International Journal of New Technology and Research (IJNTR)
ISSN:2454-4116, Volume-2, Issue-2, February 2016 Pages 71-77

Application of Supercritical Carbon Dioxide for the Preservation of Fresh-Like Carrot Juice

Gabriele Di Giacomo, Francesca Scimia, Luca Taglieri

Abstract*—Supercritical carbon dioxide has been suggested and is under development for non-thermal pasteurization of food and perishable beverages by virtue of its effectiveness in microflora and enzyme inactivation. In comparison to competitive technologies, it is particularly attractive at industrial scale since the liquids can be continuously processed with recirculating carbon dioxide. One of the most attracting application is the preservation of increasingly interesting fresh-like juices of fruits and vegetables, particularly the low acidic ones like carrot juice, for which the shelf life can be enhanced of about one order of magnitude (from two or three days to about three weeks or more) while maintaining the main characteristics of the fresh squeezed equivalent. Most of this exciting result is directly related to the unique reversible acidification action of this technology. In this study it has been found that Pectin methylesterase and Peroxidase are actually the solely responsible for cloud stability disappearance and off-flavor, browning and other enzymatic-related undesirable changes occurring during the storage of fresh-like carrot juice, under refrigerated conditions. In fact, the level of Polyphenol oxidase activity is very low and the level of fat in this juice is also very low, thereby nullifying the potential negative action of those enzymes that may cause adverse effects by acting on fats. In this contest, it has been found, using a semi-continuous laboratory apparatus, that a micro-bubbled stream of supercritical carbon dioxide at 313 K, 25 MPa and juice/CO_2 ratio (w/w) equal to 0.33, significantly inactivated the labile isoform fraction of PME (above 70%) and of POD (almost completely) in about one hour. The values of the stable isoform dimensionless activity of the above mentioned enzymes and of the inactivation constant were calculating by fitting experimental data with a plain, semi-empirical equation obtaining the following results: 0.0894 and 0.00345 min^{-1} for PME, and 0.1072 and 0.04035 min^{-1} for POD.

***Index Terms*—Carrot, Fresh-Like Carrot Juice, Supercritical CO_2 Treatment,Enzyme Activity, Cloudiness Stabilization.**

I. Introduction

A proper diet implies the daily consumption of fruits and vegetables (F&V); the quantity depends on several factors and can be estimated, knowing the nutrient composition of the food, using the dietary reference intakes (DRIs) tables or similar methods. Traditionally, F&V were consumed as fresh, after simple homemade operations: elimination of roots, seeds and other uneatable parts, washing, peeling, cutting, and others. However, in developed countries, the use of ready-to-use F&V products became very popular as consequence of lifestyle: the ready-to-eat minimally processed F&V (MPF)/fresh like F&V, are prepared at industrial scale, packed, stored at about 4 °C and distributed. It is worth to underline that the shelf life of these MPF is significantly higher than that of the corresponding fresh product after homemade preparation for eating. An alternative way to supply the body with the necessary nutrients is to drink the juice obtained from the F&V. In the most general sense, juice is defined as the extractable fluid contents of cells or tissue [1], while Codex Alimentarius [2] defines juice as "unfermented but fermentable, intended for direct consumption, obtained by mechanical process from sound, ripe plant material, preserved exclusively by physical means." Traditionally industrially made F&V juices are strongly concentrated and pasteurized in order to insure safety and to reduce handling and transportation costs. However, organoleptic, nutritional and functional properties of the F&V reconstituted are very far from that of the corresponding fresh squeezed [3]. On the other hand, F&V juices are very perishable with a shelf life under refrigerated conditions of 2 to 4 days. Very recently the F&V marked registered an unpredictable interest for "cold pressed" juices which are a kind of minimally processed beverages (MPB). Actually this product, characterized by a shelf life of about 3 or even 4 weeks under refrigerated conditions, is a single strength F&V juice pasteurized by a non thermal technology which implies the use of very high hydrostatic pressure in the range from 400 to 1000 MPa (HHP). In the technical and scientific literature there are a lot of information regarding the application of HHP to a variety of food and beverages [4], [5], [6], [7], [8] with special emphasis on the preservation of thermo-labile useful compounds. Anyway, although the results are often conflicting, HHP is usually considered better than thermal pasteurization and consequently became very popular in the food industry [9], [10]. However, it can only be applied in the batch mode using very costly apparatus while, for liquid foods and beverages, one is naturally led to imagine a continuous process, possibly under operating conditions less drastic. Therefore, a number of non-thermal pasteurization technologies for the food and beverages industry were proposed and are currently under investigation/development [11], [12], [13], [14], [15[, [16]. Among these, the one that uses supercritical carbon dioxide (HPCD) in a range of pressure between 10 to 40 MPa appears very interesting for continuous non thermal pasteurization of single strength F&V juices, aiming to obtain a significant extended shelf life (ESL), while preserving most of the nutritional, sensorial, and functional properties of the raw extracts [17], [18], [19]. The purpose of this research is to

Gabriele Di Giacomo, Department of Industrial and Information Engineering and of Economics (DIIIE), University of L'Aquila, Italy
Francesca Scimia, Department of Industrial and Information Engineering and of Economics (DIIIE), University of L'Aquila, Italy,
Luca Taglieri, Department of Industrial and Information Engineering and of Economics (DIIIE), University of L'Aquila, Italy,

contribute to show the technical feasibility of HPCD for the industrial production of single strength, minimally processed, carrot juice (CJ) to be commercialized as safe and high quality fresh-like products. The first reason for focalize on CJ is the consequence of the "familiarity" of the authors with carrots and derived products [20], [21], [22] with the obvious consequence that a very rich baggage of knowledge concerning cultivation, harvesting, composition, up-stream of the raw material, products derivable from it, technologies, nutritional and functional properties, and other information may be used for the purposes of this work. Moreover, carrot is one of the top ten vegetable cultivated in huge amount almost all around the world and is rich in bioactive compounds like carotenoids with appreciable level of several other functional component having significant health-promoting properties [23], [24], [25], [26]. Some important preliminary considerations and results of this study were already anticipated at Food to Life, IV International Conference on Foodomics [27].

II. Experimental Section

A. HPCD Pasteurization

CJ treatment was performed with a semi-continuous HPCD micro-bubbles system [28], [29] schematically shown in Figure 1. The AISI 329 stainless still reactor with a volume of 250 ml (NOVA-WERKE, Zurich, Switzerland) was designed to withstand a pressure of 70 MPa, at 733 K (Figure 2). A weighted amount (190-200 ml) of de-aerated CJ was introduced into the reactor that was previously warmed at 313 K by circulating the water from the thermostatic bath in the reactor jacket. Then the reactor was closed and sealed to avoid leaking and the carbon dioxide stream was continuously fed, as refrigerated liquid, at the desired pressure using a membrane metering pump (MILTON ROY: rate 0-3 Kg/h, P_{max} = 35 MPa). This allows one to obtain a good and stable regulation of the rate of the stream, while avoiding cavitation. A mass-flow-meter (MICROMOTION, D6) located at the exit of the expansion vessel, was used to measure and to detect continuously the rate of the carbon dioxide stream; in this way it was also possible to collect the very small amount of liquid that condensate during the expansion of the carbon dioxide stream, from the beginning to the end of the treatment. For each experimental test, the rate of the carbon dioxide stream was adjusted to a value such as the ratio (w/w) of the CJ charged into the reactor to the total amount of carbon dioxide circulated from the beginning to the end of the run, is equal to 0.33. The micro-bubbles were obtained using a fritted glass disk fixed at the end of the small pipe, immersed in the liquid, used to inject the carbon dioxide into the CJ under treatment. To this purpose, it is worth to underline that the carbon dioxide flow very slowly and, consequently, it enters in the liquid as gas, although it is fed as refrigerated liquid. Moreover, a variable rpm magnetic stirring system, with adjustable speed, operated from the outside was used to enhance the contact between the liquid and the gas phase. The values of P and T inside the reactor were continuously measured and monitored by a pressure transducer (HAENNI, EOR430) and a type J thermocouple, respectively, with an accuracy of 0.15%. The feed and the extraction of the carbon dioxide stream, as well as the insertion of the thermocouple and of the pressure transducer inside the reactor were made using the three holes located on the top of the autoclave, as shown in Figures 1 and 2. At the end of each ran the reactor was slowly depressurized and suddenly opened to take the treated CJ that was collected in two sterile glass bottles and refrigerated at 275 K before the analysis. Each experimental HPCD test was made in duplicate. Abatement of the microbial charge was not quantified since, it was recently well established that, under operating conditions even less severe than those used in this study, the HPCD treatment was effective in inactivating indigenous microbes in low acidic juices [30], [31]. On the contrary, the identification of the tissue enzymes that actually affect negatively the cloudiness stability (one of the most important quality factor for CJ) and other sensorial properties, need to be further investigated.

B. Carrot juice-control sample (CS)

PGI (Protected Geographical Indication) carrots (Daucuscarota L.) officially named "Carote del Fucino" were taken, just harvested, from a local farmer that also operate a large industrial plant for juicing carrots and vegetables. After pretreatment (destalking, washing and peeling), carrots were stored at 275 K for a time never longer than three days until used for producing the juice by a juice extractor (MULINEX FRUTTI-PRO XL). The cloudy juice was divided in three parts, bottled, sealed and stored at 275 K; one part was used as CS, the others were used to make the HPCD experiments. The same procedure was repeated for each HPCD experimental test.

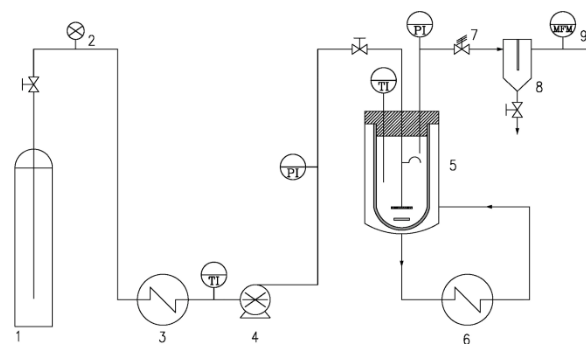


Figure 1 - Schematic diagram of HPCD apparatus: 1. CO_2 tank; 2. Pressure gauge; 3.Cooling unit; 4. High pressure pump; 5. Jacketed reactor, with CO_2 diffuser, magnetic agitator and splashguard; 6. Thermostatic bath; 7. Micrometric valve; 8. Expansion vessel; 9. Mass flow meter.

Figure 2 – Reactor details.

C. Analysis

The CS and of the two treated samples (TS) were analyzed as soon as possible (CS within max 24 h and TS within max 60 h, from juicing) in order to quantify the value of pH along with the activity of selected enzymes which can deteriorate the quality of the juice during the storage. For the carrot juice, according to the literature [32], these are the Polyphenol oxidase (PPO, EC 1.14.18.1), the Peroxidase (POD, EC 1.11.1.7) and the Pectin methylesterase or Pectinesterase (PME, EC 3.1.1.11). In fact the activity of Lipoxigenase (LOX), Lipase and Protease is not relevant since the concentration of fat in the carrot is very low (about 2% on water free-basis and much lower in the juice); for this reason these three last mentioned enzymes were not considered in this research. Before being analyzed, both the CS and the TS were filtered through 4-lyers cheesecloth.

D. Determination of pH

The pH of CS and TS was measured at 298 K with a Thermo Orion 868 pH meter (Thermo Fisher Scientific, Inc., MA, U.S.A). Values around 6.5 were always found regardless of different CS, and of treated and untreated samples, as consequence of the reversibility of the CJ acidification.

E. PME activity assay

Principle - Evaluation of carboxylic groups that are released as a result of the action of pectinesterase on pectin. Determination of pectinestase activity was carried out with autotitrator on 10 g of product to which 20 ml of pectin suspension was added. Titration occurs automatically by recording the amount of 0.1 N NaOH supplied over time, which is necessary to maintain pH at 7.5. Analysis lasted 30 min or until all pectin was hydrolyzed. Temperature was kept constantly at 303 K.

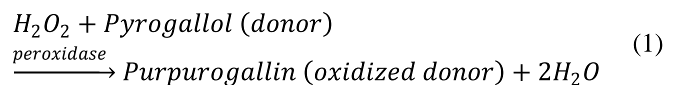
Apparatus - Automatic titrator with pH-Stat option (Metrohm).

Reagents – Ethanol 95% (A); NaCl 0.15 M (B); *Pectin solution*: to 2 g of pectin (P9135 Sigma) add 4 g sucrose, 4 ml Ethanol 95% and 200 ml of NaCl solution. Check the pH solution and adjust to pH=7.5 with NaOH 1 M and 0.1 N (C); *Sample solution:* mix 10 g of sample with 200 ml of NaCl solution. Check the pH solution and adjust to pH=7.5 with NaOH 0.1 N (D).

Calculation - Pectinesterase activity was expressed as U/g of sample(P · 0.1 · 1000/g, with P=ml NaOH/min and 0.1=Normality of NaOH used for titration), where 1 U corresponds to 1 U_{eq} of acid released by pectin in 1 min.

F. POD activity assay

Principle:



$$H_2O_2 + Pyrogallol\ (donor) \xrightarrow{peroxidase} Purpurogallin\ (oxidized\ donor) + 2H_2O \quad (1)$$

Conditions - T=293 K; pH=6; A_{420nm}; Light path=1 cm.Method - Continuous Spectrophotometric Rate Determination.Reagents -100 mM Potassium Phosphate Buffer, pH=6 at 293 K (A); 0.5% (w/w) Hydrogen Peroxide Solution, freshly prepared (B);5% Pyrogallol Solution, freshly prepared and keep from light (C);Sample solution: prepare 40 ml in Reagent A at pH=6 using 20 g of homogenized sample, filter with filter paper and then use filtered solution for enzymatic assay (D).

Procedure - Prepare a reaction cocktail, to be used for blank and for test, by pipetting the following volume (ml) of reagents into a suitable container: Deionized water (2.10); Reagent A, Buffer, (0.32); Reagent B, H_2O_2, (0.16); Reagent C, Pyrogallol, (0.32). Mix by inversion and equilibrate to 293 K. Then add 0.10 ml of Buffer and 0.10 ml of sample solution.

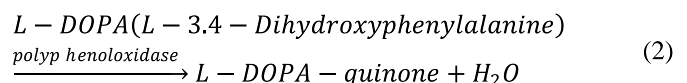
Mix again and record the increase in A_{420nm} for five minutes in order to obtain the ΔA_{420nm}/20 seconds using the maximum linear rate for both the test and blank.

Calculation-Units/ml enzyme=(ΔA_{420nm}/20s test-ΔA_{420nm}/20s blank)·3·fd/(12·0.1), with fd=diluition factor; 3=Volume of assay (ml); 12=Extinction coefficient of 1 mg/ml of Purpurogallin at 420 nm; 0.1=Volume of enzyme used (ml).

Unit Definition – One unit will form 1.0 mg of Purpurogallin from Pyrogallol in 20 s at pH 6 at 293 K.

G. PPO activity assay

Principle:



$$L - DOPA(L - 3.4 - Dihydroxyphenylalanine) \xrightarrow{polyp\ henoloxidase} L - DOPA - quinone + H_2O \quad (2)$$

Conditions - T=298 K; pH=6; A_{480nm}; Light path=1 cm.Method - Continuous Spectrophotometric Rate Determination.Chemicals:Sodium phosphate monobasic 0.2 M; di-Sodium hydrogen phosphate 0.2 M; L-DOPA; NaOH 0.1 M.Reagent A: mix 438.5 ml of sodium phosphate monobasic 0.2 M with 61.5 ml of di-Sodium hydrogen phosphate 0.2 M. Check the pH of solution and adjust to pH=6 with NaOH 0.1 M;L-DOPA solution: 20 ml made of Reagent A and 80 mg of L-DOPA;Sample solution: 50 ml made of Reagent A and 5 g of homogenized sample, filtered on filter paper.

Procedure - Prepare a reaction cocktail, to be used for blank and for test, by pipetting the following volume (ml) of reagents into a suitable container; for blank: 1 ml of Reagent A and 1 of ml L-DOPA solution; for test: 1 ml of L-DOPA solution and 1 ml of Sample solution. Mix and record the increase in A_{480nm} for five minutes in order to obtain the ΔA_{480nm}/min using the maximum linear rate for both the blank and the test.

Calculation - Units/ml enzyme = ($\Delta A_{480\,nm}$ /min test - $\Delta A_{480\,nm}$ /min blank)·fd/0.001, with fd=diluition factor.

Unit Definition – One unit will cause an increase of 0.001/min at pH 6 at 298 K in 2 ml of reaction medium containing polyphenoloxidase.

The raw experimental data for CS resulting from the analysis and those for TS resulting from the treatments and analysis, are reported in table I.

III. Fitting of Experimental Data, Results and Discussion

A. *Kinetics of PME and POD inactivation*

As can be seen in Table I, the activity of PPO in both CS and TS is always very low and, consequently, the fitting of experimental data was restricted to POD and PME.

This was done by the following equation

$$\boldsymbol{a_j = a_{sj} + (1 - a_{sj}) \cdot e^{(-k_j \cdot P_r \cdot t)} \quad j = POD\ or\ PME} \quad (3)$$

where:

$\boldsymbol{a_j}$ is the dimensionless residual activity of POD or PME in TS defined as $\boldsymbol{A_j/A_{0j}}$;

$\boldsymbol{a_{sj}}$ is the dimensionless stable isoform activity of POD or PME defined as $\boldsymbol{A_{sj}/A_{0j}}$;

$\boldsymbol{A_j}$ is the residual activity of POD or PME in TS, (U/g);

$\boldsymbol{A_{0j}}$is the activity of POD or PME in CS, (U/g);

$\boldsymbol{A_{sj}}$ is the stable isoform activity of POD or PME, (U/g);

$\boldsymbol{k_j}$ is the kinetics constant of POD or PME ($\boldsymbol{min^{-1}}$), at 313 K and CJ/CO_2 = 0.33 (w/w);

$\boldsymbol{P_r}$ is the operating reduced pressure of supercritical CO_2 stream, for each test;

$\boldsymbol{t}$ is the HPCD treatment time (min).

Equation 3 was obtained by applying the fundamental equation to the labile isoform fraction of the enzyme $\boldsymbol{j}$ and assuming a first-order kinetics [33], [34]. Furthermore, considering that at 313 K or less the influence of the temperature is negligible [35], at least for the duration of the treatment, the rate of inactivation is affected only by the reduced pressure of the carbon dioxide stream, for any fixed value of CJ/CO_2 (w/w). Unlike what normally done [36], the above mentioned influence was expressed with a plain constitutive equation given by the product between the temperature independent kinetic constant, $\boldsymbol{k_j}$ ($\boldsymbol{min^{-1}}$) and the always positive dimensionless reduced pressure $\boldsymbol{P_r}$ of the supercritical CO_2 stream.

The main motivation for this choice, instead of using the linearized Eyring equation [37], is that, unlike the HHP treatment, the effect of HPCD on microorganisms and enzymesis mainly related to the supercritical CO_2 solvent power, or density, and to its reversible solubility in the CJ, with the consequent pH lowering during the treatment.

The values of the two adjustable parameters in equation 3 (a_{sj} and k_j) were found minimizing the following equation

$$S(a_{sj}, k_j) = \sum_{i=1}^{N} \left(a_{i,j}^{exp} - a_{i,j}^{cal}\right)^2 \; j = POD\ or\ PME \quad (4)$$

where N is the number of the experimental tests.

The values of a_{sj} and k_j and their corresponding significance at 95%, were reported in Table II, along with the most significant statistical parameters that are usually calculated to assess the goodness of the fitting:

- the values of the Percent Average Absolute Error

$$PAAE_j = 100 \cdot (1/N) \sum_{i=1}^{N} \left(\left|a_{i,j}^{exp} - a_{i,j}^{cal}\right| / a_{i,j}^{exp}\right) \quad j = POD\ or\ PME \quad (5)$$

- the values of the Maximum Percent Error ($EPMAX_j$)
- the values of the bias factor [38]

$$B_{f,j} = 10^{(1/N)\sum_{i=1}^{N} log\left(a_{i,j}^{cal}/a_{i,j}^{exp}\right)} j = POD\ or\ PME \quad (6)$$

- the values of the accuracy factor [39]

$$A_{f,j} = 10^{(1/N)\sum_{i=1}^{N} \left|log\left(a_{i,j}^{cal}/a_{i,j}^{exp}\right)\right|} \; j = POD\ or\ PME \quad (7)$$

- the values of the coefficient of multiple correlation

$$R = \sqrt{\sum_{i=1}^{N} \left(a_j^{cal} - \bar{a}_j\right)^2 / \sum_{i=1}^{N} \left(a_j^{exp} - \bar{a}_j\right)^2} \quad j = POD\ or\ PME;\ \bar{a}_j = \sum_{i=1}^{N} a_j^{exp}/N \quad (8)$$

B. *Results and discussion*

Table III and Figure 3 show, in more details, a comparison between the measured dimensionless residual activity for POD and PME and the corresponding calculated value, for each experimental test.

Table I - Raw experimental results, at 313 K (CS: Control Sample; TS: Treated Sample).

test	sample	P [bar]	treat. time [min]	activity [U/g]		
				PME	POD	PPO
1	CS	-	-	-	0.319	-
	TS	100	40	-	0.067	-
2	CS	-	-	0.690	0.310	-
	TS	100	45	0.580	0.057	-
3	CS	-	-	0.780	-	-
	TS	150	40	0.610	-	-
4	CS	-	-	-	0.219	< 10
	TS	150	70	-	0.029	< 10
5	CS	-	-	0.920	-	-
	TS	150	75	0.600	-	-
6	CS	-	-	-	0.250	17
	TS	150	80	-	0.029	< 10
7	CS	-	-	-	0.383	-
	TS	150	90	-	0.030	-
						-
8	CS	-	-	1.140	0.427	-
	TS	200	75	0.590	0.048	-
9	CS	-	-	-	-	18
	TS	200	90	-	-	< 10
10	CS	-	-	-	0.154	22
	TS	200	110	-	0.016	< 10
11	CS	-	-	0.800	-	-
	TS	250	60	0.410	-	-
12	CS	-	-	-	-	< 10
	TS	250	80	-	-	< 10
13	CS	-	-	0.800	-	< 10
	TS	250	90	0.340	-	< 10

Table II – Adjustable parameters with significance at 95% and most significant statistical parameters.

Enzyme	a_s	k	*PAAE*	*EPMAX*	B_f	A_f	R
POD	0.10720±0.01314	0.04035±0.00472	10.35	37.58	1.01	1.10	0.94
PME	0.08941±0.02632	0.00345±0.00148	3.38	5.49	0.99	1.03	0.99

As can be seen, the agreement is quite good considering the complexity and the variety of the experimental measurements required to obtain a value of $a_{i,j}^{exp}$.
Finally, Figure 4 shows the behavior of POD and PME inactivation as function of the treatment time and for different value of the reduced pressure of the CO_2 stream.
It can be observed how the experimental values of the dimensionless residual activity always follow the calculated behavior in the whole range of $\boldsymbol{P_r}$ and t. Furthermore, the figure shows that the HPCD treatment, as performed in this study, is significantly more effective for the POD inactivation than for the PME inactivation. In fact, the level of inactivation of POD is about 90% after 30 min and 20 MPa while, a treatment time three times higher and a pressure of 25 MPa are required to reach a significant level of inactivation for the PME.

Table III– Experimental and calculated dimensionless residual activity for each test.

Enzyme	test	a^{exp}	a^{cal}	percent error
POD	1	0.2100	0.2074	1.25
	2	0.1787	0.1834	2.66
	4	0.1324	0.1101	16.88
	6	0.1160	0.1085	6.49
	7	0.0783	0.1078	37.58
	8	0.1124	0.1075	4.41
	10	0.1039	0.1072	3.19
PME	2	0.8406	0.8270	1.61
	3	0.7821	0.7770	0.64
	5	0.6522	0.6272	3.83
	8	0.5175	0.5406	4.46
	11	0.5125	0.5406	5.49
	13	0.4250	0.4070	4.23

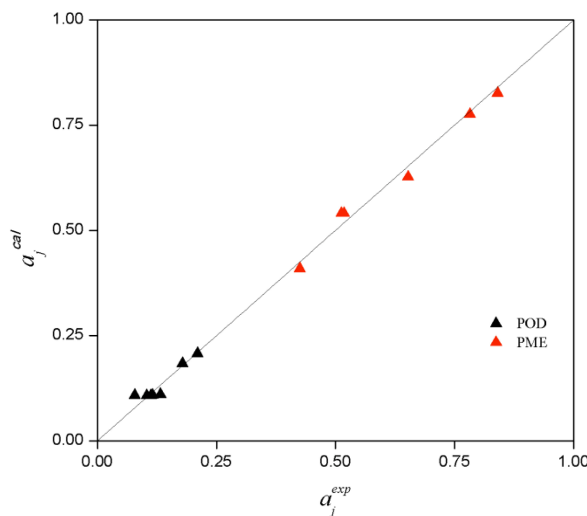


Figure 3 –Comparison between experimental and calculated dimensionless residual activity.

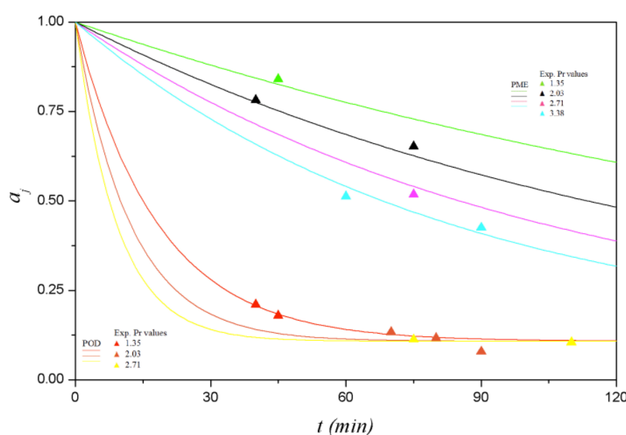


Figure 4 – Behavior of POD and PME inactivation as function of the treatment time and for different value of the reduced pressure of the CO_2 stream.

IV. Conclusions

At 25 MPa, treatment time of about one hour and carrot juice/carbon dioxide (w/w) equal to 0.33, non-thermal pasteurization of fresh squeezed carrot juice with a semi-continuous high pressure carbon dioxide micro-bubbles laboratory system is effective. In particular, under these conditions, the labile isoform fraction of Peroxidase was almost completely inactivated, while that of Pectin methylesterase was reduced of more than 70%.

This is important since these two endogenous enzymes are actually the solely responsible for cloud stability disappearance and off-flavor, browning and other enzymatic-related undesirable changes occurring during the storage, under refrigerated conditions, of fresh-like carrot juice.

Overall, supercritical carbon dioxide pasteurization at 313 K is a promising technique to obtain high quality fresh-like carrot juice as save alternative to the corresponding fresh squeezed.

Further work is required to optimize the operating process parameters using a continuous pilot plant with recirculating CO2.

Acknowledgment

We thank the national ministry of the university (MIUR) for the financial support and all the people that collaborated to perform the laboratory tests, and did the analysis.

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