in the OnVu™ form the basis for the pigment that is used in the formation of the water paint, which enables the color change, which in this case is perceptible with the change from colorless to dark blue after irradiation with UV [21].

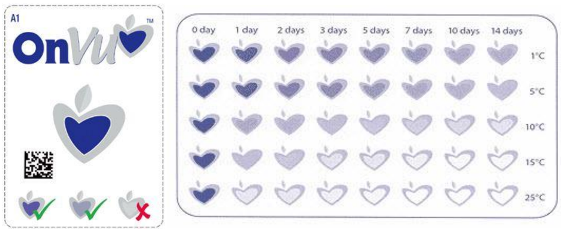


Figure 4. OnVu ™ solid-state photochromic TTI [27].

4. MICROBIAL KINETICS: DEDUCTION FOR THE SPECIFIC CASE OF CHERRY

During the post-harvest period, cracks and bruises are the most frequent damages in cherries, and are a direct consequence of the rupture and collapse of the mesocarp cells, caused by the impact and compression of the fruits during development, harvesting, transport and consequent handling [15], [28].
Pathogenic fungi arising in the post-harvest period of the cherry are one of the main causes of losses in the fresh market. In sweet cherries, the main pathogenic microorganisms responsible for the alteration of the cherry quality parameters are: *Penicillium expansum*, *Monilinia fructicula* (Spp), *Botrytis cinerea*, *Alternaria* spp and *Rhizopus stolonifer*, of which some of its effects on fruit [15].

4.1. Micro-organisms: Particular case of the genus *Monilinia*

The genus *Monilinia fructicola* was first observed at the beginning of October 2015, in peaches imported from Italy and Spain. Visually an early brown spot (rot) was observed, which rapidly evolved and spread throughout the fruit in the form of conidia, which reproduce asexually, emerging as spores, as shown in Figure 5. Afterwards, the studies carried out were able to isolate M11 and M13 genes, based on molecular morphological characteristics. Pathogenicity was tested by incubating mature peaches and conidia sterilized. Five days after incubation, typical rot symptoms developed in incubated fruits, unlike control fruits, which remained healthy [29]. Moniliosis can cause damage to the fruits, after harvesting and during the storage period. Although the fruits are susceptible to all phases, the vulnerability reaches its peak at the beginning of maturation. In the presence of *Monilinia fruticula*, the fruits are completely rotten, covered with spores, exhibiting the appearance "creamy white" characteristic and easily recognized in about five days [30]. In the particular case of the cherry, it can be infected from other cherries in direct contact or through spores transported by air, rain, or insects that penetrate the flowers. However, the main factors that contribute to cherry infections are the injuries caused by rain, hail, wind, cracks, and contact with insects and other fruits. Again, favorable conditions such as humidity and mild temperatures provide fungal proliferation [15], [30].

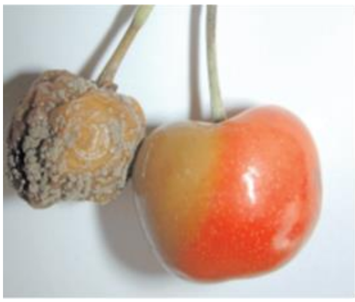


Figure 5. Effect the fungus genus *Monilinia* in cherries.

4.2. Database of quantified microbial responses in diverse food environments: ComBase

ComBase is an online database of quantified microbial responses in diverse food environments that allows predicting microbial behavior in food [31]. It intends to describe how the microorganisms survive and proliferate, depending on certain predefined conditions, which directly influence the food [32]. The most serious pathology is bacterial canker caused by *Pseudomonas syringae*. Through ComBase, the microbial kinetics of the logarithmic function that translates bacterial growth as a function of time can be predicts. The main objective is to verify when the infectious dose is reached, and then to predict when the degradation process begins. The values of initial and infectious dose for several microorganisms are shown in Table 1. As shown in Figure 6, the effective dose is reached substantially after 29 hours, which ignores that from this moment, monitoring with greater acuity should be performed.

Table 1. Dose limit values for pathogenic microorganisms.

Microorganism	T_{min} [ºC]	Initial colony [log10 cells/g]	Infecting colony [log10 cells/g]
Bacillus cereus	5.0	1000	>10^5
Escherichia coli	10.0	100	>10^6
Listeria monocytogenes	1.0	20	>10^2
Staphylococus aureus	7.5	20	>10^5
Salmonella	7.0	100	>10^5
Yersinia enterocolitica	-1.0	100	>10^7
Brochothrix thermosphacta	0.0	100	>10^7
Pseudomonas spp.	0.0	100	>10^7

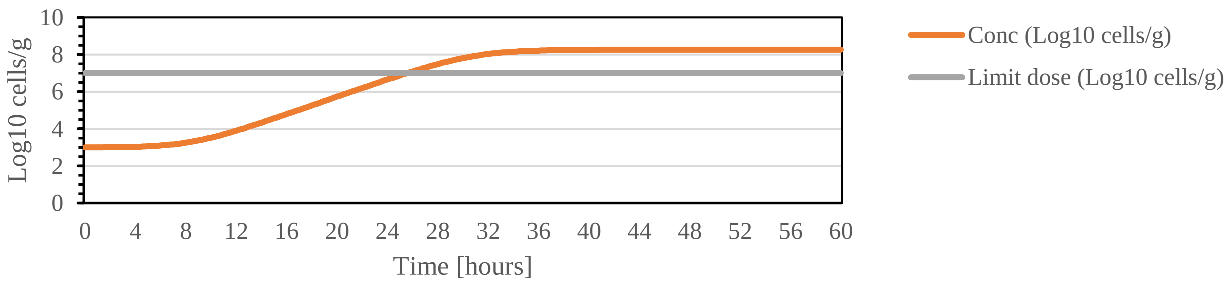


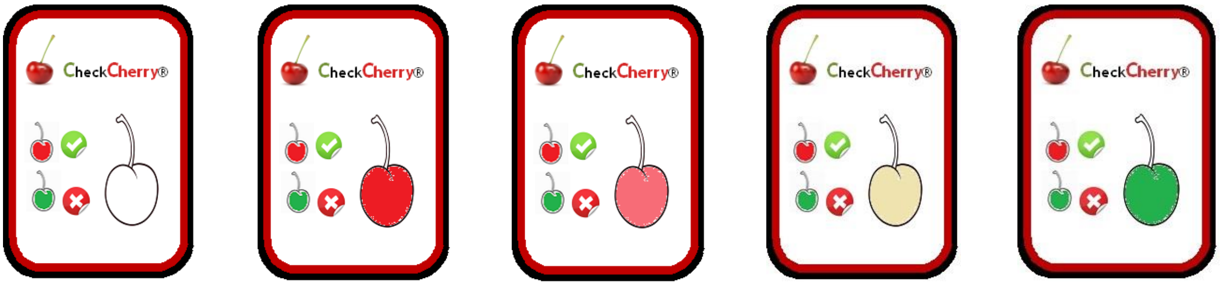
Figure 6. Graph illustrating the growth kinetics of the bacteria *Pseudomonas*, for a temperature of 20ºC, pH 7 (maximum value), initial dose of 1000 CFU/g (Colony forming unit) during a period of 60 hours.

5. DEVELOPMENT/APTNESS OF PARAMETER MONITORING TECHNOLOGIES

Refrigeration is the preservation method considered necessary not only for the cherry, but also for all minimally processed fruits and vegetables. However, as with all beneficial processes, it also has its drawbacks, namely temperature abuse during the time interval after packaging and subsequent distribution and transportation ending with storage. In order to monitor the quality and condition of the cherry, it is proposed to create a specific TTI.

5.1. TTI Prototype

The use of TTI in the case of cherry is particularly useful not only for manufacturers but also for transport operators. However, the final consumer has an extremely important role and therefore has the right to be able to check what product conditions are about to be consumed, namely tracking the location and extent of exposure to adequate temperatures. A proposal for a TTI for the cherry is thus presented, as shown in Figure 7, named, CheckCherry. As the TTI indicates, when stored under suitable temperature conditions, the TTI will keep the inside of the cherry red in color, its usual color.



(a) (b) (c) (d) (e)

Figure 7. Scheme that translated the overall cherry quality development: a) TTI present "not activated", therefore not valid for evaluation. b) indicates the ideal consumption status of the cherry; Maximum quality detected. c) provides a non-TTI visual indication that the cherry has entered the beginning of the degradation process; Dates can be sent with maximum attention; d) indicates an aggravated degradation process with mass degradation of the phenolic compounds. Finally, e) provides an indication of cherry consumption and is not recommended.

The enzymes present in the TTI will alter the color of the latter as a function of accumulation of the time-temperature history for the microorganisms described previously, taking into account the growth curves.
This TTI is based on a physiological principle, visible, that is, it translates into a color change, which is due to an oxidation reaction. As regards the detected enzymatic process, degradation of the phenolic compounds occurs, which are substrates of the polyphenol oxidase enzyme, whose hydroxylation reaction of a monophenol in o-diphenol culminates in oxidation to o-Quinone. The polyphenol oxidase enzyme catalyses the oxidation of polyphenols in quinones that react non-enzymatically, producing colored pigments.
The fact that polyphenoloxidase and its substrates are present in different cell compartments, enzymatic darkening, is a direct consequence of tissue disintegration. The integrity of the membrane is lost after damage to the tissues during senescence or injury resulting in the destruction of the biological barrier between the polyphenoloxidase and its substrates, leading to rapid oxidation of phenols and consequent production of dark pigments.

6. CONCLUSIONS

It was suggested that, according to the enzymatic reactions that occur, the enzymatic-type TTI would be the most appropriate to fit the specific case of Cova da Beira's cherry. Phenolic compounds are thus the key so that an enzymatic substrate can be detected, indicating to the producer, distributor and consumer the actual state of conservation of the cherry, leaving aside "random" predictions. Although there are many microorganisms that affect the cherry, they are not easily detectable by TTI, also because there are many other environmental conditions that directly influence them, and it is not possible to predict with certainty that the detection by the labile substance of the TTI would be totally reliable. In short, the food waste and the safety of the cherry may be predicted by the use of TTIs, ensuring control of all phases of the cherry chain. The objective of promoting product profitability and investigating ways and procedures to provide as much information as possible in real time is thus achieved, while awaiting the future implementation of this technology in Portugal. In the future, the implementation of TTIs either in Portugal and worldwide should be a priority, not only for the case of Cova da Beira's cherry as for all products. Priority should be given to TTIs for the most perishable products, such as meat, fish, all types of fruit and vegetable and shellfish, and later for other products where the shelf life is often merely indicative and bureaucratic as regards marketing.
For intelligent packaging systems, they are considered as an asset that helps predicting the degradation of perishable products in general. However, more studies are needed to combine atmospheric components that destroy the development of certain microorganisms, but also not increasing the proliferation of others.
Finally, the joint use of TTIs with the new packaging will be certainly the future of trade, although costs need to be revised.

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