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Abstract

This work aimed to investigate the supercritical CO_2 ($ScCO_2$) drying of strawberries and its effect on enzymatic, chemical and microbial stability.

Process conditions influenced the final weight loss, water activity and the inactivation of polyphenol oxidase (PPO) and peroxidase (POD). At 40°C, an efficient drying (WL>92%, a_w<0.34) and a complete enzymatic (POD and PPO activity) inactivation can be achieved using several combinations of pressure, time and flow rate. $ScCO_2$ dried strawberry at 40°C, 13.3MPa, 7h and 19kg/h flow rate maintain the total content of Vitamin C (358.5 mg/100g), 95% of total anthocyanin (61.68 mg/100g) and 76% of total flavonoids (25.85 mg/100g) in comparison with fresh samples. Foodborne pathogens (*E.coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes)* inoculated at high concentration

(≥6 log CFU/g) were undetected after the process. Overall results are promising for the development of a novel low temperature drying process for the production of healthy and safe snack.

Key words: strawberry; supercritical drying; carbon dioxide; microbial inactivation; nutritional evaluation

Highlights

- $ScCO_2$ process parameters influenced the final weight loss and water activity
- PPO and POD can be completely inactivated after $ScCO_2$ drying at 40°C
- The total content of Vitamin C was maintained
- *E.coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* were completely inactivated

1. Introduction

Berries are one of the most important functional food categories and they are rich in both nutritive and non-nutritive compounds (e.g. vitamins, minerals, polyphenols) (Afrin et al., 2016). Among berries, strawberries (*Fragaria* × *ananassa*) are the most popular and year-round available berries in the world. The global production of fresh strawberries in 2020 was evaluated to be 8.8 million tons (FAOSTAT, 2022). In the same year, the relative market size was USD 18370 million and it is estimated to increase in the next five years with a CAGR of 3.4%, until reaching the value of USD 23210 million (360ResearchReports, 2021) .

Daily consumption of about 150-200 g of fresh strawberry is associated with several benefits for human health (Tulipani et al., 2011), like lower incidences of cancer, age-related neurodegenerative disorders, metabolic alterations, cardiovascular disease and inflammation (Tulipani et al., 2014). Long-term conservation of strawberries is a challenge. Indeed, fresh strawberries are easily affected by mechanical damages and fungal infection that can rapidly reduce the fruit quality. The shelf-life of fresh strawberries in cold storage (0°C) is around two weeks, thus making marketing a challenge (Gol et al., 2013). Frozen strawberries can be used as a substitute for fresh products. However, the high cost for storage and delivery as well as the textural change calls upon specific applications like frozen cakes, smoothies and ice cream. Drying is an efficient alternative to promote the preservation of strawberries over time. In the past, the drying of strawberries has been extensively investigated and, among different techniques, freeze-drying (Harguindeguy & Fissore, 2020), microwave vacuum drying (de Bruijn et al., 2016) and convective drying (Krzykowski et al., 2020) are some of the most extensively used industrially. Innovative hybrid techniques have also been investigated and they are promising for the improvement of current technologies (Onwude et al., 2017). Despite their benefits, these processes might lead to an alteration of product quality, especially referring to the health-promoting components (Méndez-Lagunas et al., 2017; Wojdyło et al., 2009). There is evidence that temperature has a strategic role in the preservation of bioactive molecules in strawberries. Specifically, conventional air-drying of strawberries, that combines i) the presence of oxygen, ii) high

temperatures (T>50°C) and iii) long process time, facilitates enzymatic and non-enzymatic degradation reactions, especially for anthocyanins and vitamin C (Méndez-Lagunas et al., 2017; Patras et al., 2010; Rahmawati & Bundjali, 2012). On the contrary, when low temperatures are used, i.e. freeze drying and vacuum microwave's samples have higher retention of phenolic compounds, anthocyanins, ascorbic acid and carotene (Wojdyło et al., 2009) compared to convective drying. Regarding food safety, it is worth noticing that strawberries have been shown to have a high risk of contamination by bacteria such as *Escherichia coli* and *Salmonella*, which are very resistant and can survive in the dried state (Beuchat & Mann, 2014). Currently, available drying technologies have a limited inactivation power against microorganisms (Bourdoux et al., 2016). For these reasons, the interest in alternative technologies to increase the safety of the dried products while maintaining a high quality of the food is growing. In this contest, Supercritical Carbon Dioxide ($ScCO_2$) has demonstrated to be an efficient technology for alternative drying of food. The first investigation was reported by Brown et al. for carrots, where $ScCO_2$ was used alone and in combination with ethanol co-solvent (Brown et al., 2008). More recently it was demonstrated that the process can achieve the drying and microbial inactivation simultaneously. Examples were recently published for coriander leaves (Bourdoux et al., 2018; Michelino et al., 2018; Zambon et al., 2021), apple slices (Zambon et al., 2021), strawberry slices (Zambon et al., 2022) and chicken breast (Morbiato et al., 2019) for both natural present microorganisms and pathogens (*Salmonella enterica, Listeria monocytogenes, Escherichia coli* O157:H7). Specifically in coriander leaves, the technology was able to completely inactivate yeasts and molds below the limit of detection (<10 CFU/g) and decrease up to 4 log the total mesophilic bacteria count (Zambon et al., 2018). Pathogens were strongly reduced even at mild process conditions after the pressurization and depressurization phase (Bourdoux et al., 2018). Similarly, inoculated pathogens resulted under the detection limit in apple slices after a $ScCO_2$ treatment (Zambon et al., 2021). Studies on chemical stability showed a good retention and preservation of nutrients in dried apples, which were comparable with freeze-dried samples (Tomic et al., 2019). The sensorial quality and acceptance by the consumers of the $ScCO_2$ dried product are

also promising for the development of the technology at a commercial scale (Djekic et al., 2018; Tomic et al., 2020). However, the state-of-the-art is still limited to a few case products and additional studies are needed to demonstrate the potential of the technology to produce high-quality products.

In this contest, this work aims to study the supercritical drying of berries, and in particular strawberry (*Fragaria* × *ananassa*) slice, focusing on the effect of the process variables (i.e. pressure, time and flow rate) on the drying efficiency (final weight loss and water activity) and the activity of the enzymes (polyphenol oxidase (PPO) and peroxidase (POD)). Trials include the inactivation of pathogenic microorganisms (*Listeria monocytogenes, E. coli O157:H7 and Salmonella enterica*) and the characterization of secondary metabolites (Anthocyanins, Polyphenols and Vitamin C) after drying at an optimised process condition and a proof of concept consumers' test.

2. Materials and methods

2.1 Sample preparation

Fresh strawberries (*Fragaria* × *ananassa*) were purchased from a local market in Padova (Italy), stored at 4°C and treated within 3 days after the purchase. Unwashed fruits were cut into slices (about 5 mm thickness) before processing. For each test 10 g ± 0.1 g of product was equally distributed in 10 metallic baskets (1 g per basket).

2.2 Lab scale reactors

A new lab scale reactor with recirculation (Separex S.A.S., Champigneulles, France) equipped with a drying and regenerative cylindrical vessel of about 150 mL (internal diameter 2.5 cm) and 600 mL (internal diameter 6 cm), respectively, was used. The plant includes: a CO_2 tank (purity 4.0, Rivoira, Italy) kept at room temperature; a chiller reservoir (M418-BC MPM Instruments, Milan, Italy); a membrane pump (Lewa, EK01, Germany) used to pressurize the apparatus; a centrifugal pump (Separex, P300, France) to recirculate the $ScCO_2$ between the two reactors; a heat exchanger to preheat the fluid before entering the drying vessel. Temperature and flow rate are controlled through a control panel, while pressure is controlled with a back-pressure regulator valve. The plant is automated and controlled by Labview software. A schematic representation of the plant is reported in Supplementary Fig S1.

2.3 $ScCO_2$ drying procedure and optimization study

Before each experiment, the vessel was cleaned with pure ethanol (Sigma Aldrich, 99.8%) and washed with sterile distilled water. The vessel was then flushed with CO_2 for 2 minutes in order to remove water residues. The samples were weighed inside the metallic baskets (2 cm width, 3 cm height) that were previously cleaned with absolute ethanol and burned with a Bunsen flame. The baskets were then inserted inside the drying vessel. The treatment consists in three main phases i) pressurization; ii) drying and iii) depressurization. Pressurization was set at 0.4 MPa/min, while depressurization was achieved in 40 min as previously used (Zambon et al., 2018).

A face-centered central composite design (Montgomery, 2012) has been used to plan the experiments with the purpose of finding the optimal operating domain of the process to guarantee the desired quality of the dried strawberries. In this study, the strawberries quality was investigated through 4 main types of responses *y*: the effect of drying (weight loss y_1 and water activity y_2) and the inactivation of oxidative enzymes (residual activity (RA%) of POD y_3 and PPO y_4). The impact of 3 factors on these responses was studied: pressure, drying time and pump flow rate. Pressure x_1 was varied in the range 10-14 MPa; drying time x_2 in the range 4-8 h and pump flow rate x_3 in the range 5-25 kg/h. Table S1 in the Supplementary summarizes the variation of the factors in the experimental design. Each tested condition was repeated once, except for the central point (three replicates). To understand the functional relationship among the factors, response surface empirical models were built through second-order regression models with interactions:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} x_i x_j + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \varepsilon \quad (1)$$

where i and j = 1, 2, 3 are related to the three factors pressure, drying time and pump flow rate, respectively, which are coded in the interval $x_1, x_2, x_3 \in [-1,1]$, while ε is the error which is minimized in the least-squared sense. The first term of Equation (1) determines the intercept β_0, the second term identifies the so-called main effects, the third identifies the interactions between variables, and the fourth the quadratic effects. Matlab was used to analyze the outcomes of the experimental measurements and estimate the parameters β.

2.4 Mass-loss and water activity analyses

The mass loss before and after the process was calculated as

$$mass\ loss = \left(1 - \frac{m_{dry}}{m_{fresh}}\right) * 100\% \quad (2)$$

where m_{dry} and m_{fresh} indicate the mass of the sample after and before the process, respectively (Zambon et al., 2018). After each experiment the weight loss was calculated for each basket; the mean and standard deviation was then calculated for each condition from an average of 10 measurements. Water activity (a_w) was measured (Hygropalm Rotronic, Bassersdorf, Switzerland) at the end of the

process for the sample in the basket placed at the bottom, center and top of the vessel in order to calculate the mean and standard deviation from three independent measurements and consider the variability thought the length of the vessel.

2.5 Enzymatic activity

2.5.1 Enzyme extraction

The extraction of PPO and POD enzymes was performed as described previously with some modifications (Marszałek et al., 2015). Fresh strawberries (10 g) were washed and the calix was removed. Samples were pureed with a hand blender for 3 min and then were mixed in ratio 1:1 with extraction buffer (0.2 M phosphate buffer, pH 6.5, 1 M NaCl, 1% Triton X-100 (Sigma Aldrich, Milan) and 4% polyvinylpolypyrrolidone (PVPP, Sigma Aldrich, Milan)) with gentle shaking for 1 h at 4°C. For $ScCO_2$ dried strawberries the amount of water loss during the treatment was replaced with extraction buffer. The mixtures (from fresh or $ScCO_2$ dried strawberries) were homogenized for further 3 min with potter at 1800 rpm on ice and then incubated at 4°C for 1 h with gentle shaking. Samples were then centrifuged at 14000 x *g*, 20 min at 4°C (Avanti™ J-25, Beckman). Supernatants were collected and filtered through a cloth layer. The obtained extracts were analyzed for the PPO and POD activity.

2.5.2 Determination of enzymatic activities

PPO and POD enzymatic activities were determined spectrophotometrically as described by Siguemoto and Gut (Siguemoto & Gut, 2017), with minor changes. The reaction mixture for PPO assay was made by mixing 40 μL of extract with 160 μL of a solution 0.07 M pyrocatechol (Sigma Aldrich, Milan) in 50 mM sodium phosphate buffer (pH 5). The blank sample was prepared by using phosphate buffer instead of the extract. PPO activity was monitored by measuring the change in absorbance at 420 nm with Infinite M200 PRO NanoQuant absorbance microplate reader (TECAN,

Switzerland). Absorbance was measured at intervals of 1 h for a total of 8 hours. The evaluation of absorbance was performed in triplicate for each sample.

POD activity was determined by mixing 50 μL of extract with 100 μL of 50 mM sodium phosphate buffer (pH 6.5) and 25 μL of 1% *p*-phenylenediamine (Sigma Aldrich, Milan). The blank sample was prepared using phosphate buffer instead of the extract. The reaction started by the addition of 25 μL of 1.5% H_2O_2. The activity of POD was monitored by measuring the change in absorbance at 480 nm with Infinite M200 PRO NanoQuant absorbance microplate reader (TECAN, Switzerland). Absorbance was measured at intervals of 30 s for a total of 10 min.

The activity of PPO or POD in the sample was defined by the slope generated by fitting the absorbance obtained during time with a linear regression in a pseudo 0 kinetic model, as reported previously (Manzocco et al., 2017). The stationary phase of the kinetic curve was not included in the data fitting. The enzymatic residual activity (RA%) was calculated as the percentage of the ratio between the slopes obtained for the treated samples (k) and the untreated ones (k0).

2.6 Microbial analyses

2.6.1 Bacterial strains and inoculum preparation

The stock cultures of *Escherichia coli* O157:H7 (ATCC 700728), *Salmonella enterica* (serovar Thompson RM1987) and *Listeria monocytogenes* (LMG 23192) were kindly provided by Prof. Frank Devlieghere, Ghent University, and used for contaminating the matrix and for the $ScCO_2$ drying test. The inoculation procedure was adapted from a previous work (Bourdoux et al., 2018). The strains were revived by transferring a loopful of bacteria from the slant cultures in 1.5 mL of Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company) for 6 h at 37°C. After incubation, 0.1 mL of broth cultures were plated on specific selective agar media: Mac Conkey agar with Sorbitol, Cefixime, and Tellurite (CT-SMAC, Sacco, Italy) supplemented with 50 μg/mL of nalidixic acid (Sigma Aldrich, Milan) for *Escherichia coli* O157:H7; Xylose Lysine Deoxycholate agar (XLD,

199 Biolife, Italy) containing 50 μg/mL of nalidixic acid for *Salmonella enterica*; Ottaviani Agosti (O.A.)
200 Listeria Agar (Liofilchem, Italy) for *Listeria monocytogenes*. Plates were incubated at 37°C for 24 h.
201 Each microorganism was cultured separately by taking a colony from agar plates and transferring it
202 into 3 mL of BHI; after 6 h at 37°C, working cultures were prepared diluting 50 μL of each inoculum
203 into 5 mL of BHI broth and incubating at 37°C for 18 h. One mL of each culture was transferred in
204 an eppendorf tube and centrifuged at 2900 rpm for 10 min. Pellet was washed twice in phosphate
205 buffer saline (PBS) (Sigma Aldrich, Milan) and resuspended in 1 mL of PBS.
206 2.6.2 Contamination of matrices with pathogenic bacteria and analysis
207 Strawberry slices were contaminated by the addition of 16 ± 4 μL of the bacterial suspension per
208 gram of fresh product, in order to obtain an inoculum concentration of 6.0 ± 0.5 log CFU/g. Each
209 inoculum was poured over strawberries' slides and left for 30 min in a biosafety cabinet to allow the
210 bacteria attachment to the surface of the product. For each experiment, a non-contaminated sample
211 was included and adopted as background. One contaminated sample was used to determine the initial
212 load of the bacteria and other three contaminated samples were treated with $ScCO_2$.
213 After treatment, all strawberry samples were diluted in Buffer Peptone Water (BPW, Sacco System,
214 Italy) at a ratio of 1:10 and mixed by vortexing for 1 min. After mixing, ten-fold dilution was prepared
215 and 0.1 mL of each sample was plated in duplicate on selective media described above. Plates with
216 *E. coli* O157:H7 and *S. enterica* were incubated at 37°C for 24 h, while plates with *L. monocytogenes*
217 were incubated at 37°C for 48 h. All experiments were performed by spread plate technique at least
218 in duplicate for each condition.
219 The inactivation degree was expressed as $\log_{10}(N/N_0)$, where N_0 (CFU/g) and N (CFU/g)
220 corresponded to the number of CFU/g initially present in the untreated sample and those estimated
221 after the treatment, respectively. The limit of quantification and detection was set at 2000 CFU/g and
222 100 CFU/g respectively. When the microbial count was below the detection limit, an enrichment was
223 performed by incubation for 24 h at 37°C the first dilution before plating. 0.1 mL of samples were
224 then plated onto a selective agar medium, according to the specific pathogenic bacteria (as previously

225 described in section 2.6.1). After the 24 h incubation at 37°C, an absence of countable colonies
226 indicated that the residual microbial count in the sample was below 1 CFU/g.

227 **2.7 Chemical analyses**

228 Reagents are from Sigma Aldrich (Milan, Italy) when not specified. Fresh and dried strawberries
229 were analyzed for the total content in vitamin C, anthocyanins and polyphenols. Each sample was
230 homogenized and grinded (IKA grinder model A11) before extraction. For each experiment, 8 g of
231 fresh strawberry and 0.5 g of dried strawberry were weighed and 10 mL of a solution of formic acid
232 (0.1%) for vitamin C or HCl (1%) for anthocyanins and polyphenols, was added. The solution was
233 placed in the ultrasonic bath for 10 minutes and then centrifuged (13000 rpm for 5 min) to collect the
234 supernatant for the analysis.

235 2.7.1 Analysis of polyphenols

236 Quantitative analysis of phenolic derivatives was obtained by HPLC-DAD-MS^n. The measurements
237 were performed with an Agilent 1260 chromatograph (Santa Clara, CA, USA) equipped with 1260
238 diode array (DAD) and Varian MS-500 ion trap as detectors. Separation was achieved using an
239 Agilent Eclipse XDB C-18 (3.5 × 150 mm) 3.0 μm as stationary phase. The mobile phases were water
240 0.1% formic acid (A) and acetonitrile (B). The elution gradient started at 90% A then decreased to
241 0% over 36 min, flow rate was 0.5 mL/min. At the end of the column a T connector splitted flow rate
242 to DAD and MS. The DAD detector was used to quantify flavonoids and rutin, chlorogenic acid and
243 gallic acid were used as reference compounds. The chromatograms were monitored at 280, 330 and
244 350 nm and UV-Vis spectra were acquired in the range of 200-650 nm. The sample injection volume
245 was 10 μL. MS spectra were recorded in negative mode in 50–2000 Da range, using ESI ion source.
246 Fragmentation of the main ionic species was obtained by the turbo data depending scanning (TDDS)
247 function. Identification of compounds was obtained based on fragmentation spectra as well as the
248 comparison of fragmentation patterns with the literature and injection of reference compounds when
249 available. Quantification of phenolic constituents was obtained with the method of calibration curve:

rutin (Sigma Aldrich, St. Louis, MO, USA) was used as external standard for flavonoid quantification, chlorogenic acid (Sigma Aldrich) was used for caffeoylquinic acid derivatives, gallic acid (Sigma Aldrich) was used for phenol derivatives. Calibration curves were as follows rutin y =28.732x + 315.78 (R^2=0.988); caffeoylquinic acid y=79.285x - 268.61 (R^2=0.964); gallic acid y=118.79x - 76 (R^2=0,999).

2.7.2 Analysis of cyanidins

Quantitative analysis of anthocyanin was performed with HPLC-DAD-MS^n on the same system described above. Analyses were performed on Zorbax poroshell C-18 (3.0 X 100mm) 5 µm column as stationary phase and water 1% formic acid (A) and methanol (B), as mobile phase. The elution gradient started at 95% A then decreased to 45% at min 38^{th}, 0% at min 48^{th}, and then 7 min at 95% A. Flow rate was 1 mL/min. Cyanidin chloride (Phytolab) was used as reference standard (y=170.84x - 424.56; R^2=0,993). At the end of the column, a T connector splitted flow rate to DAD and MS. The chromatograms were monitored at 550 nm and UV-Vis spectra were acquired in the range of 200-650 nm. Fragmentation of the main ionic species was obtained by the turbo data depending scanning (TDDS) function. Identification of compounds was obtained based on fragmentation spectra as well as the comparison of fragmentation patterns with the literature and injection of reference compounds when available.

2.7.3 Analysis of vitamin C

Quantitative analysis of vitamin C was performed with HPLC-DAD on the same system described above. Analyses were performed on Zorbax SB C-3 (4,6 X 150 mm) 5 µm as stationary phase water 1% formic acid (A) and acetonitrile (B) as mobile phase. The elution gradient started at 90% A then decreased to 60% at min 30^{th}, and then 0% at min 35^{th}. Flow rate was 1 mL/min. The chromatograms were monitored at 280 nm and UV-Vis spectra were acquired in the range of 200-650 nm. Vitamin C was used as reference standard (y=23.815x - 7.1344; R^2=0,999).

2.8 Consumers' sensory test

A consumer test was carried out between strawberries treated with three different drying techniques: i) Sc-CO_2 drying (13.3 MPa, 7 h, flow rate of 19 kg/h), ii) vacuum-freeze-drying (Coolsafe 95/55-80 Freeze Dryer, Labogene) with a pre-freeze at -40°C and for a total drying of 48h, iii) air-drying (Melchioni Babele, 250W) at 50° for 10h. Twenty-two students (12 females and 10 males, 25-32 years old) were selected to conduct an acceptance test between the samples. The main criterion for the selection was if they were relatively frequent (from time to time) users of dried fruit snacks and the absence of an allergic reaction to strawberries. Overall liking, appearance, flavour, taste, and firmness were assessed using a 5-point hedonic scale (1-dislike extremely; 2-dislike slightly; 3-neither like nor dislike; 4-like slightly; 5-like extremely). Order presentation of the three samples was balanced, so that they appeared in the same position an equal number of times, to minimize any bias caused by the order of presentation. Three cups containing about 3 g of dried strawberries randomly selected from each treatment and labelled with 3-digit random numbers, were presented to each student. The panelists observed and rated in order the appearance, then the flavour, taste, firmness and overall liking. The acceptance rating differences among treatments were explored through analysis of variance (ANOVA) per attribute.

3. Results and discussion

3.1 Optimization of drying

The optimization was performed analyzing the effects of the process factors (pressure x_1, time x_2 and flow rate x_3) on the responses. An optimal operating domain is one that guarantees at the same time the obtainment of specific product characteristics. For this reason, the process optimization was performed for both the drying efficiency and the enzymatic residual activity. Weight loss and water activity are indicators of drying efficiency, while enzyme activity is important for the overall product quality and the preservation over time. During the experimental campaign, the temperature was set at 40°C to avoid thermos-degradation of sensitive molecules caused by heat and high temperatures.

299 Supercritical CO_2 is characterized by a critical point close to ambient temperature (31°C), therefore
300 40°C ensures supercritical conditions and should not affect chemical degradations.
301 3.1.1 Weight loss
302 For the weight loss y_1 the response surface model which best fits the experimental data is:
303 $$y_1 = \beta_0 + \beta_3 x_3 + \beta_{23} x_2 x_3 + \beta_{33} x_3^2 + \beta_{233} x_2 x_3^2 .$$
304 The determination coefficient is R^2=0.93, meaning that the fit of the experimental data provided by
305 the model is accurate. The response surface parameters β_i are shown in Table 1, which indicates that
306 the main effects on the weight loss are related to the flow rate (x_3). The flow rate seems to have a
307 strong linear impact on the weight loss: the higher the flow rate is, the higher the velocity of the
308 $ScCO_2$ that increases the mass transfer of the process is. However, the increase of weight loss is also
309 related to the decrease of the squared value of the flow rate (x_3^2) and the decrease of the interaction
310 between time and flow rate ($x_2 x_3$). The complex relation of the time and flow rate $x_2 x_3^2$ seems to
311 have the most important effect on weight loss. The time (x_2) showed a complex interaction with the
312 flow rate, while pressure (x_1) seems to play a marginal role in the supercritical drying, probably
313 because in the range of the investigation the solubility of water in CO_2 at 40°C, is approximately
314 constant (Wang et al., 2018).
315 **Table 1**: values of the selected parameters of the response surface models fitted on the data of the available experiments
316 (n.s. = not selected).-y1 refers to weight loss, y2 water activity, y3 POD, y4 PPO-

Parameter	y_1	y_2	y_3	y_4
β_0	91.38±1.51	0.36±0.03	0±0.61	13.12±13.18
β_1	n.s.	n.s.	-1.68±0.43	n.s.
β_2	n.s.	-0.08±0.03	-1.8±0.43	25.25±15.97
β_3	4.14±1.26	-0.11±0.03	n.s.	-16.47±15.97
β_{12}	n.s.	n.s.	n.s.	-22.50±15.97
β_{13}	n.s.	n.s.	1.46±0.43	27.81±15.97
β_{23}	-4.23±1.41	0.09±0.03	-1.32±0.43	n.s.
β_{11}	n.s.	n.s.	n.s.	n.s.
β_{22}	n.s.	0.07±0.05	n.s.	n.s.
β_{33}	-3.60±1.97	0.08±0.05	3.33±0.74	n.s.
β_{233}	4.74±1.41	n.s.	n.s.	n.s.

Through the response surface model, it is possible to identify a domain, which is able to achieve optimal weight loss. In particular, the response surface of Figure 1A shows how the weight loss is dependent on the main effects of the flow rate and the time at a constant value of the pressure (12 MPa). Since pressure has not a relevant effect, the response surface at different values of the pressure are similar (data not shown). At 40°C, the pressure was also not influencing the drying in the case of supercritical drying of red bell pepper (Zambon et al., 2020) at a short drying time (6h), which is similar to the maximum value of time in this investigation. A high weight loss is achieved for a wide range of condition: $y_1 > 90\%$ (yellow part of the surface) for different combinations of flow rate and time. Only the combination of low flow rate and low processing time determines low weight loss (blue part of the surface). A more detailed visualization of this domain is shown in the contour plot of Figure 1B; it demonstrates the complex nonlinear interaction between the flow rate and the time (at constant intermediate pressure, 12 MPa). A weight loss higher than 90% is highlighted in full color. Figure 1C shows the response in the entire three-dimensional experimental domain: the blue dots are related to combinations with y_1<90.5%, the green circles are related to combinations with $y_1 \epsilon [90.5\%, 92\%[$, while the magenta dots identify the combination related to weight loss $y_1 \geq 92\%$. The shape of the most promising domain for weight loss (green and magenta circles) do not change significantly with pressure and ensures a good weight loss with a complex nonlinear interaction of intermediate and high flow rate and processing time. As result, an optimal drying can be reached with short processing time (e.g., $x_2 = 4$ h), while with long processing time ($x_2 > 7.5$ h) almost all the levels of flow rate in the inspected range guarantee a good drying performance. According to the model, the optimum water loss (maximum weight loss) WL=93.5% is obtained at the following conditions: pressure=12 MPa, time=4 h, flow rate=20.02 kg/h. However, the results are affected by uncertainty. Since the standard deviation among replicates of the same experiment is 1.62% of weight loss, it is worth identifying as an optimal operating domain and not simply a single processing point. For this reason, a relatively wide part of the explored domain is identified as optimal. This is the part

of the domain that is shown in the contour plot of Figure 1B whose weight loss is higher than 90%. The choice of the operating point within the optimal domain in Figure 1B can be selected including all the outcomes. Considering also the results achieved on the final water activity (3.1.2), the model was validated at pressure=13.3 MPa, time=7 h, and flow rate=19 kg/h by confirmatory experiments. Validation is important to verify that the model prediction error is lower than the standard deviation among replicates of the same experiments. Confirmatory validation was performed with strawberries from different batches. Table S2 shows the value of weight loss obtained for the confirmatory experiments. In our case, the model prediction indicates $\hat{y}_1 = 92.4 \pm 2.85\%$ (where $\pm 2.85\%$ indicates the 95% confidence limit of the model uncertainty in prediction) while the real outcome of the experiment is y_{1b1}=91.09 $\pm 1.35\%$ (where $\pm 1.35\%$ indicates the 95% confidence limit of uncertainty obtained by the replicates of the validatory experiments) for batch 1, y_{1b2}=92.62±0.8% for batch 2 and y_{1b3}=90.15±2.86% for batch 3, respectively. The difference between the predicted value and the real value $(\hat{y}_1 - y_1) < 1.96\sigma$ is lower than the variability among replicates of the same processing conditions, meaning that the model performance is satisfactory for the three batches tested. Furthermore, the real value y_1 falls always well within the uncertainty interval of the model prediction $\hat{y}_1$. These results are promising for the possible use of the model in the prediction of the final weight loss of strawberry from different locations and cultivars. Additional experiments should be performed to demonstrate the goodness of the model using different strawberry cultivars and from different geographical locations.

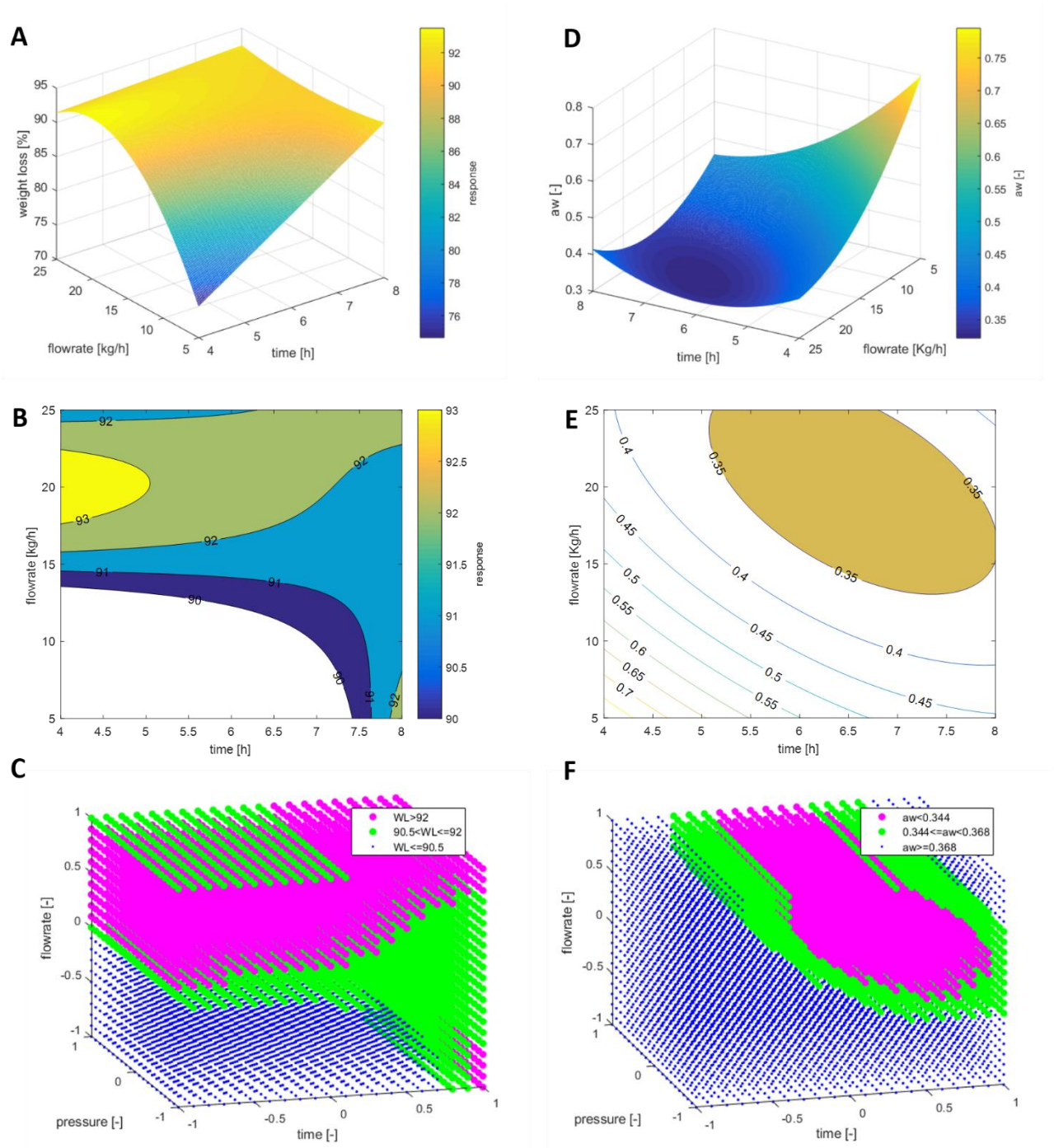

Figure 1. *Response surface model for the weight loss (A, B, C) and the water activity (D, E, F). (A, D) response surface depending on time and flow rate at a constant pressure of 12 MPa; contour plot of the weight loss (B) and water activity (E) as a function of time and flow rate at a constant pressure of 12 MPa and optimal processing domain (full color area); (D, F) visualization of the optimal processing domain in the space of time, pressure and flow rate.*

3.1.1 Water activity

Water activity y_2 is a good indicator for the achievement of a drying state and its value is important for the inhibition of microbial growth (Troller, 2017). Most bacteria, yeasts, and molds are unable to

grow below 0.87, 0.88, and 0.80, respectively (Bourdoux et al., 2016). It is worth noticing that mass loss and water activity are highly inversely correlated (i.e., the correlation coefficient is ρ =-0.88), meaning that they change with similar behaviour, but when mass loss increases, water activity decreases (and vice versa). The response surface model for the water activity is:

$$y_2 = \beta_0 + \beta_2 x_2 + \beta_3 x_3 + \beta_{23} x_2 x_3 + \beta_{22} x_2^2 + \beta_{33} x_3^2$$

This response surface has very good fitting performance (R^2=0.95). Similar to the case of weight loss, the main effects affecting water activity are related to the time and flow rate (both linear and quadratic) and their interaction. The response surface of Figure 1D shows that the lowest levels of water activity are related to high levels of flow rate and time. Figure 1E shows the detail of the variability of water activity as a function of time and flow rate (at intermediate values of the pressure), identifying the optimal domain to guarantee the minimum in the yellow zone. As can be seen in Figure 1F, the shape of this domain does not change using different levels of processing pressure.

The prediction performance of the response surface model was tested in the validation point at pressure x_1=13.3 MPa, processing time x_2=7 h, and flow rate x_3= 19 kg/h. The confirmatory experiment (Table S2) demonstrated that the model performance is satisfactory. In fact, the model prediction is $\hat{y}_2 = 0.3244 \pm 0.0835$, which is not far from the average of the water activity of the confirmatory experiments ($y_{2b1} = 0.3710$; $y_{2b2} = 0.2550$ $y_{2b3} = 0.3870$). Also in this case the error is comparable to the width of the variability σ of the experiment replicates. In our previous work with red bell pepper at pilot scale, $ScCO_2$ dried samples at 40°C reached similar values for a_w after 6 hours of drying (Zambon et al., 2020). Lower a_w can be reached at long drying time (16 hours), suggesting that also in the case of strawberry lower a_w could be obtained by changing the process parameter. Further study at semi-industrial scale should be performed to build a mathematical model able to predict the final a_w at industrial level. Moreover, analysis on the correlation between a_w with the texture and sensorial attribute should be included in further study taking into account the target industrial application of such technology (e.g. snacks, topping, and flour).

3.2 Inactivation of Enzymes

Enzymes inactivation during drying has been associated with retention of the quality overtime. To the best of our knowledge, there is no evidence about the effect of $ScCO_2$ drying on the enzyme activity of fruits. However, the previous results achieved on the sensory aspects for apples (Tomic et al., 2019), pepper (Zambon et al., 2020) and beetroots (Tomic et al., 2020) are promising indicators of enzyme stability and inactivation with $ScCO_2$ drying. Previous studies on enzyme inactivation with $ScCO_2$ were mainly focused on juice products, and they showed a high dependence on the food matrix and process variable (Silva et al., 2020). Here we have performed a 2^3 full-factorial design of experiment with 3 replicates of the center point for a screening study on the inactivation of enzymes. The most representative response surface for POD activity y_3 is:

$$y_3 = \beta_1 x_1 + \beta_2 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2$$

with a very accurate fitting (R^2=0.99). The most important effects are related to pressure, time, and their interaction with flow rate and squared pressure. In particular, the quadratic term of the pressure has the main effect on POD. Figure 2A shows the domain that guarantees a complete inactivation of POD with magenta points.

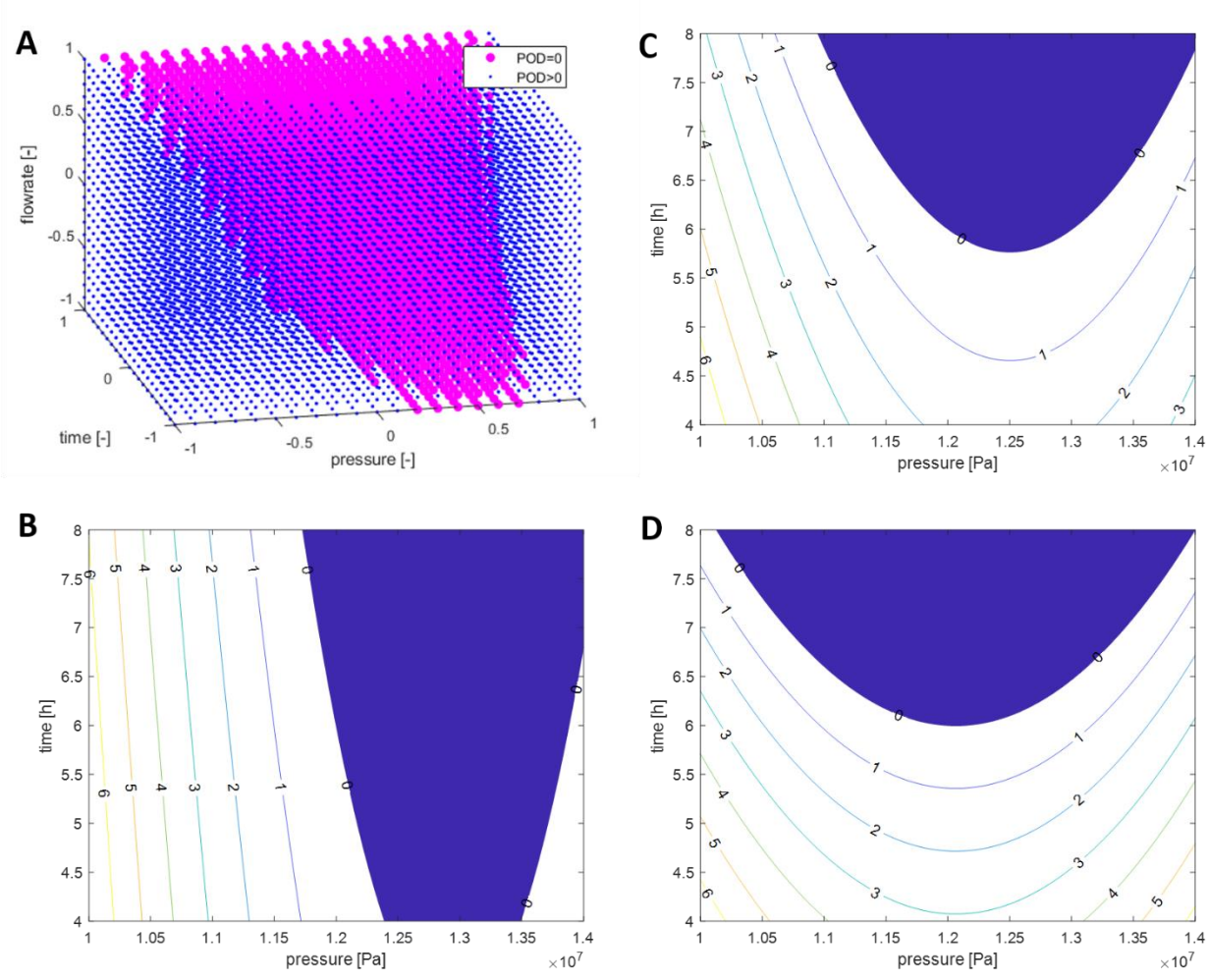

Figure 2. *Response surface model for POD: (A) visualization of the optimal processing domain in the space of pressure, time, and flow rate, and contour plot of pressure vs. processing time at constant flow rate of: (B) 5 Kg/h; (C) 15 Kg/h; (D) 25 Kg/h. The blue domain indicates the conditions that guarantee POD=0.*

In particular, it shows a strong interaction of pressure with time and flow rate, together with a quadratic behaviour of the POD with the pressure. Furthermore, Figures 2B, 2C and 2D show the domain of the conditions in terms of pressure and processing time which guarantees POD=0 at different levels of flow rate (blue area). Typically, with low flow rates (5 Kg/h) medium-high pressures are required to guarantee POD=0 whatever the treatment time. When the flow rates are medium or high (15-25 Kg/h) a wide range of pressure conditions can be utilized to obtain POD=0, but only with a long treatment duration (>6 h). POD in strawberry was found to be thermos-sensitive and completely inactivated in less than 5 min at 70°C (Terefe et al., 2010). A complex interaction between process parameters for the inactivation of POD in strawberry puree was also observed in high pressure-thermal process. Similarly, a dependence of pressure, time and a second level order of

pressure was observed together with a quadratic dependence with pressure in the case of a combined high pressure–thermal processing. Terefe et al. also showed that the highest inactivation was achieved at the highest pressure (690 MPa) and temperature used (90°C), while at ambient temperature a residual 30% of activity was observed even at the highest pressure. Previous studies on $ScCO_2$ pasteurization of strawberry juice (Marszałek et al., 2015) showed a resistance of POD to short treatment (maximum 30 min) neither at the highest pressure (60 MPa). This is consistent with what was observed with other juices after $ScCO_2$ treatment, where enzyme inactivation was not completed and was dependent on process parameters (Marszałek et al., 2016, Marszałek et al. 2017).

The most representative response surface for PPO activity y_4 is:

$$y_4 = \beta_0 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3$$

with good fitting performance (R^2=0.87). The most important effects which make PPO decrease are related to the flow rate and the interactions between time and pressure. On the other hand, time and the interaction between pressure and flow rate make PPO increase (with increasing $x_1 x_3$). The functional dependence of PPO on the three factors is complex and there is not the predominance of one specific factor. The optimal domain that guarantees a complete inactivation of PPO (PPO=0) is represented in Figure 3A (blue dots). As a general outcome, when medium-high flow rate is used, a wide domain of conditions in terms of pressure and time can guarantee to obtain PPO=0 (Figures 3C and 3D). In general, the higher the flow rate, the larger the time domain where it is possible to inactivate the PPO. When high pressure is used, the inactivation is possible only at low flow rate and for a short drying time (Figure 3B). Previous works on the inactivation of PPO showed also a dependence with the process parameters (Hu et al., 2013). The inactivation was also matrix dependence; PPO activity, for example, decreased with the increase of the pressure in graham flour (Hojnik Podrepšek et al., 2020). In mushrooms (Marszałek et al., 2019) and fresh-cut carrots (Spilimbergo et al., 2013) an increment of pressure and temperature was associated with a decrease in PPO residual activity. In both cases, complete inactivation was not possible after the maximum time, 30 and 45 min for mushrooms and carrots, respectively. Also in the case of carrots and celery

juices treated with $ScCO_2$, the enzymes resulted to be sensitive to prolonged time (Marszałek et al., 2016). In our cases, the long processing time facilitated the achievement of a large domain area in which a complete inactivation was possible.

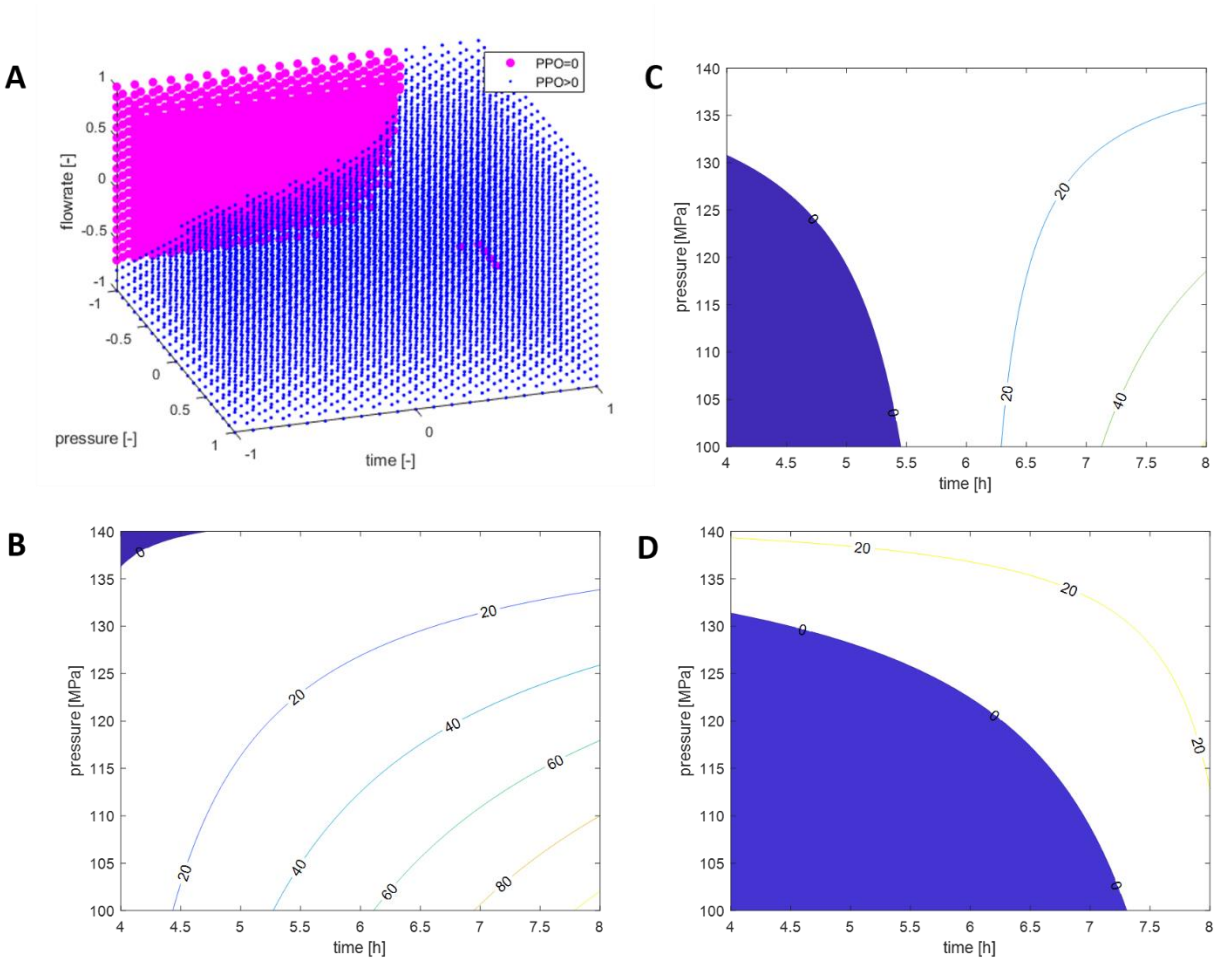

Figure 3. *Response surface model for PPO: (A) visualization of the optimal processing domain in the space of pressure, time, and flow rate, and contour plots of flow rate vs. processing time at constant flow rate of B) 5 Kg/h; (C) 15 Kg/h; (D) 25 Kg/h.. The blue domain indicates the conditions that guarantee PPO=0.*

However, since a complex interaction was highlighted by our model, a simple correlation between the PPO inactivation and process parameters is not possible. Further analysis and studies are needed to understand the relationship between the inactivation with $ScCO_2$ drying process and different food products. The inactivation of enzymes is important to avoid browning and undesirable changes in chemicals and sensorial properties (Chisari et al., 2007). Overall, our results highlighted the possibility to obtain a complete inactivation of PPO and POD using a proper combination of process

parameters. A low enzyme activity is promising for the long preservation over time of the chemical and sensorial properties of dry strawberry. Additional further studies should also investigate the stability of the product during storage under different conditions (e.g. temperature, humidity, light).

3.3 Microbial inactivation

Inoculated samples were dried at 40°C, 13.3 MPa for 7 h at 19 kg/h flow rate. The initial inoculated count were 6.29±0.02, 6.80±0.07 and 5.75±0.01 log CFU/g for *E.coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes*, respectively. A complete inactivation was achieved after the $ScCO_2$ drying and no viable colonies were detected with standard plate count technique as previous observed using a semi-continuous drying apparatus (Zambon et al., 2022). Similar funding was also observed for *Salmonella* in our previous study on chicken breast (Morbiato et al., 2019). *Salmonella* was found to be completely inactivated after 45 min of treatment. $ScCO_2$ drying was efficient on the microbial inactivation of coriander, for both natural present microorganisms (Zambon et al., 2018) and inoculated pathogens (Bourdoux et al., 2018). Also in the case of apple slices, the treatment was successful for the inactivation of pathogenic microorganisms after the pressurization and depressurization phase (Zambon et al., 2021). This finding is important to demonstrate the potential of the treatment to increase the product safety over a wide range of food products and using different types of high-pressure apparatuses. Confirmatory studies at semi-industrial scale should be performed to demonstrate the efficacy of the technology at a larger scale. Moreover, studies on the inactivation of viruses should be performed to investigate the effect of the $ScCO_2$ drying to reduce the risk of infection. Indeed, high risk in berries is associated with norovirus and hepatitis A virus (Bouwknegt et al., 2015). Currently, $ScCO_2$ technology alone has shown limited inactivation capacity against viruses (Hu et al., 2013). However, no evidence is present in the literature about the use of $ScCO_2$ for drying and inactivation of virus.

3.4 Chemical analysis

Strawberry fruit is a rich source of antioxidant compounds such as vitamin C, with an extremely high content of secondary metabolites (Bermúdez-Oria et al., 2020). The possible role of these metabolites

495 in the antioxidant activity of strawberry was studied by Tulipani et al., who indicated that vitamin C
496 was responsible for more than 30% of the activity, followed by anthocyanins contributing to 25 to
497 40% (Tulipani et al., 2008). The others were mainly related to the presence of ellagitannin derivatives
498 and flavanols. Vitamin C is an essential vitamin, which is highly unstable, sensitive to oxygen and
499 temperature. Vitamin C has to be ingested because it cannot be synthetized by human metabolism
500 (Drouin et al., 2011). The effect of $ScCO_2$ process on the retention of bioactive molecules in
501 strawberries were evaluated through chemical analysis of vitamin C, anthocyanins and flavonoids on
502 fresh and dried strawberries at the optimized conditions (13.3 MPa, 7 h, 19 kg/h). Vitamin C was
503 397.2±35.3 and 358.5±56.6 mg/100g of dry material in the fresh and $ScCO_2$ dried strawberries,
504 respectively. Results show a similar concentration of Vitamin C between the fresh and $ScCO_2$ dry
505 sample. This effect on Vitamin C content can be explained by two main factors: the low temperature
506 of the process and the absence of oxygen during drying process. Indeed degradation of Vitamin C
507 can occur under aerobic conditions (Santos & Silva, 2008), thus not happening in CO_2 environment.
508 Previous work reported a decrease in Vitamin C content in strawberries after freeze-dried and air-
509 dried when compared to frozen samples (Asami et al., 2003). Similar behaviour was achieved after
510 vacuum freeze-drying combined with ultrasound pre-treatment (Xu et al., 2021).
511 The average amounts of anthocyanin and flavonoids in fresh and processed strawberries are reported
512 in Table 2. Strawberry samples contained different cyanidin derivatives, mainly pelargonidin
513 glucoside, pelargonidin rutinoside and pelargonidin malonyl glucoside. For flavonoids, the main
514 constituents were apigenin derivatives, quercetin glucuronide and kaempferol glucuronide, as
515 reported by the literature (Ornelas-Paz et al., 2013). The anthocyanin and flavonoid contents
516 expressed as mg/g of dried weight were comparable in the $ScCO_2$ dried samples and in the fresh ones,
517 although some reductions were observed for some molecules. Specifically, a significant reduction
518 was observed in the processed samples for cyanidin, malvidin, petunidin derivative, apigeinin
519 hexoside, quercetin and kaempferol glucuronide. Degradation appears to be more evident in the
520 flavonoids compared to the anthocyanins; specifically, the flavonoid glucuronides resulted to be the

most sensitive to process degradation. This observation may be related to hydrolytic processes that can be promoted in the acidic conditions generated by the $ScCO_2$ environment. On the contrary, the acidic environment can play a role in stabilizing the anthocyanin derivatives as recently described (Idham et al., 2021).

Anthocyanins stability depends on the processing conditions (light and oxygen), temperature and intrinsic properties of the products, such as pH, and the presence of enzymes. Anthocyanins are degraded enzymatically in the presence of PPO and these enzymes could play a role in the degradation of anthocyanins in strawberry (Méndez-Lagunas et al., 2017). Inactivation of PPO by the effect of $ScCO_2$ drying process may be a co-factor for the retention of anthocyanin when lower temperatures are used. Results indicated that $ScCO_2$ drying processes reduce cyanidins in limited amount while flavonoids contents resulted significantly reduced in the dried strawberry. Indeed, the total amount of anthocyanins and flavonoids in the dried samples decreased of 4,6% and 24%, respectively. The different behaviour of anthocyanins and flavonoids can be explained mostly to the mild conditions and the acidic pH that are favourable for the anthocyanins. On the other hand, some authors have previously studied the effect of $ScCO_2$ and revealed that pressurized carbon dioxide could serve as a catalyst for the hydrothermal degradation of hesperidin, a flavonoid glycoside (Ruen-Ngam et al., 2012), suggesting this possible explanation for the flavonoid glycoside reduction in strawberry.

Overall, $ScCO_2$ drying process maintained the strawberry nutritional content, minimizing degradation processes. Indeed, a higher degradation of Vitamin C and polyphenols were observed in previous studies (Kowalska et al., 2018) after freeze-drying and convective drying. However, more studies should be performed including strawberries from different varieties, seasons and geographical regions to confirm the results. Pre-treatment might also be coupled to increase the preservation of bioactive molecules (Kowalska et al., 2018; Macedo et al., 2021). Previous results on apples dried with $ScCO_2$ showed similar behaviour in the retention of bioactive molecules (Tomic et al., 2019). However further analysis of the chemical stability should be performed to confirm the behaviour over-time. In addition, analysis on the consumer's acceptance (sensory evaluation score) should be performed to

demonstrate also the acceptance of the dried strawberry in practical conditions as previously reported for apple (Djekic et al., 2018) and beetroot (Tomic et al., 2020).

Table 2: quantification of anthocyanin and flavonoid derivative in strawberry sample and comparison between fresh and Sc-CO_2 dried strawberry. Data are reported as mg of compound on 100g of dried material. One-way analysis of the variance (ANOVA) was performed to establish significant differences ($p < 0.05$, Turkey's post hoc test) for fresh and Sc-CO_2 dried strawberries and the significance (Yes or No) between the fresh and dried sample is reported.

tr	**[M-H]+**	**Identification**	**Fresh [mg/100g]**	**ScCO_2 dried [mg/100g]**	**Significance ($p<0.05$)**
9.22	449	cyanidin glucoside	2.11±0.32	0.92±0.63	Yes
10.2	433	pelargonidin glucoside	29.0±3.90	26.75±6.10	No
10.7	579	pelargonidin rutinoside	14.5±8.88	16.90±8.78	No
10.9	331	malvidin	1.22±0.11	0.83±0.52	Yes
12.4	465	petunidin derivate	0.66±0.14	0.33±0.05	Yes
12.8	519	pelargonidin malonyl glucoside	17.44±5.44	15.95±9.95	No
		Total content	64.9	61.68	-
tr	**[M-H]-**	**Identification**	**Fresh mg/100g**	**ScCO_2 dried [mg/100g]**	**Significance ($p<0.05$)**
6.6	431	apigenin hexoside	18.0±1.5	15.70±2.10	Yes
7.8	401	apigenin pentoside	0.15±0.10	0.16±0.14	No
7.9	473	apigenin derivative	6.33±2.56	4.32±2.74	No
9.8	477	quercetin glucuronide	6.33±0.93	2.97±0.83	Yes
10.1	593	kaempferol rutinoside	1.22±0.21	1.16±0.01	No
10.7	461	kaempferol glucuronide	2.10±0.33	1.54±0.25	Yes
		Total content	34.1	25.85	-

3.5 Sensory test

In order to prove the acceptance of the treated products, a proof of concept consumer test was carried out between strawberries treated with three different drying techniques. Consumers' acceptability by the ratings for appearance, flavour, taste, texture and overall liking of strawberries subjected to different drying treatments are reported in Table S3. From univariate analysis of variance (ANOVA), no differences ($P > 0.05$) were identified on appearance, flavour, taste and texture between the three drying techniques. Significant differences ($P < 0.05$) were identified on overall liking ratings, whereas ScCO_2 dried strawberries showed slightly lower ratings than air-dried strawberries and freeze-dried strawberries. In the previous study conducted on red-bell pepper (Zambon et al., 2019), higher

preference was given to the freeze-dried product, followed by Sc-CO_2 and last air-dried samples. In the case of apple slices (Tomic et al., 2019), no significant difference in the acceptance of the product dried with the three drying techniques was found, confirming the results achieved with strawberries. In a study conducted on beetroot Tomic et al. reported a higher acceptance by consumers for the $ScCO_2$ dried product compared with the freeze dying ones (Tomic et al., 2020). This study was performed on a small scale because only a few amounts of sample could be dried with a lab-scale reactor, therefore our finding should be confirmed with products dried with a bigger size plant thus including more people in the test. However, these preliminary results are promising to confirm the good quality and acceptance of the product dried with $ScCO_2$ technique as previously reported.

4 Conclusions

This work explored the use of $ScCO_2$ for the drying and simultaneous inactivation of enzymes and pathogens in strawberry slices. At 40°C, an efficient drying (WL>92%, a_w<0.34) and a complete enzymatic (POD and PPO activity) inactivation can be achieved using several combinations of pressure, time and flow rate. *Salmonella enterica, Listeria monocytogenes* and *Escherichia coli* O157:H7 were reduced up to 6 log CFU/g in the dried products. The total Vitamin C and anthocyanin content was maintained after the drying, while flavonoid one was slightly reduced. This result suggests the preservation of the high nutritional value of the original fresh samples. The preliminary sensory test showed good acceptance by the consumers. Overall, results are promising for the development of a sustainable green drying technology for the obtaining of safe and healthy products. Additional studies should be performed to demonstrate the stability of the dried product overtime and a larger consumer's acceptance by trained or semi-trained panels. Moreover, economic and financial analysis should be considered to foster the process scale up of the technology, comparing the energy consumption with other conventional drying technology like freeze-drying and air-drying.

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