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Isothermal calorimetry protocols to monitor the shelf life and aftermarket follow-up of fresh cut vegetables

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Abstract
Protocols and guidelines were assessed in order to apply isothermal calorimetry as a complementary/alternative method to monitoring, during the shelf life and the microbial growth/metabolism in commercial fresh cut vegetables with random initial microbial population. Moreover, the endogenous microbial population was used as a biosensor to check the modifications occurred during long storage for aftermarket characterization in the frame of vegetable waste treatments. Validation was obtained following ready-to-use carrots highlighting the effects of the different exposed surfaces (cylinders, sticks and à-la-julienne cut) on the overall spoiling process during shelf life and green salad stored up to 14 days with regard to the aftermarket characterization.

Keywords Isothermal calorimetry · Shelf life · Ready-to-use vegetables, microbes

Introduction

Ready-to-use fresh vegetables became so far quite appealing for consumers and food service companies thanks to the mild and quick processing treatments (trimming, peeling, washing and disinfection) required to provide commodities with sensory and nutritional properties close to those of the fresh products as defined by the International Fresh Cut Produce Association (IFPA). However, these treatments accelerate deterioration for various reasons (for instance, the cut treatment implies some cell disruption with the release of enzymes that sustain production of off-flavors and browning) [1–3]. Among them, a relevant factor of early spoiling is the enhancement of microbial contamination and proliferation due to the nutrients released by wounded vegetables and the increased exposed surface area of these products [4–6]. Most of these contaminants come from machineries, environment and hand manipulations, and despite washing and bland disinfection procedures, often, the commercial fresh cut vegetables result to be more contaminated than the original products [7].

The microbial species most found on in fresh cut vegetables and responsible of alterative process are Gram-negative aerobic psychrotrophic rods belonging to genus *Pseudomonas* and facultative anaerobic rods as *Erwinia carotovora.* These microbial forms are characterized by a strong pectinolytic activity that is the principal alterative phenomenon present on these products [8–10].

Microbial growth and metabolism during shelf life can be slowed by using improved packages (e.g., inert atmosphere, etc.) [11–13] which, however, do not avoid a still impressive food waste [14].

Fruit and vegetable waste (FVW), including pre- and aftermarket phases, poses environmental problems due to its high biodegradability and represents a loss of valuable biomass and an economic cost for companies. Different reduction, reuse and recycle strategies to tackle FVW have been proposed including the extraction of specific functional compounds that is one of the most studied field in the last years [15, 16]. In this frame, many efforts are devoted to the characterization and monitoring of these products with regard to both the shelf life prediction and the

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aftermarket status in order to define the waste processing [14–16].

Shelf life of fresh cut vegetables can be monitored and/or predicted either controlling the driving agents of damages (growth of microbial populations, enzymatic activities, concentration of reactive compounds, etc.) or monitoring their effects, like changes in pH, color, texture, nutritional value and the presence/absence of peculiar compounds. However, in these complex multi-parameter systems, there is no simple correlation between cause and effect and direct determination of the relevant parameters often is needed. Such a practice is expensive since it requires repeated chemical, biochemical and microbiological tests and implies collection of several food specimens and preliminary treatments of each sample, including the simplest ones, like pH determination [17, 18]. The characterization of the wastes becomes even harder [14–16, 19–21].

As regards, the microbial growth and metabolism monitoring, isothermal calorimetry (IC or ITC in the case of titration), represent a suitable complementary/alternative to the traditional approaches and have already been applied to the study of the metabolic activity of bacteria in opaque liquids, on surfaces and in solids in a wide range of conditions [21–36]. The instrumental output, i.e., the heat flow (HF), is a non-specific neat signal that reflects the overall energetic and kinetic picture of the bacteria activity in the real system under investigation, i.e., accounting for all the chemical and physical processes in the sample [37–39].

However, many issues are still to be addressed in order to apply this technique to the case of commercial fresh cut vegetables in order to discriminate the cut treatment effects in a complex system where a number of factors and processes are occurring simultaneously and partially overlap to one another. One of the main issues is that the samples of commercial fresh products may have different initial bacterial loads depending on many factors as mentioned before, precluding a straight comparison of the calorimetric data. Furthermore, the vegetable matrix may present differences even in the case of the same type of vegetable depending on the cultivation season, region, etc. [40].

In order to overcome these problems, definition of some main contour conditions and initial setup/normalization IC protocols are needed. The present work addresses this issue. For this purpose, carrots were selected as an example of fresh cut vegetable, since they allow preparation of ready-to-use products with different exposed surfaces (cylinders, sticks and à-la-julienne cut), and IC normalization protocols were assessed in order to monitor the evolution of the microbial activity during the shelf life. Furthermore, IC protocols suitable for vegetable waste characterization was also assessed following the case of ready-to-use green salad stored up to 14 days and using the endogenous microbes as biosensors. Indeed, green salad may well represent a model of the vegetable wastes since it represents the major amount in such a category [14].

Materials and method

Raw material and sample preparation

Carrots

Fresh carrots used in the present work were purchased from a local producer (Northern Italy), who applies a simple entrance/storage routine: The carrots are manually selected according to their size (medium-sized 19.5 ± 1.0 cm length, 3 ± 0.1 cm diameter), washed with hydrogen peroxide (1%), cooled to about 4 °C and finally delivered to the market (in our case to the laboratory) in large polyethylene plastic bags at 0 °C and approx. 100% relative humidity. The carrots were then peeled using a sterile manual peeler, the top and tip were removed and the remainder cut into cylinders (13 mm of diameter and 6 cm in length), sticks (2 mm of thickness, 12 mm of width and 6 cm in length) and "Julienne" slices (2 mm of thickness and 6 cm in length).

Carrot juice was also prepared, for the preliminary assessment, from hand-peeled carrots using a household juice machine. The pH was first decreased to 4 ± 0.1 using juice lemon in order to inactivate the native microbial flora (simulating the commercial practice) and then was adjusted to 7 ± 0.1 (that is close to the native condition of carrots) for the IC measurement.

Green salad

Hundred gram of fresh ready-to-use green salad "*songino*" (*Valerianella locusta Laterr*) packaged in PVC bags were obtained from the same producer as for carrots. The salad bags were transported to our laboratory immediately after packaging, under refrigerated conditions at 4 °C. The products were stored up to 14 days (the arrival time was considered as reference zero time) under controlled conditions, i.e., at 10 °C and relative humidity of 82%. The storage conditions were chosen to simulate an average stressed environment that a commercial product may be experiencing during distribution [13, 18].

Isothermal calorimetry

The instrument used for the IC investigations was the "Calorimètre E. Calvet pour Microcalormétrie, DAM" (Setaram, Lyon, France) equipped with 10 cm^3 stainless

steel cells. Calibrations were performed using the Joule effect calibrator EJ2 (Setaram, Lyon, France). Measurements were performed at 30 °C. Calorimetric cells were sterilized before every sample load. Typical sample mass was 5 g and 2 g for carrots and salad, respectively. Constant air environment was assured in the headspace of the cell through a capillary air circulation system (Microlab 500, Hamilton Company, USA). Some measurements at 10 °C were also performed using the Thermal Activity Monitor 2277 (TAM) instrument (Thermometric, Sweden) equipped with 20 mL cells. The heat flow versus time raw signal was integrated to obtain the overall thermal effect $\Delta H_{\text{overall}}$. Errors were evaluated on the basis of at least three replicates. The $\Delta H_{\text{overall}}$ error was below 6%.

Microbiological analysis

Microorganisms test and inoculum preparation for carrots juice

Pseudomonas fluorescens ATCC 13525 was used as microorganism test. A fresh overnight culture of microorganism test was inoculated [10^2–10^3 colony-forming unit (CFU) ml^{-1}] in 5 mL carrot juice incubated at 30 °C for 48 h. As control, Tryptic Soy Broth (TSB) [41] inoculated in the same conditions was used. At establishing time, microbial count was performed by plating on Tryptic Soy Agar (TSA) [41] and incubation for 2 days at 30 ± 2 °C.

Green salad total bacterial count

Ten grams of samples from each stored package were drawn and homogenized with 90 mL of sterile trypton salt solution at 0.85%, into a sterile Stomacher bag, by the use of a Colworth Stomacher 400 blender for 2 min. Decimal progressive dilutions were prepared, and the bacteriological determination of total bacterial count (TBC) was carried out according to the standard procedure (ISO 4833, 2003).

All microbiological analyses were carried out in triplicate, and the results were expressed as the mean of CFU/gram.

Results and discussion

Shelf life monitoring: preliminary assessment

In order to assess the microbial growth in the selected fresh cut vegetable system (carrots in our case) and discriminate the influence of the cut treatment through IC calorimetry, a preliminary assessment had to be performed since the commercial samples may have random initial microbial load N_0.

The main idea to overcome this problem is to take advantage of the detectability threshold of the calorimeter. Indeed, in a previous work [42] we demonstrate that, in the case of the same matrix simple systems, the initial exponential behavior of the microbial growth quickly overcomes the experimental setup peculiarities and the signal onset time mainly depends on the N_0 and, more importantly, the corresponding microbial population, namely the lowest cell population, N_{onset}, that produces a detectable calorimetric signal, is constant. This last statement is not so obvious in the case of complex matrices such as fresh cut vegetables, and an experimental validation in well-controlled conditions is needed. For this purpose, particular microorganisms that may represent the overall microbial spoilage must be selected. In our case, *Psedomonas fluorescens* was the microbial strain selected to represent the overall endogenous microbial load of carrots [43].

Figure 1 reports the raw exothermic calorimetric traces obtained from two liquid samples (5 mL) of cultures of *P. fluorescens* with same starting population at about 600 CFU mL^{-1}, in TSB medium and in carrot juice at pH 7, i.e., close to the native condition of carrots. The corresponding parallel plate counts are also reported. The profile of the IC signal reflects the kinetics and energetics of the overall growth/metabolism process (the end tail of the signal reflects the microbial metabolism not coupled with cell duplication), while the area beneath it represents the overall heat released, which in turn is related to the extent of the overall process occurred.

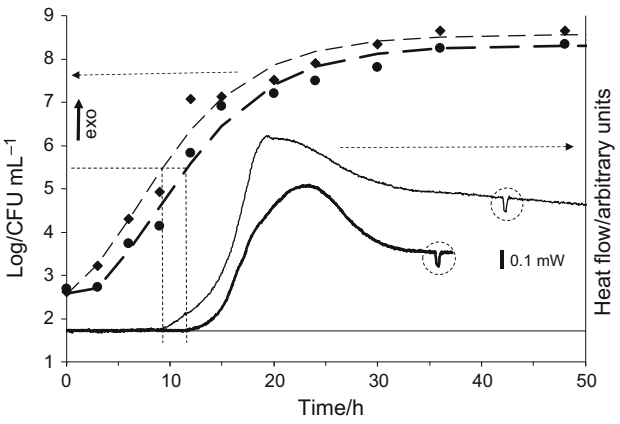

Fig. 1 Report (right side) on the raw exothermic IC traces obtained from two liquid (5 mL) samples of cultures *of P. fluorescens* with starting population at about 600 CFU mL^{-1}, in TSB medium (thin continuous line) and in carrot juice at pH 7 (bold continues line). Corresponding parallel microbiological plate counts are also reported (left side, thin and bold dashed lines for TSB and carrot juice, respectively). In the circles, the calibration signals (0.1 mW for 1200 s) are also reported

The choice of liquid samples with the same volume and N_0 was applied to normalize as much as possible the other factors that may influence the curve (sample geometry, etc. [42]) in order to highlight the substrate differences due to nutrient availability and type [3, 40]. We observe that both the onset time and the calorimetric profile are influenced by the nutritional composition of the substrate. Nonetheless, the microbial population at the onset time, namely the lowest cell population, N_{onset}, that, in a given medium, produces a detectable calorimetric signal and remains constant and apparently independent of the specific media (about 3.2 10^5 CFU mL^{-1} in our experimental conditions). This observation allows to overcome any small difference of the commercial products (composition of vegetable products varies according to season, cultivation region, etc. [40]) and also gives the opportunity to normalize the random initial microbiological load in the systems to be compared. Indeed, if we shift the thermograms' time axis in order to overlap the different onset times to a common virtual initial time $t_0 = 0$ we may compare the evolution of the microbial growth and metabolism at the same initial load $N_0 = N_{onset}$. Of course, the reliability of this conclusion with regard to the real systems depends on how much the microbiological strain/s selected to perform this preliminary test is representative of the overall microbial population (see below application range and limits).

Therefore, this t_0 normalization procedure allows the comparison of curves for commercial samples to take into account the other parameters that can affect the microbial growth in the fresh cut vegetables (exposed surface ratio, other cut effects, etc.).

Application: exposed surface and cut effect in commercial cutting treatments

Figure 2 shows the comparison of the t_0 normalized calorimetric traces obtained from fresh carrots which had undergone a different cutting treatment. We observe that major the accessible surface area major the microbial growth/metabolism rate in the systems. We may note that these curves represent a reliable starting point for further theoretical modeling [44, 45] to compare the cut effects since, according to our normalization, all samples have the same initial contamination N_0 and the same nutritional matrix (carrots). However, this exploitation is beyond the scope of this paper and we limit here to highlight the immediate evidences. We observe that in all cases the traces return to the baseline. This indicates that the microbial initial random contamination of the samples was predominantly constituted of aerobic forms (like *Pseudomonas fluorescens* that is an obligate aerobe, Gram-negative bacillus) in line with the literature [46]. The effect observed depends, accordingly, on the accessible surface area that influences the respiration rate and consequently determines the depth of contamination in the samples. The overall enthalpy was of 28.2, 20.9 and 9.8 J g^{-1} for the systems shaped as "Julienne," stick and cylinder, respectively, and relatively quantifies these effects that are of major importance in the packaging research sector (application of modified atmospheres, etc.) [13, 47].

Aftermarket characterization (green salads): preliminary assessment

Figure 3 shows the calorimetric traces obtained at 10 °C from fresh salad samples (hosted in the TAM calorimeter). Delays in growth and microbial metabolism were observed, as expected at this low temperature and eventually the

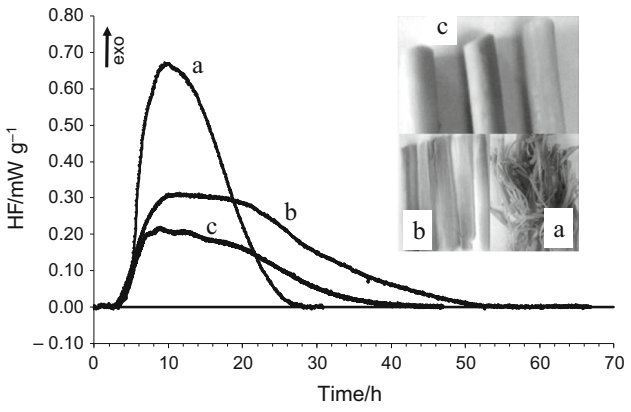

Fig. 2 Normalized IC records (exothermic) from carrots with different cut treatments (**a** Julienne, **b** sticks and **c** cylinders)

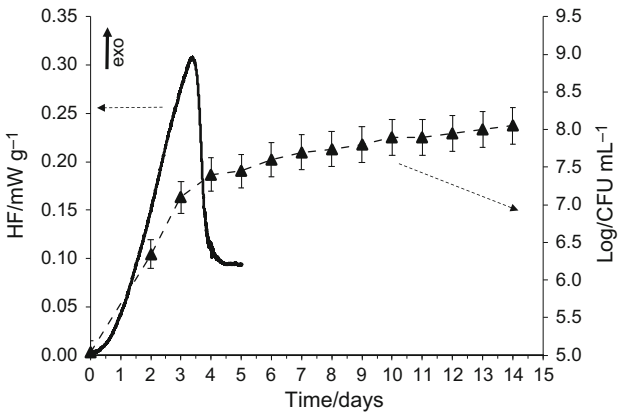

Fig. 3 IC curve (exothermic, bold line) obtained at 10 °C from fresh green salad sample. Corresponding parallel microbiological plate counts (TBC) are also reported (full triangles)

signal attains a plateau after about 4 days corresponding to when the microorganism enters the stationary phase (stationary phase is induced by increased bacterial cell density, depletion of nutrition in media and accumulation of toxic secondary metabolic wastes). Parallel microbial assessment was performed for the samples stored at 10 °C, and the total microbial count data (TBC) are also reported in the same figure to display the trend of the overall aftermarket microbial population in the system during the 14-day storage period. We observe a good matching with the calorimetric signal at the early stage of storage. After 4 days, the total microbial count data (TBC) remain almost steady in the $10^{7.5}$–$10^{8.0}$ CFU mL^{-1} range confirming the stationary phase status.

During such a long storage, the fresh cut salad undergoes some deterioration not necessarily correlated directly with microbial activity (chemical, textural, etc. [48, 49]) that eventually compromises the aftermarket recovery possibilities [14–16].

In order to evaluate the overall status of the product during such a long storage through IC calorimetry, the basic hypothesis is that the food spoilage, i.e., the vegetable cellular damages, produces a release in microbial nutria that become easily accessible [8, 50]. Considering the almost steady level attained by the endogenous microbial population, we may use this population as a biosensor to check the modifications occurred in the salad leaves during long storage. In other words, the hypothesis is that if we stimulate a further microbial activity in such systems, for example by rising the temperature, the rate of this activity will depend on the matrix nutrients availability that, in turn, may be correlated with the damage and waste status [50].

Application

To this aim, the salad samples that had been stored for different time lapses at 10 °C were calorimetrically investigated in isothermal conditions at 30 °C. These samples undergo the IC run with an initial microbial load in almost steady condition in the $10^{7.5}$–$10^{8.0}$ CFU mL^{-1} range, that is, well above the 10^{5} CFU mL^{-1} signal detection threshold. The temperature rise stimulates the overall microbial activity, which, however, would be re-activated in different matrices according to the extent of deterioration experienced during the previous storage. The time of this re-activation to produce a detectable IC signal was not taken into account because it is difficult to discriminate between environmental factors and microbe peculiarities (lag time, initial load small differences, etc.) [51].

Figure 4 shows the calorimetric traces at 30 °C relevant to salad samples that had been stored at 10 °C for 4, 7, 9, 11 and 14 days. We observe that the initial heat flow rate indicates an increasing trend which follows the age of the samples, i.e., it is in line with our hypothesis that the overall microbial activity may depend on the matrix nutrients availability which increases during the storage period of time.

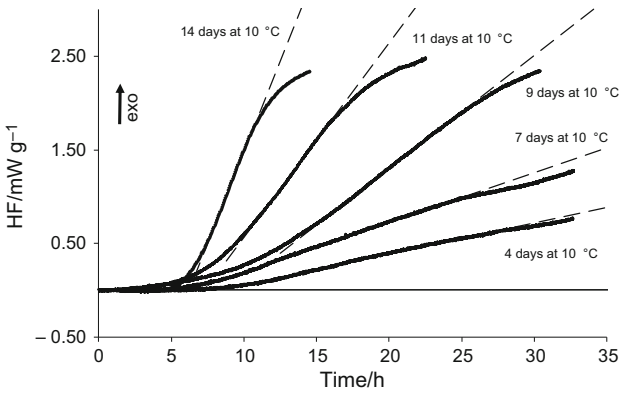

Fig. 4 IC traces at 30 °C from salad samples that had been stored at 10 °C for 4, 7, 9, 11 and 14 days

Indeed, the trend of the IC trace slope versus storage time, presented in Fig. 5, follows an exponential low that is in line with the increase in substrate availability in stochastic microbial activity [52].

In summary, this function, obtained using the IC method, may be used as a phenomenological relative index of the fresh cut vegetable's overall spoilage. However, correlation with the other complementary indexes is needed, depending on the peculiar case, in order to be integrated in a control protocol [53–56].

Overall application range and limits

(a) The onset time normalization implies that the initial contamination is under the detectability of the

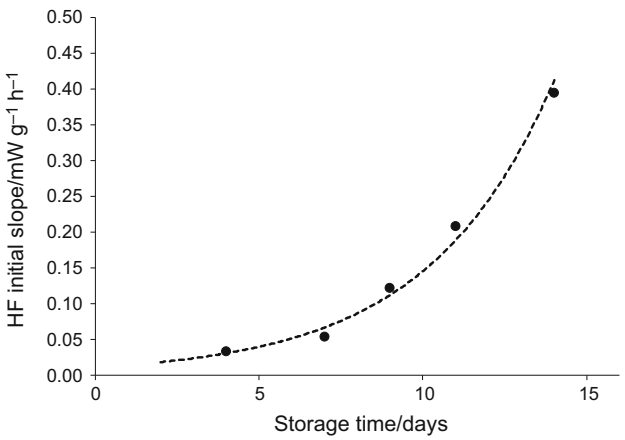

Fig. 5 HF initial slope obtained from the curves presented in Fig. 4 versus storage time

instrument. However, the procedure of time normalization may be applied also taking into account another reference point of the HF signal (higher than zero) if the corresponded $N_{reference}$ is proved constant at the initial assessment.

(b) In our case, we used, for demonstration purposes, a selected microorganism to represent the overall vegetable microbial population. Fresh vegetables are mainly contaminated by the same or few general classes of microorganisms with regard to the calorimetric point of view, i.e., the energetic behavior of these microbes [9, 57]. However, depending on the system and the research purposes, other choices and/or the overall load method may be applied without changing the concept of the initial assessment, i.e., to prove that the corresponded N_{onset} is constant.

(c) The effects of cut treatment (carrots) were monitored through IC measurements performed at 30 °C acceleration regime in order to avoid deterioration side effects by a long stay of the samples in the calorimetry. We may note, however, that temperature is a relevant factor in the microbial growth and metabolism kinetics and a more accurate inspection is necessary if we intend recognize the combined effect of temperature and time of conservation on the microbial activity [5, 13, 58].

(d) The long time storage of the green salad product was performed with the samples in the original sealed bags, under standard microbiology laboratory-controlled conditions, and the total bacterial count (TBC) was regularly monitored to confirm the microbial stationary status. These conditions seem unlike the contamination and proliferation of external microbial contaminants at a relevant level to strongly influence the IC curves at the early stage.

However, for application in real systems, this issue has to be taken into account applying more severe microbiological control in the method setup phase to assess experimentally how external microbial contamination, during storage, may influence the IC trace.

Conclusions

Protocols and guidelines were assessed in order to apply isothermal calorimetry as a complementary/alternative method to monitor, during the shelf life, the microbial growth/metabolism in commercial fresh cut vegetables with random initial microbial population. Moreover, the endogenous microbial population was used as a biosensor to check the modifications occurred during long storage for aftermarket characterization in the frame of waste treatments. Validation was obtained following ready-to-use carrots highlighting the effects of the different exposed surfaces (cylinders, sticks and à-la-julienne cut) on the overall spoiling process during shelf life and green salad stored up to 14 days for the aftermarket characterization.

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