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Supercritical CO_2 for the drying and microbial inactivation of apple's slices

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ABSTRACT

Supercritical CO_2 (Sc-CO_2) drying has been recognized as a promising low temperature drying technique for food products. In this regard, this work focuses on the feasibility of Sc-CO_2 drying of apple's slices: both the microbiological stability and mechanical behavior of the test product after the process have been investigated in dependence from different process parameters, namely drying time, pressurization time, and depressurization time. The microbiological stability was determined for both inoculated pathogenic bacteria (*Escherichia coli* O157:H7, Salmonella, and *Listeria monocytogenes*) and naturally present microorganisms (yeasts and molds, mesophilic bacteria and spores and Enterobacteriaceae). Results demonstrated a complete inactivation of pathogenic bacteria under the detection limit (<1 CFU/g) just after the pressurization (10 min) and depressurization (20 min) phases. After the same steps, a strong reduction of vegetative bacteria and yeasts and molds was also observed in comparison with air drying and freeze drying samples. As regards the mechanical behavior, the Young Modulus, measured before and after the CO_2 processes to provide a measurement of samples' stiffness, resulted dependent from the final water activity, but independent from the length of pressurization and depressurization phases at longer drying time. Overall, these results are promising to foster the development of the technology at industrial level.

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1. Introduction

Food safety is a worldwide challenge and current trends in food-related outbreak[1] indicate that available technologies and consumer's practices need to be improved. The consumption of dried products is increasing globally,[2] and even if growth of spoilage and pathogenic microorganisms is inhibited at dried state, the risk for the consumer remains high.[3] The risk is higher when the food is consumed without effective heat treatment. *Salmonella*, a common Gram-negative pathogen, is known to be one of the most resistant bacteria in the desiccated state.[4] Recent outbreaks involving dried food[5] pointed out the need for effective solutions. Currently available drying technologies have a limited inactivation power against microorganisms.[6,7] In the case of spices and herbs, known to be case sensitive products, additional decontamination steps, like irradiation, are often performed to increase the safety of the product. Recently, the use of supercritical CO_2 (Sc-CO_2) drying alone or in combination with high power ultrasounds has shown to be able to dry and inactivate microorganisms in coriander leaves[8–10] and chicken breast[11] simultaneously. However, the current state-of-the-art demonstrating the microbial inactivation for Sc-CO_2 drying is very limited and additional studies on microbiological inactivation considering different food matrices are needed to assess the feasibility of the process to a wider food range.

CO_2 is a non-polar, nontoxic molecule that has been defined as *Generally Recognized As Safe* (GRAS) by FDA. It is used in many beverages and as modified atmosphere for packaging. When at supercritical state (above 73.8 bar and 31.1 °C) it exploits physical properties in between a gas and a liquid and it is extensively used as extracting solvent.[12] During supercritical drying, the Sc-CO_2 is pumped through to

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the vessel containing the food product and it gradually extracts the water. The vapor–liquid interfaces are avoided thus helping the preservation of the original microstructure. While there are several studies on the mechanical characterization of hydrogel produced by Sc-CO_2,[13] little is known about mechanical properties of Sc-CO_2 dried products. Djekic et al.[14] were the first to show texture profile analysis on Sc-CO_2 apple slices, however the study did not take into account different process conditions like depressurization time. Depressurization has been shown to be responsible to the dimension of mesopore in aerogel,[15] therefore it might also play an important role for the mechanical properties of the dried food. Indeed, traditional drying technologies have been already shown to influence the microstructure after drying.[16,17]

In this contest, the main goal of this work is to determine the feasibility of the supercritical CO_2 drying of apple slices for the microbial inactivation and the mechanical properties, evaluating the effect of process parameters like pressurization, depressurization, and drying time. Pathogenic (Escherichia coli O157:H7, *Salmonella, and Listeria monocytogenes*) and naturally present microorganisms were taken into account for this study. For the microbiological stability of naturally present microorganism, air drying and freeze drying techniques were used as control.

2. Materials and methods

2.1. Inactivation of pathogens

2.1.1. Bacterial strain

Three strains of *E. coli* O157:H7 (ATCC 700728, BRMSID 188, LFMFP 846), *L. monocytogenes* (LMG 23192, LMG 23194, LMG 26484), and *Salmonella enterica* (serovars Thompson RM1987 and Typhimurium SL1344, LFMFP 883) were used as target pathogenic bacteria. Details of used bacterial strains with their respective selective medium, antibiotic resistance and origin are reported in our previous work.[8]

2.1.2. Inoculation procedure

Every test was made using an inoculation mixture composed of the three strains of a single microorganism per time. Different microorganisms were not mixed together during the same experiment. The strains were obtained in form of stock cultures provided by Ghent University;[8] they were revived by placing a loopful of the slant culture in 10 ml of fresh Brain Heart Infusion broth (BHI, Fluka analytical) for 6 h at 37 °C. After this first incubation step, 100 µL of the obtained solutions were further subcultured in 10 ml of BHI broth for 18 h at 37 °C obtaining working cultures. From each strain, a 500 µL volume of the working culture was taken into 2 ml sterile Eppendorf tube, mixed for 15 s using a vortex (Velp scientifica, Usmate, Italy) and centrifuged for 10 min at 2900 rpm. The supernatant was removed and substituted with 500 µL of phosphate buffered solution (PBS; Sigma Aldrich, Italy). The cells and the solution were again vortexed for 15 s. The mixture for each inoculum was prepared by mixing together the three strains per inoculum in the ratio 1:1:1 and adding 500 ml of PBS in order to obtain a final volume of 2 ml. The correct mixing ratio has been obtained by an empiric correlation between plate count and the optical density of the solution (data not shown). Each inoculation solution has been tested in term of microbial load by plate count. The target cell concentration was of 8.0 log CFU/ml.

Aseptic inoculation was performed under a biosafety cabinet. Fresh apple was cut in slices (5 mm thick) and then cut in smaller pieces of about 0.5 g each. Each sample consisted in 1 g ± 0.1 g that was inoculated by adding drops of the inoculum solution on the external pulp. The samples were previous placed inside a sterile Petri dish and 16 ± 4 µL of the inoculation broth were used per gram of fresh product to obtain an initial load of 6.0 ± 0.5 log CFU/g. After inoculation, the samples were left 30 min to dry in the bio-safety cabinet at 22 °C, allowing the attachment of the inoculated microorganism on the surface.

2.1.3. Inactivation of pathogens within supercritical CO_2 drying

For the investigation of the supercritical CO_2 drying capacity to inactivate pathogens, a semi-continuous lab scale reactor (Separex S.A.S., Champigneulles, France) with an internal volume of 50 mL was used. More information on the plant are described in our previous works.[9,18] Before each treatment, the vessel was cleaned by filling a mix of ethanol and water (7:3) for 10 min, rinsed with sterile distilled water and then flushed with CO2. The samples were inserted inside a metal basket that was previously cleaned with ethanol and burned with a Bunsen flame. The reactor was preheated at 40 °C before starting the experiment. The pressurization step starts when the CO_2 tank is opened and the pressure in the system increases up to 60 bar (which corresponds to the pressure value of CO_2 inside the tank). At 60 bar, the pump is turned on to reach the operative set-up pressure of 100 bar.

Table 1. Summary of the three drying techniques and conditions used for the microbial stability.

Drying technique	Pressure	Temperature	Time
Sc-CO_2	125 bar	50 °C	15 h
Air drying	–	60 °C	8 h
Freeze drying	0.2 mbar; desorption	40 °C	24 h
	0.05 mbar; sublimation	4 °C	

Pressurization from 60 to 100 bar was achieved in 10 min (Pressurization rate of 4 bar/min). Once 100 bar was reached, the process was stopped and the depressurization was achieved in 20 min with a constant rate of 5 bar/min. Pressure, temperature, pressurization, and depressurization rate were the same used for coriander in our previous work with pathogens.[8] At the end of depressurization, the sample was transferred into a sterile stomacher bag for further microbial enumeration. Each experiment included one Sc-CO_2 treated inoculated sample, one inoculated control sample, and one non-inoculated control sample and was performed in triplicate.

2.1.4. Enumeration of the inoculated pathogens

Microbial load before and after the treatment was analyzed by means of the standard plate count techniques. In stomacher bags, treated samples were diluted in steril MIlliQ water with a ratio of 1:10. After stomaching for 1 min, 10-fold dilutions were prepared and plated on the selective media. The appropriate dilutions were spread-plated on Cefixime-Tellurite Sorbitol MacConkey Agar (CT-SMAC, Sacco, Italy) containing nalidixic acid (50 µg/mL, Sigma-Aldrich, Germany) and CT-SMAC containing kanamycin (100 µg/mL, Sigma-Aldrich) for *E. coli* O157:H7. *Salmonella* was enumerated on Xylose Lysine Deoxycholate agar (XLD, Biolife, Italy) containing nalidixic acid (50 µg/mL), XLD containing kanamycin (100 µg/mL), and XLD containing streptomycin (100 µg/mL, Sigma-Aldrich). *Listeria monocytogenes* was enumerated on Listeria Agar (Liofilchem, Italy). The use of antibiotics for *Salmonella* and *E. coli* O157:H7 permitted the enumeration of the specific strain based on their antibiotic resistance.[8] The incubation was performed at 37 °C for 24 h for *E. coli* O157:H7 and *Salmonella*, and at 37 °C for 48 h for *L. monocytogenes*. The enumeration was referred to the weight of initial fresh product and expressed in $\log_{10}$ CFU/g. The limit of detection (LOD) for the spread plate was 100 CFU/g, respectively. In the case that the results of quantitative microbial analysis were below of LOD, the plating was also done from the first dilution of the sample that was incubated for 24 h at 37 °C providing the enriched sample decreasing the detection limit up to 1 CFU/g.

2.2. Influence of drying on the microbial stability

The influence of drying on the microbial stability of apple's slice was performed testing different drying techniques (Table 1). Process conditions were chosen based on recent published works.[14,19] Apples (Elstar cultivar) were harvested during the 2016 harvest season in a commercial orchard in the Netherlands and stored for around 1 month in normal atmosphere at 1 ± 0.5 °C and 90–95% relative humidity before processing. Apples of approximately uniform size and without obvious sunburn were cut into semi-circular slices ca. 50–55 mm in length and 2.2–2.5 mm thick, without removing the skin. Table 1 summarize the process conditions of the three drying methods. Air-drying in a stagnant belt dryer at 60 °C during 8 h; freeze-drying under pressure of 0.2 mbar during sublimation and 0.05 mbar during desorption, at the temperature of −5 °C during sublimation which was gradually increased up to 40 °C during desorption (total drying time 24 h); and supercritical drying using CO_2 (Sc-CO_2-drying) under pressure of 125 bar at 50 °C during 16 h. Water activity of the apples after drying was 0.19 ± 0.01, 0.18 ± 0.01, and 0.14 ± 0.01 for Sc-CO_2-dried, air-dried, and freeze-dried samples, respectively. Dried apples were packed under 100% nitrogen with in multi-layer polyethylene supplemented with aluminum (Alu-PE) package. Each package contained approximately 25 g of dried apples. Sealed packages were stored at room temperature, in a dark environment up to 12 months. Water activity was measured with a TH-500 AW SPRINT (Novasina, Switzerland).

The microbiological quality of the samples was assessed in terms of total plate count (TPC), mesophilic aerobic spores, yeasts and molds, and Enterobacteriacea. Ten grams of dried apples were re-hydrated in 20 ml buffered peptone water (BPW, Oxoid, UK) and left for 15 min before stomaching for 2 min. For the mesophilic spores, this first suspension was treated at 80 °C for 12 min. Then, 10-fold dilutions were prepared and plated on appropriate media: Plate Count Agar (Oxoid) for TPC and mesophilic spores, Yeast Glucose Chloramphenicol (Bio-Rad, Belgium) for yeasts and molds, and Rapid'Enterobacteriacea (Bio-Rad) for Enterobacteriacea. Plates were incubated for 3 d at 30 °C, for 4 d at 22 °C, and for 2 d at 37 °C, for TPC and mesophilic spores, yeasts and molds, and Enterobacteriaceae, respectively. Experiments were performed on two different samples.

2.3. Mechanical characterization of CO_2 dried apple

Testing machine (MIDI 10 by Messphysik Materials Testing) was used to perform compression tests for the mechanical characterization of the apple samples. Tests were conducted under quasi-static conditions at a rate of 0.01 mm/s. Around 1-cm disk samples were prepared using a scalpel from the center of the fruits slice. Young's modulus values were calculated from the slope of the stress–strain curve at about 20% strain for all the samples. Supercritical drying was performed on slices (2–3 mm thickness) at 100 bar and 40 °C from 4 to 18 h. Two different pressurization and depressurization times were used (10 or 40 min). After the supercritical drying, samples were sealed in Alu-PE bag using nitrogen as modified atmosphere. Bags were sent to the University of Trento (Italy) to be analyzed within 1 month from delivery.

3. Results and discussion

3.1. Influence of supercritical CO_2 drying on the microbial inactivation

Supercritical CO_2 has been extensively studied as innovative low temperature pasteurization for liquid and solid products showing to be effective against spoilage and pathogenic microorganisms.[20,21] The inactivation mechanism is product dependent and it occurs thought several steps those start with the solubilization of the CO_2 in the water and the permeation trough the cellular membrane.[21] Sc-CO_2 has been extensively studied in batch system with the goal to maintain the original features and structure of the unprocessed products. During Sc-CO_2 drying, CO_2 acts as a solvent that gradually extract the water; as results, the product changes its water content during the process becoming lighter and dry.[22] Simultaneously the product undergoes microbiological inactivation as it happens for the traditional Sc-CO_2 pasteurization. Since the Sc-CO_2 drying operates at high pressures, it is composed of three main steps: pressurization up to the desired process pressure, holding and depressurization to ambient pressure. The inactivation of pathogenic bacteria was performed based on preliminary experiments with coriander.[8,9] Trials started evaluating the inactivation after just the pressurization and depressurization of the drying vessel. Table 2 shows the initial load of pathogenic microorganisms in apple's slices. After 10 min of pressurization up to 100 bar at 40 °C followed by 20 min of depressurization, no viable colonies were detected for all the types of microorganisms. Enriched samples decreased the detection limits to 1 CFU/g supporting the complete inactivation after Sc-CO_2 drying. These data confirmed the inactivation capacity of supercritical CO_2 drying already observed for coriander, making the Sc-CO_2 drying a robust technology able to dry and pasteurize the food in a single step. As observed with coriander,[7] the inactivation capacity was independent from the type of strains. It is worth noticing that the inactivation of *E.coli* O157:H7 resulted similar to the one achieved with coriander, but the inactivation of *L. monocytogenes* and *Salmonella* was higher in case of apple. Specifically, the inactivation on coriander samples was below the enumeration limits (150 CFU/g of fresh product) for *Salmonella,* while *L. monocytogenes* was inactivated up to 5 log CFU/g. This evidence was also observed in previous published work, in which different food matrices showed different microbial inactivation after similar process conditions.[20] Since a complete inactivation was already achieved after the pressurization and depressurization steps, experiments at a longer drying time were not performed.

Table 2. Initial and final counts of Escherichia coli O157:H7, *Salmonella,* and *L. monocytogenes* inoculated on apple's slices and inactivated with Sc-CO_2 at 100 bar, 40 °C, 10 min pressurization, and 20 min depressurization.

Microorganism	Initial count	Final count
Escherichia coli O157:H7		
BRMSID 188	5.56 ± 0.17	<DL
NCTC12900 & LFMFP 846	4.91 ± 0.25	<DL
Salmonella		
Salmonella Thompson RM1987	5.51 ± 0.20	<DL
Salmonella typhimurium SL 1344	5.63 ± 0.22	<DL
Salmonella typhimurium LFMFP 883	5.42 ± 0.19	<DL
Listeria monocytogenes		
LMG 23192. LMG 23194 & LMG 26484	7.41 ± 0.28	<DL

Note: DL refers to the detection limit of the technique (1 CFU/g after sample enrichment).

Once confirmed the capacity to inactivate pathogenic microorganisms inoculated on the pulp of the fruit, we focused on the microbiological stability over time. For this study a comparison with traditional air-drying and freeze-drying was used as control to confirm previous results.[8,9] Microbiological count on dried apples was assessed after drying (time 0) and during shelf life after storage (3, 6, and 12 months). Figure 1 shows the final count achieved at different time points. Samples dried with Sc-CO_2 resulted in lower counts for mesophilic aerobic bacteria (Figure 1(A)), yeasts and molds (Figure 1(C)), and Enterobacteriaceae (Figure 1(D)). Spores were found in samples dried with all three techniques (Figure 1(B)) confirming that they are

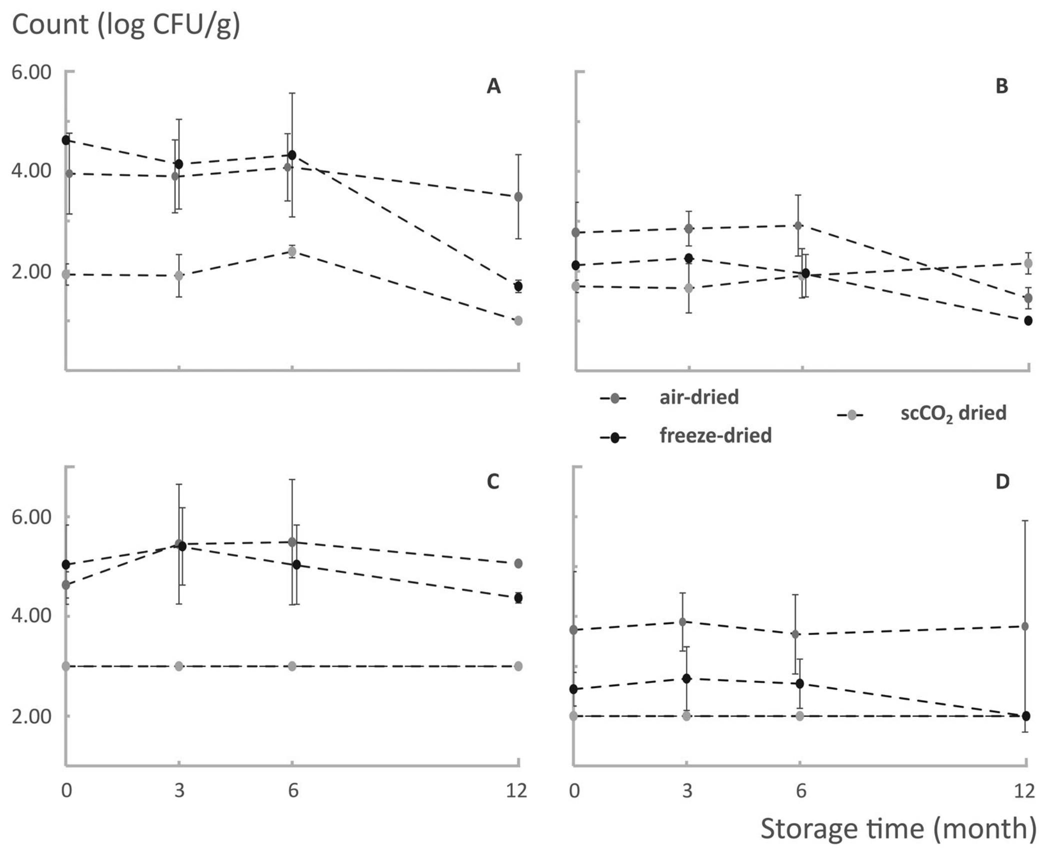

Figure 1. Microbial counts over time for total plate count (A), mesophilic aerobic spores (B), yeasts and molds (C), and enterobacteriaceae (D). Detection limit was 100 CFU/g for the yeasts and molds and 10 CFU/g for the others.

Table 3. Water activity measured during the storage (t_0 refers to the measurement at the beginning of shelf life).

Drying technique	t_0	3 months	6 months	12 months
Sc-CO_2	0.19 ± 0.01	0.23 ± 0.01	0.28 ± 0.04	0.27 ± 0.03
Air drying	0.18 ± 0.01	0.22 ± 0.01	0.28 ± 0.01	0.26 ± 0.02
Freeze drying	0.14 ± 0.01	0.19 ± 0.02	0.22 ± 0.02	0.21 ± 0.02

less sensitive than vegetative cells to Sc-CO_2 drying, as previous observed with coriander.[8,9] This evidence confirmed previously established results in which it was found that Sc-CO_2 drying induced a higher reduction of vegetative cells when compared to freeze-drying[8] and oven drying.[9] With apple slices it was confirmed that yeasts and molds were particularly sensitive to Sc-CO_2 as they were inactivated below the detection limit. The microbiological quality of the samples was found to be very stable and no increase occurred after 12 months of storage. This suggests actual inactivation and no sub-lethal injury, which was already expected based on data from enrichment cultures. However, even if the cells were injured they would not resuscitate at stable low water activity ($a_w < 0.3$, Table 3), which inhibits resuscitation and growth. It is worth to point it out that also sensorial and chemical stability over time are possible[19] making the Sc-CO_2 drying promising for the production of safe and good quality products. However proper design of experiments should be carried out to determine the best set of process variable able to induce high product quality as well as the investigation of combined treatment.[23,24] Nevertheless, economic analysis should be accomplished to demonstrate the sustainability as novel energy save technique.[25]

3.2. Influence of supercritical CO_2 drying on the mechanical properties

Given the specific size and morphology of the apple samples, we performed compression tests in order to derive information about their mechanical behavior after supercritical drying. Indeed, tensile tests were preliminary performed on other fruit matrices (data not shown), however the inhomogeneous nature of the dried samples made difficult to prepare samples with homogeneous shape for standard uniaxial testing machine. Compression tests were preferred because it was easier and more consistent to prepare samples with comparable shape to be used for the analysis. Furthermore, similar tests on apple and other vegetable samples can be found in the literature,[26–28] indicating compression tests as a standard method for the mechanical characterization of fruit and vegetable samples.

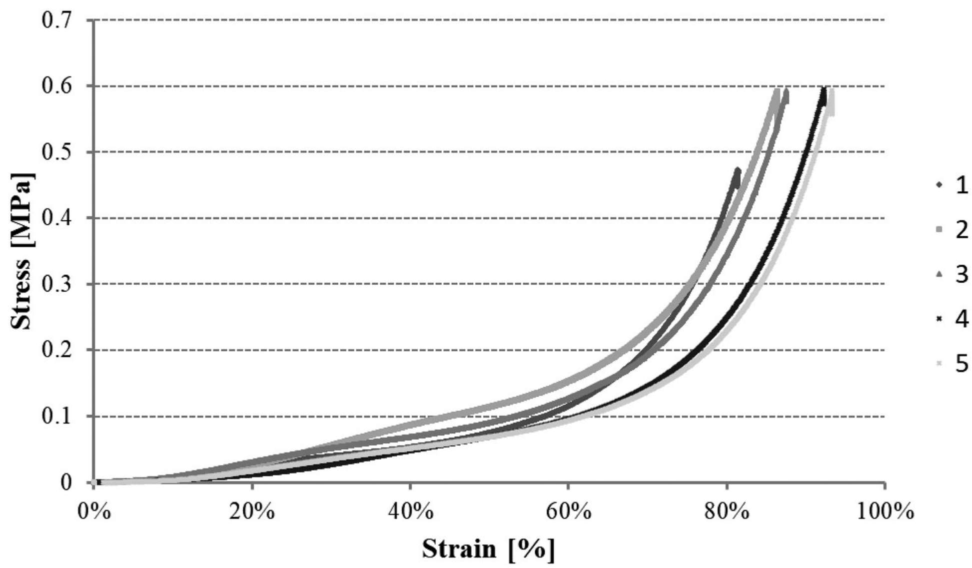

Figure 2. Stress–strain curve for samples after 4 h of drying and depressurization of 10 min. Legend's numbers refers to different samples. Similar profile for the others conditions (data not shown).

Table 4. Water activity and Young modulus (E) extracted from compression test for dried samples at different drying times (t_{drying}), pressurization times (t_{pres}), and depressurization times (t_{dpres}).

t_{drying} [h]	t_{pres} [min]	t_{depres} [min]	a_w [−]	E [MPa]
4	10	10	0.37 ± 0.02	0.194 ± 0.030
4	10	40	0.39 ± 0.02	0.098 ± 0.014
7	10	10	0.32 ± 0.03	0.237 ± 0.098
7	10	40	0.31 ± 0.02	0.177 ± 0.166
18	10	10	0.25 ± 0.02	0.439 ± 0.030
18	10	40	0.24 ± 0.02	0.452 ± 0.053
7	40	10	0.31 ± 0.01	0.246 ± 0.166
7	40	40	0.30 ± 0.01	0.194 ± 0.098

From compression tests we could derive stress–strain curves, from which the Young modulus was extracted as the slope of the initial region, which provides a measurement of samples' stiffness. Figure 2 shows the typical stress–strain curve obtained from Sc-CO_2 dried apple samples after the compression test. Because apple is naturally soft, samples tend to get extremely compacted under a compression load, with no evidence of fracture even at relatively high loads (40 N). Stress–strain curves show an initial linear part (up to 20–30% strain) and then a strongly non-linear region with higher slope. In the last part, the substrate supporting the sample starts playing an important role for the slope of the curve. Thus, only the initial part of the curve allows to extract meaningful data about sample behavior. Young modulus values were extracted from stress–strain curves of samples produced at different process conditions. In this study, we only focused on the drying time and pressurization/depressurization rate. Pressurization and depressurization profiles were chosen in accordance with the processing times that can be achieved on lab, pilot and potentially industrial scale. Table 4 reports the operative conditions (drying time, pressurization time and depressurization time), the water activity after drying and the average Young Modulus extracted from the compression test. The order of magnitude of Young modulus of Sc-CO_2 drying is consistent with literature.[29] Drying time influenced both final water activity and also the Young Modulus. At lower water activity (longer drying time) the Young Modulus was higher, suggesting that the samples were stiffer. This is consistent with the fact that water content in the dried product influences the crunchiness and crispiness of the dried fruit.[30] The depressurization time played an important role only at short drying time, while it is negligible starting from 7 h of drying. For this reason, the effect of pressurization time was tested only for 7 h drying. Similarly, with the depressurization, a longer pressurization did not change the final value of Young Modulus of the samples. These results are very important from an industrial point of view, especially in the perspective of further upscaling of the technology. When performing small scale testing, a quick pressurization and depressurization is technically feasible, but not possible at industrial scale. At larger scale, pressurization and depressurization can easily take 30–60 min since the vessels have a much larger volume compared to the lab scale reactor.

4. Conclusions

This work explored and confirmed the feasibility of Sc-CO_2 process to dry and pasteurize food products in one step. The microbial inactivation (for both pathogenic bacteria and natural present microorganisms) and the mechanical characterization of the samples have been measured as a function of different operative parameters. A complete inactivation up to 5 log CFU/g was achieved within a lab scale semi-continuous reactor for *E.coli* O157:H7 and *Salmonella*, while 7 log CFU/g for *L. monocytogenes*. The inactivation was achieved after the pressurization and depressurization step. The count of mesophilic bacteria, yeasts and molds, and Enterobactericeae were lower for the Sc-CO_2 dried product if compared with freeze-dried and air-dried sample, confirming previous evidence with coriander.[8,9] The Young Modulus of Sc-CO_2 dried apples, an an indicator for the stiffness of the sample, was analyzed at different process conditions; it resulted to be dependent from the final water activity, but independent from pressurization and depressurization time, when drying time was higher than 7 h.

In conclusion, the results achieved in the present work are very promising for the scale up of the innovative process at industrial level. Nevertheless, additional data on sensory quality as well as structure profile should be performed to validate the results reached in this investigation.

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Disclosure statement

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