Microbial inactivation efficiency of supercritical CO_2 drying process

Alessandro Zambon[a], Filippo Michelino[a], Siméon Bourdoux[b], Frank Devlieghere[b], Stefania Sut[c], Stefano Dall'Acqua[c], Andreja Rajkovic[b], Sara Spilimbergo[a]*

[a]Department of Industrial Engineering, University of Padova, via Marzolo 9, 35131 Padova, Italy

[b]Department of Food Technology, Safety and Health, Faculty of Bioscience Engineering, Coupure 653, B-9000, Ghent, Ghent University

[c]Department of Pharmaceutical and Pharmacological Science, University of Padova, Via Marzolo 5, 35131 Padova, Italy

*Corresponding author: sara.spilimbergo@unipd.it. FAX +39 049 8275461

Abstract

Conventional drying of spices, as hot air treatment, often needs an additional downstream inactivation step to decrease the microbial load of the dried product and improve its microbial safety and microbial quality.

In this regard, the present work explored the possibility to dry and decontaminate food in a single step by the use of supercritical carbon dioxide ($scCO_2$) as a drying agent. A case study was focused on the drying of herbs and the antimicrobial effects were evaluated on the naturally present microbiota. For this purpose, experiments were carried out on coriander leaves using a high pressure vessel at 10 MPa, at two different temperatures (40 and 50 °C) with drying time of 0 and 150 min

to establish the influence of each parameter on the microbial inactivation.

Yeasts and molds appeared to be the least resistant to $scCO_2$ as they could never be detected after the treatment (< 2 log CFU/g). Mesophilic bacteria were also significantly reduced, up to 4 log CFU/g, but remained above the limit of quantification. The quality of the dried product was comparable with the quality of air dried samples in terms of phenolic constituents. Overall, the results indicated that $scCO_2$ drying was a promising green drying technique combining both drying and microbial inactivation in a single step with a relevant impact on safety and costs.

Key words: supercritical drying; carbon dioxide; microorganism inactivation; coriander

1. Introduction

Lowering the water activity of food is among the oldest and the most common forms of microbial food preservation[1]. Although microbial growth is prevented or retarded in dry products, a sufficient number of surviving pathogenic microorganisms may still pose a threat to the consumer, especially with ready-to-eat foods or foods that can be insufficiently cooked. Indeed, once in a desiccated state, microbial growth is inhibited, but vegetative cells and spores can remain viable for months [2] featuring quick spoilage when rehydrated and increasing the risk of foodborne illness [3].

Conventional food drying operations may expose the product to a wide range of microbial contaminants and (cross)-contamination routes during post-harvest handling that add to the prior pre-harvest contamination [4,5]. An additional antimicrobial treatment (e.g. irradiation) is currently often applied to ensure microbial safety on spices, but increase the total cost of food production and may have negative perception with the consumer. For other dry products, such as dried vegetables and fruits, an additional decontamination step is not usually required but would be desirable to reduce the incidence of microbial contamination during processing and storage.

It has been shown that high pressure carbon dioxide in the supercritical phase ($scCO_2$) is a green solvent with the potential to be applied in several fields of food processing [6].

Concerning food drying, $scCO_2$ has been successfully applied on basil [7], carrot [8], agar gels [9], apple slabs [10] and recently on mango and persimmon [11], exhibiting great potential as an alternative drying agent. It is worth noting that $scCO_2$ has bactericidal properties [12, 13], making it an attractive alternative non-thermal technique to classic pasteurization. The effect of $scCO_2$ on microorganisms' inactivation has been investigated for both liquid [14] and solid [15] foodstuffs, but so far no study had been undertaken to investigate the effect of $scCO_2$ on microorganisms' survival during the drying process.

The present work focused on the inactivation of spoilage microorganisms on coriander (*Coriandrum sativum* L.) leaves, during $scCO_2$ drying. The inactivation potential was verified for the natural microbiota with most relevant microbial parameters comprising mesophilic bacteria, mesophilic bacterial spores and yeasts and molds. Experiments were carried out on lab scale using a semi-continuous reactor at 10 MPa, evaluating the effect of temperature (40 and 50 °C) and drying time of 0 and 150 min. A comparison of the microbial inactivation with hot air-drying (80 °C in oven) was made to compare the inactivation of $scCO_2$ drying versus conventional drying. Furthermore, to demonstrate the quality of the $scCO_2$-dried coriander, the phenolic constituents were also analyzed.

2. Materials and methods

2.1 Sample preparation

Coriander (*C. sativum* L.) was purchased from a local market in Padua (Italy), stored at 4 °C and treated within 3 days after the purchase. Experiments were carried out with coriander leaves, selected by similar dimension and color. For each trial 1 g ± 0.1 g of product was inserted inside a metallic net basket. The basket was sterilized before each operation by immersion in pure ethanol (Sigma Aldrich, 99.8%) followed by burning with a Bunsen flame.

2.2 Lab scale semi-continuous reactor

The high pressure carbon dioxide apparatus consists of a sapphire high pressure visualization cell (Separex S.A.S., Champigneulles, France) with an internal volume of 50 mL designed to withstand up to 400 bar and 100 °C. The plant includes a CO_2 tank, kept at room temperature, a chiller reservoir (M418-BC MPM Instruments, Milan, Italy), a HPLC pump (307 Gilson, Milan, Italy), and a thermostatic water bath (ME-Julabo, Seelbach, Germany) to keep the vessel at the desired temperature. A micrometric valve is installed on the outlet line to control the depressurization of the vessel. This valve is maintained closed during the pressurization of the vessel. Once the operative pressure was reached inside the vessel, it was partially open to allow the drying of the product

at a constant flow rate. Further details of the reactor were reported elsewhere [16].

2.3 scCO$_2$ drying procedure

Before each experiment, the vessel was cleaned with pure ethanol and washed with sterile distilled water. The vessel was then flushed with CO_2 for few minutes in order to remove water residues. Coriander leaves were inserted inside the sample holder and placed into the vessel.

The scCO$_2$ treatment consists in three main phases i) pressurization; ii) drying and iii) depressurization. Pressurization up to 100 bar was reached from 60 bar (the pressure inside the CO_2 tank) within 20 minutes using 2 mL/min flow rate that corresponds to a rate of 2 bar/min. Experiments were carried out at the final pressure of 100 bar at 40 or 50 °C. When drying occurred, CO_2 flow rate was set at the maximum pump flow rate (23 mL/min) up to 150 min. Depressurization was achieved in 40 min as reported in Table 1. At the end of the process the coriander was removed from the dryer and placed in a sterile tube for further analysis. Water activity was measured (Hygropalm Rotronic, Bassersdorf, Switzerland) at the end of the process.

The mass loss was calculated as

$$= 1 - \frac{\quad}{\quad} * 100\% \qquad 2.1$$

where and indicate the mass of the sample after and before the process, respectively.

2.4 Oven drying procedure

Coriander was also dried using a static oven G-Therm (F.lli Galli, Milan, Italy) with a digital PID controller at 80 °C for 5 h at atmospheric pressure. The samples were placed inside a sterile petri dish, covered with aluminum foil. The relative humidity was not controlled during the experiment. $scCO_2$-dried and air-dried samples were compared in terms of microbial and chemical characteristics.

2.5 Microbial analyses

Mesophilic bacteria, mesophilic bacterial spore, and yeasts and molds counts were assessed before and after the treatment by means of the standard plate count techniques as previously reported [17,18]. Phosphate buffer saline (Sigma Aldrich, Milan, Italy) was used for serial dilution with a weight ratio of 1:10. Mesophilic bacteria and spores were cultured using total plate count agar (Microbial Diagnostici, Catania, Italy) at 30°C within pour plate, while yeasts and molds were cultured with DRBC agar (Bitec S.r.l., Grosseto, Italy) supplemented with chloramphenicol at 22°C within spread plate. For the enumeration of mesophilic spores, the first dilution tubes were inserted in a thermostatic bath at 80°C for 10 min before

plating. The incubation time for mesophilic bacteria and spores was 72 h, while 72-120 h for yeast and molds.

The enumeration was referred to the weight of initial fresh product and expressed in CFU/g. Reductions are expressed as $\log_{10}(N_0/N)$ where N_0 was the number of initial microorganisms in the untreated sample and N the number of viable microorganisms after the treatment, in CFU/g of fresh product.

Each experimental condition was repeated at least in triplicate and the results were calculated as mean value. Error bars in the graphs shows standard deviations. The Limit of Quantification (LOQ) was 200 CFU/g for the mesophilic bacteria and mesophilic bacterial spores, 2000 CFU/g for yeast and molds while the Limit of Detection (LOD) was < 10 CFU/g < 100 CFU/g respectively. Results were analyzed with one-way analysis of variance to compare effects of the different treatments with significance at $\alpha = 0.05$.

2.6 Chemical analyses

The determination of phenolic content was performed as described by Iannarelli and others (2017) [23]. Briefly dried leaves were grinded using a IKA mod A11. Grinded material (100 mg) was weighted and used for the extraction. The extraction procedure took place for 5 min in a ultrasound bath by adding 5 mL of extraction solvent (methanol (Scharlab, Milano, Italy) and water 50% v/v). The surnatant was collected

and the extraction was repeated twice on the residue material. The extracted volume was then adjusted to 25 mL in a volumetric flask. The final volume was used for HPLC-DAD-MS analysis. For the analysis, a chromatograph with diode array detector (Agilent 1260 series) connected to an Ion Trap Mass Spectrometer MS500 (Varian, Palo Alto, CA, USA) was used. The ion source was Electrospray ionization (ESI) operating in negative ion mode. Separations were obtained on an Agilent Eclipse plus C-18 2.1 x 150 mm (3.5 μm). For the analysis of polar constituents the mobile phases were acetonitrile (A) and water with 0.1% of formic acid (B). The gradient started with 10% A and in 20 min reached 54% of A, then in 23 min 100% A. Re-equilibration time was 8 min and the flow rate was 200 mL/min. ESI parameters were: capillary voltage 70 V, needle voltage 5000 V, RF loading 100%, nebulizing gas pressure 20 psi (N_2), drying gas pressure 15 psi, drying gas temperature 350 °C. Mass range was 50-2000 Da. Fragmentation patterns of eluted compounds were obtained using the turbo detection data scanning (TDDS®) function of the instrument. For identification of constituents fragmentation spectra were compared with the literature [24,25] and reference compounds were used when available. Gallic acid, rutin and chlorogenic acid were used as reference compounds for the quantification. Calibration curves were obtained in the range 0.5–20 μg/mL at four different concentrations. Gallic acid

calibration curve was (area Y vs. concentration X) Y = 1335,2 X + 43 (at 280nm), rutin calibration curve was (area Y vs. concentration X) Y = 244,2 X + 51 (at 280 nm), chlorogenic acid one was Y = 325,2 X + 25 (at 330 nm). Measurements were obtained in triplicate and data expressed as average and standard deviation.

3. Results and discussion

The initial microbial contamination for mesophilic bacteria, mesophilic bacterial spores, and yeasts and molds was 7.67 ± 0.32, 3.68 ± 0.39 and 5.69 ± 0.60 log CFU/g of fresh coriander, respectively. As the coriander was heavily contaminated with naturally occurring microorganisms as reported elsewhere [28], it represents a good case product for the evaluation of microbial inactivation by $scCO_2$. When $scCO_2$ is used as drying agent, it is continuously applied to solid matrices causing water extraction and therefore reducing the water content and the water activity of the product. Table 2 reports the mass loss and the water activity at different drying times: as expected, longer drying times led to higher water loss. After 90 min of drying the water loss reached a plateau value of 82% and no further weight reduction could be observed. Similar results were obtained for water activity that reached a stable value of 0.42 after 90 min of drying. Since the moisture content of fresh coriander is about 87.9% [26] we were not able to extract all the moisture within 150 minutes of supercritical drying. This

can be further improved by introducing CO_2 recirculation systems with adequate absorption materials [7,27]. Further optimization could be made by using larger drying chambers. Namely, the small dimension of the drying chambers used in this trial influenced the variability of the final water activity since the leaves might have been affected by squeezing during their insertion into the chamber. However, the main objective of the present study was the inactivation capacity on microorganisms and not the drying efficiency. Hence, this fact did not represent a significant issue for a given experimental setup.

Figure 1 shows the microbial reduction in terms of mesophilic bacteria, mesophilic bacterial spores, and yeasts and molds at 40 and 50 °C after the pressurization and depressurization (no drying) and 150 min of drying. For all the experiments, after the process, yeasts and molds were reduced below the detection limit, confirming that these microorganisms are particularly sensitive to $scCO_2$ treatment. For the mesophilic species, a 4 log CFU/g reduction was reached for bacteria, while a reduction lower than 1 log CFU/g was observed for spores, independent of the process conditions. Spores are very resilient to inactivation with $scCO_2$ suggesting that additional treatments are needed in combination with (or after) the $scCO_2$ process [21.22].

Data obtained highlight that the constant pressure during 150 min of drying did not introduce an additional inactivation, suggesting that the main inactivation occurs during the pressurization and depressurization phases. This might be a consequence of water losses, as microbial metabolism is inhibited below a certain value of water activity [2,19] and it could affect the inactivation kinetic during the drying phase. Otherwise it simply means that a complete inactivation of $scCO_2$ sensitive microorganism was already reached during the pressurization step, which has been already proven to affect microbial inactivation during scCO2 process [20]. Pressurization and depressurization are certainly important phases that cannot always be chosen arbitrarily because they highly depend on the volume of the vessels, the pump capacity and overall equipment design. Investigation on these phases is also very important for the process scale up, since lab scale apparatus allows fast pressurization and depressurization step that are not guaranteed upon scale up of the process.

Since $scCO_2$ inactivation is influenced by the temperature [17, 21], experiments were performed at 40 and 50 °C as previously reported for carrots [8]. Inactivation was not significantly different for both mesophilic bacteria and mesophilic spores under the conditions tested. This result confirmed that the vegetative bacteria, which are sensitive to $scCO_2$, were already inactivated at 40 °C during the pressurization (and

depressurization) phases. Probably a greater inactivation could be reached at higher temperatures, but this is not appealing for food industry and the consumers, as it would induce degradation of nutritional and thermolabile compounds in the products.

In order to confirm the potential of $scCO_2$ drying against traditional drying methods, a proof of concept experiment was performed with coriander air-dried at 80 °C in a drying oven. As shown in Figure 2 the treatment at 80 °C for 5h (corresponding to a water loss of 83.0 ± 0.4 % and water activity of 0.40±0.01) resulted in a lower inactivation for mesophilic bacteria, mesophilic bacterial spores, and yeast and molds compared to the $scCO_2$ treatment at 40°C.

Regarding the quality in terms of retention of phytochemical constituents HPLC-MS analysis showed that several flavonol derivatives, mainly quercetin and kaempferol glycosides, as well as quinic and chlorogenic acid were identified during extract fingerprinting (Table 3). The data indicated that $scCO_2$ dried product contains identified coriander phytoconstituents. The amount of polyphenols classes is reported in Table 4, comparing the hot air dried sample with the $scCO_2$ dried one. There were no significant differences in the quantitative analysis of phytoconstituents, meaning that both techniques are comparable for the retention of polyphenols.

4 Conclusions

The experiments performed on a lab-scale equipment with coriander leaves showed that $scCO_2$ drying alone is an efficient technology to reduce the natural contamination on the dried product. Indeed, up to 4 log reduction was achieved for mesophilic bacteria while yeasts and molds were completely inactivated. However the most resistant bacteria and spores were still detected after the process, suggesting that a complete inactivation might require additional combined technologies.

The reductions at mild temperature condition (40 °C) were higher than those reached with conventional air-drying at higher temperature (80°C). This highlights the great potential of this innovative process when the higher temperature should be avoided. This study also demonstrated that inactivation was not influenced by the drying time confirming that inactivation of microorganisms present on coriander is mainly achieved during the pressurization and depressurization phases. However, additional experiments should be carried out to clarify the mechanism of inactivation of microorganisms and have a deeper understanding of the effect of pressurization and depressurization time/rates on inactivation efficiency. Further analyses are also needed to investigate the effect of $scCO_2$ drying on the inactivation of specific pathogens known to be resistant to pasteurization in a desiccated state, such as *Salmonella*,

$ScCO_2$ drying is a promising technology that combines both drying and microorganism inactivation in a single step, making it attractive for industrial applications, as it is in the same time cost effective, safe and able to preserve a good quality of the product.

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Table 1 - Depressurization rate

time (min)	Pressure (bar)
0	100
5	70
10	40
15	30
20	22
25	15
30	10
35	6
40	1

Table 2 – Percentage of mass loss and water activity measured at different drying time for the Sc-CO2 drying process at 100 bar and 40°C

time (min)	**Mass loss [%]**	**Water activity [a_w]**
0	-	0.96±0.01
15	54±11	0.95±0.02
30	67±10	0.80±0.04
45	77±4	0.60±0.09
60	80±3	0.46±0.10
90	82±4	0.42±0.08
150	82±2	0.42±0.11

Table 3 - main phytoconstituents identified in the dried samples of C.sativum in both Sc-CO2 and Oven treatment. Compounds indicated with * have been also compared with authentic standard.

Retention Time	Compound	[M-H]	Fragments
2.6	Quinic Acid*	191	111- 67
4.3	Chlorogenic acid *	353	191
5.0	Dimethoxycinnamoyl hexoside	369	189-127
5.6	Rutin	609	301
6.3	Quercetin 3-*O*-glucuronide*	477	301
6.8	Quercetin 3-O-glucoside*	463	301
9.2	Kaempferol-rutinoside	593	285
12.5	Quercetina acetil hexoside	505	301
13.6	Kaempferol-glucuronide	461	285

Table 4 – quantification of the main phytoconstituents identified in the dried samples of *C.sativum* related to the Sc-CO2 drying (40°C, 100bar, 150min) and oven dried (80°C, 5h).

Sample	**Flavonoid as rutin [mg/g]**	**Cholorgenic acid derivatives [mg/g]**	**Gallic acid derivatives [mg/g]**
Oven	2.28 ± 0.04	2.95 ±0.01	2.82 ±0.02
Sc-CO_2	2.31 ± 0.01	2.65 ±0.01	2.14 ±0.02

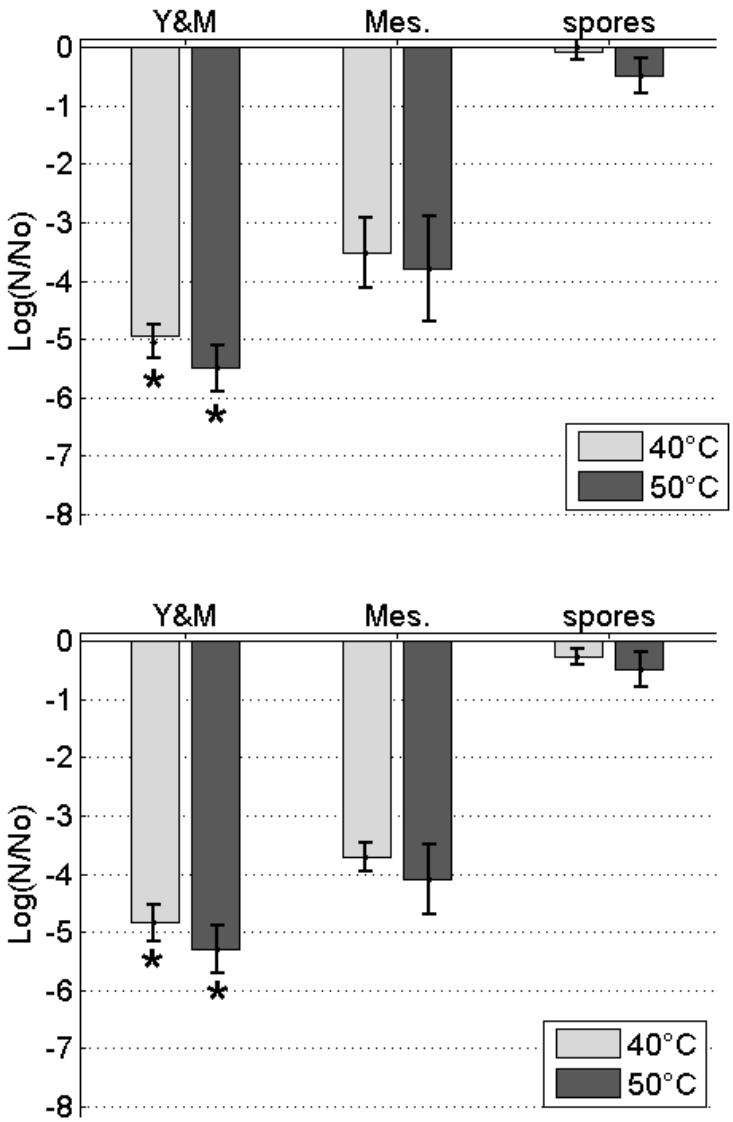

Figure 1 - Inactivation of mesophilic bacteria, mesophilic spores and yeast and mold as a function of temperature (40 and 50°C) after 150 min (Top) and 0 min (Bottom) drying time; * indicates below the detection limit.

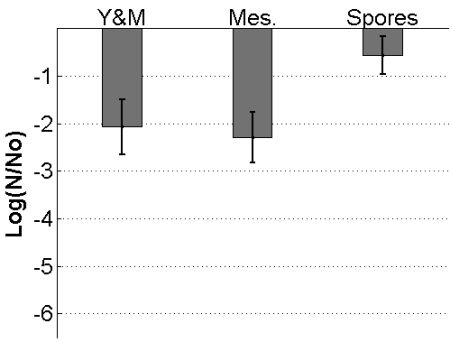

Figure 2 - Inactivation of mesophilic bacteria, mesophilic spores and yeast and mold after thermal drying (80°C).