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ANALYSIS OF THE EFFECTIVENESS OF ULTRA-HIGH PRESSURE HOMOGENISATION, SHORT-WAVE ULTRAVIOLET RADIATION AND THEIR COMBINATION ON THE HYGIENIZATION OF APPLE JUICE AND THEIR EFFECT ON THE QUALITY AND NUTRITIONAL ASPECTS.

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HACEN CONSTAR que JEZER NOÉ SAUCEDA GÁLVEZ ha realizado, bajo nuestra dirección, en el área de Higiene de los Alimentos de la Universitat Autònoma de Barcelona (UAB), el trabajo titulado "*Analysis of the effectiveness of ultra-high pressure homogenisation, short-wave ultraviolet radiation and their combination on the hygienization of apple juice and their effect on the quality and nutritional aspects*" que presenta para optar al grado de Doctor en Ciencia y Tecnología de los Alimentos.

Y para que así conste firmamos el presente documento en:

Bellaterra (Cerdanyola del Vallès). Septiembre de 2020

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TO BE CONTINUED...

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Summary

This work comprises the use of two different technologies for the hygienization of apple juices. Those technologies were the ultra-high pressure homogenisation (UHPH) and short-wave ultraviolet radiation (UV-C).

UHPH was applied with pressures of 100, 200 and 300 MPa, and UV-C treatments were performed at different doses between 1.8 – 200 J/mL, sometimes in more than one pass through the system (only UV-C) or combined with different inlet temperatures (InT). The two technologies were tested in order to elucidate their microbicidal capacities in different types of apple juices (clear and cloudy, with or without the addition of ascorbic acid or chia seed extract) and in other matrices adjusted to simulate some of the interferences that apple juice can oppose to UV-C like the absorption coefficient at 254 nm (α_{254}) and turbidity. In order to validate those treatments, spores of different species of bacteria (*Bacillus subtilis*, *Alicyclobacillus acidoterrestris*, *Geobacillus stearothermophilus*) and moulds (*Aspergillus niger*, *Talaromyces macrosporus* and *Neosartorya spinosa*) were inoculated into the matrices. Shelf-life studies over the microbial stability of the juice were also performed, and for those the endemic microbiota of the product was also taken into consideration.

UHPH was effective in inactivating the endemic microbiota of the product and *A. niger* spores with pressures of 200 and 300 MPa at 20 ºC InT with inactivation rates of 4 - 5 Log respectively, while the bacterial spores *of B. subtilis* and *A. acidoterrestris* could be inactivated in more than 4 Log only after 300-MPa UHPH and at least 70 ºC of InT.

UV-C treatments were effective in the inactivation of bacterial spores inoculated in matrices where turbidity and α_{254} values were closer to 0, and when the doses and number of passes increased, lethality rates also became higher, but it varied according to the type of microorganism, being the spores of *A. niger* who showed the highest resistance against those treatments.

A combination of UHPH and UV-C had an additive effect in the inactivation of microorganisms in most of the assayed combinations, except for the fungal spores of *T. macrosporus* and *N. spinosa* where a synergistic effect between those technologies was observed.

The effect of these technologies in the physicochemical and quality aspects of cloudy apple juice were analysed as well. It was observed significant changes by the effect of the UHPH at 200 and 300 MPa in the parameters of ºBrix, colour, particle size, turbidity and in α_{254} of the product. The activity of the enzyme polyphenol oxidase was not detected after the treatment of 300 MPa, and the residual activity of pectin methylesterase was reduced in more than 70%. The antioxidant capacities of cloudy apple juice and clarified apple juice with chia extract processed by UHPH and UV-C were measured, as well as a quantitative analysis of the antioxidants profile in juice with chia extract.

A sensory analysis test was performed in order to know the preferences between juices treated by UHPH, UV-C and without treatment. Changes in their sensory attributes were detected mainly in odour and flavour of the treated juices.

Resumen

En este trabajo se ensayan dos tecnologías diferentes para la esterilización de zumos de manzana. Las tecnologías aplicadas fueron la homogeneización por presiones ultra altas (UHPH) y la radiación ultravioleta de onda corta (UV-C).

La UHPH fue aplicada con presiones de 100, 200 y 300 MPa, y los tratamientos de UV-C fueron llevados a cabo a diferentes dosis entre los 1,8 - 200 J/mL, en algunas ocasiones con más de un pase a través del sistema (únicamente UV-C) o combinados con diferentes temperaturas de entrada (InT). Ambas tecnologías fueron probadas para demostrar su capacidad microbicida en los diferentes tipos de zumo de manzana (clarificado y con fibra, con o sin la adición de ácido ascórbico o el extracto de semillas de chía) y en otras matrices que fueron ajustadas para simular algunas de las interferencias que se pueden encontrar en el zumo de manzana y que se oponen al paso de la UV-C, tales como el coeficiente de absorción a 254 nm (α_{254}) y la turbidez. Para validar esos tratamientos diferentes tipos de esporas bacterianas (*Bacillus subtilis*, *Alicyclobacillus acidoterrestris*, *Geobacillus stearothermophilus*) y fúngicas (*Aspergillus niger*, *Talaromyces macrosporus* y *Neosartorya spinosa*) fueron inoculadas en las matrices. También se realizaron seguimientos de la estabilidad microbiana del zumo durante su almacenamiento para lo que se tomó en cuenta la microbiota endémica del producto.

La UHPH fue efectiva en inactivar la microbiota endémica del producto y las esporas de *A. niger* con presiones de 200 y 300 MPa a 20ºC InT con letalidades alrededor de los 4 - 5 Log respectivamente, mientras que las esporas bacterianas de *B. subtilis* y *A. acidoterrestris* pudieron ser inactivadas en más de 4 Log únicamente con la presión de 300 MPa y al menos 70ºC de InT.

Los tratamientos de UV-C fueron efectivos en la inactivación de esporas bacterianas en matrices cuya turbidez y α_{254} eran cercanos a 0 y a medida que las dosis y número de pases se incrementaban, también la letalidad se incrementaba, pero variaba con respecto al tipo de microorganismo, siendo *A. niger* el que mostró mayor resistencia ante estos tratamientos.

Una combinación de UHPH y UV-C logró un efecto aditivo en la inactivación de microorganismos para la mayoría de las combinaciones ensayadas, excepto en

las esporas fúngicas de *T. macrosporus* y *N. spinosa* en donde se observó un efecto sinérgico entre estas tecnologías.

El efecto de estas tecnologías en los aspectos fisicoquímicos y de la calidad del zumo de manzana con fibra también fueron analizados. Se observaron cambios significativos producidos por el efecto de la UHPH a 200 y 300 MPa en los parámetros de ºBrix, color, tamaño de partícula y en la turbidez y α_{254} del producto. La actividad de la enzima polifenol oxidasa no fue detectada después del tratamiento de 300 MPa y la actividad residual de la pectin metil esterasa fue reducida en más del 70%. Se realizaron ensayos para medir la capacidad antioxidante del zumo turbio procesado por UHPH y UV-C y también en zumo clarificado de manzana adicionado con extracto de chía, así como un análisis cuantitativo del perfil de antioxidantes en este último.

Se realizaron pruebas de análisis sensorial para conocer las preferencias entre producto tratado por UHPH, UV-C y sin tratamiento. Los cambios a nivel sensorial fueron percibidos principalmente en el olor y sabor de los zumos tratados.

Resum

En aquest treball s'assagen dues tecnologies diferents per a l'esterilització de sucs de poma. Les tecnologies aplicades van ser l'homogeneïtzació per pressions ultra altes (UHPH) i la radiació ultraviolada d'ona curta (UV-C).

La UHPH va ser aplicada amb pressions de 100, 200 i 300 MPa, i els tractaments d'UV-C van ser duts a terme a diferents dosis entre els 1,8 - 200 J / ml, en algunes ocasions amb més d'un pas a través del sistema (únicament UV-C) o combinats amb diferents temperatures d'entrada (InT). Ambdues tecnologies van ser provades per demostrar la seva capacitat microbicida en els diferents tipus de suc de poma (clarificat i amb fibra, amb o sense l'addició d'àcid ascòrbic o l'extracte de llavors de chía) i en altres matrius que van ser ajustades per simular algunes de les interferències que es poden trobar en el suc de poma i que s'oposen al pas de la UV-C, com el coeficient d'absorció a 254 nm (α_{254}) i la terbolesa. Per validar aquests tractaments diferents tipus d'espores bacterianes (*Bacillus subtilis*, *Alicyclobacillus acidoterrestris*, *Bacillus stearothermophilus*) i fúngiques (*Aspergillus niger*, *Talaromyces macrosporus* i *Neosartorya spinosa*) van ser inoculades en les matrius. També es van realitzar seguiments de l'estabilitat microbiana del suc durant el seu emmagatzematge per al que es va prendre en compte la microbiota endèmica del producte.

La UHPH va ser efectiva en inactivar la microbiota endèmica del producte i les espores d'*A. niger* amb pressions de 200 i 300 MPa a 20 ºC InT amb letalitats al voltant dels 4 - 5 Log respectivament, mentre que les espores bacterianes de *B. subtilis* i *A . acidoterrestris* van poder ser inactivades en més de 4 Log únicament amb la pressió de 300 MPa i al menys 70 ºC de InT.

Els tractaments d'UV-C van ser efectius en la inactivació d'espores bacterianes en matrius quan la terbolesa i α_{254} eren propers a 0 i a mesura que les dosis i nombre de passades s'incrementaven, també la letalitat s'incrementava, però variava d'acord al tipus de microorganisme, essent *A. niger* el que va mostrar més resistència davant d'aquests tractaments.

Una combinació d'UHPH i UV-C va aconseguir un efecte additiu en la inactivació de microorganismes per a la majoria de les combinacions assajades, excepte en

les espores fúngiques de *T. macrosporus* i *N. spinosa* on es va observar un efecte sinèrgic entre aquestes tecnologies.

L'efecte d'aquestes tecnologies en els aspectes fisicoquímics i de la qualitat del suc de poma amb fibra també van ser analitzats. Es van observar canvis significatius produïts per l'efecte de la UHPH a 200 i 300 MPa en els paràmetres de ºBrix, color, mida de partícula i en la terbolesa i α_{254} del producte. L'activitat de l'enzim polifenol oxidasa no va ser detectada després del tractament de 300 MPa i l'activitat residual de la pectin metil esterasa va ser reduïda en més de 70%. Es van realitzar assaigs per mesurar la capacitat antioxidant del suc tèrbol processat per UHPH i UV-C i també en suc clarificat de poma addicionat amb l'extracte de chía, així com una anàlisi quantitativa del perfil d'antioxidants en aquest últim.

Es van realitzar proves d'anàlisi sensorial per conèixer les preferències entre producte tractat per UHPH, UV-C i sense tractament. Els canvis a nivell sensorial van ser percebuts principalment en l'olor i sabor dels sucs tractats.

Papers included in this work

The research articles this thesis is composed of were added at the end of the thesis using the following numeration:

1. Inactivation study of *Bacillus subtilis*, *Geobacillus stearothermophilus*, *Alicyclobacillus acidoterrestris* and *Aspergillus niger* spores under ultra-high pressure homogenization, UV-C light and their combination.
2. Inactivation of ascospores of *Talaromyces macrosporus* and *Neosartorya spinosa* by UV-C, UHPH and their combination in clarified apple juice.
3. Effect of single and combined UV-C and ultra-high pressure homogenisation treatments on inactivation of *Alicyclobacillus acidoterrestris* spores in apple juice.
4. Combined effects of ultra-high pressure homogenization and short-wave ultraviolet radiation on the properties of cloudy apple juice.
5. Short wave ultraviolet light (UV-C) effectiveness on bacterial spores inoculated in turbid suspensions and in cloudy apple juice.
6. Evaluation of the addition of chia seed (*Salvia hispanica*) extract in apple juice, and the impact of ultra-high pressure homogenisation, short-wave ultraviolet radiation, high-hydrostatic pressure and thermal pasteurisation on its antioxidant properties.

Table of contents

1. Introduction 1

1.1 Thermal technologies used in food industry 3

1.1.1. Ohmic heating 4

1.1.2. Microwave heating 4

1.1.3. Infrared heating 4

1.1.4. Radio-frequency heating 5

1.2. Non-thermal technologies used in food industry 5

1.2.1. High-hydrostatic pressure 5

1.2.2. Ultra-high pressure-homogenisation (UHPH) 5

1.3. Ultraviolet radiation 12

1.3.1. Types of UV-C reactors for pumpable matrices 13

1.3.2. Effect of UV-C on microorganisms 16

1.3.3. Effect of UV-C on foods 21

1.3.4. Interferences from foods against UV penetration 22

1.3.5. UV-C combined treatments with other technologies 23

1.4. Heat-, pressure- or UV-resistant microbial spores potentially present in fruit juices 24

1.4.1. *Alicyclobacillus acidoterrestris* 24

1.4.2. *Bacillus subtilis* 25

1.4.3. *Geobacillus stearothermophilus* 26

1.4.4. *Aspergillus niger* 27

1.4.5. *Talaromyces macrosporus* 27

1.4.6. *Neosartorya spinosa* 28

2. Aim of this dissertation and sampling protocol 29

2.1. Aim of this dissertation 29

2.2. Specific objectives 29

2.3. Sampling protocol 30
3. Materials and methods........ 31
3.1. UHPH equipment 31
3.1.1. Cleaning and disinfection of the UHPH equipment 31
3.2. UV-C equipment........ 31
3.2.1. UV-C Dose calculation........ 32
3.2.2. Measurement of UV-C lamp's irradiance 32
3.2.3. Reynolds number calculation........ 33
3.2.4. Cleaning and disinfection of the UV-C equipment 33
3.3. Microorganisms, spore suspension preparation and plating media........ 34
3.3.1. *Alicyclobacillus acidoterrestris* 34
3.3.2. *Bacillus subtilis* 35
3.3.3. *Geobacillus stearothermophilus*........ 35
3.3.4. *Aspergillus niger* 36
3.3.5. *Talaromyces macrosporus*........ 36
3.3.6. *Neosartorya spinosa*........ 37
3.4. Matrices 38
3.4.1. Phosphate-buffered saline (PBS) with caramel 38
3.4.2. PBS with apple fibre........ 38
3.4.3. Clarified apple juice........ 38
3.4.4. Cloudy apple juice........ 39
3.5. Physicochemical analysis 39
3.5.1. pH and ºBrix........ 39
3.5.2. Absorbance........ 39
3.5.3. Turbidity........ 39
3.5.4. Colour 39
3.5.5. Browning........ 40

3.5.6. Particle size distribution 40
3.6. Enzymatic activity 40
3.6.1. Polyphenol oxidase (PPO) 40
3.6.2. Pectin methylesterase (PME) 40
3.7. Antioxidants analysis 41
3.7.1. Total polyphenols 41
3.7.2. Antioxidant capacity 41
3.7.3. Partial characterization of phenolic compounds by HPLC 41
3.8. Statistical analysis 42
3.9. Inactivation kinetics of microorganisms 42
3.10. Sensory evaluation 43
4. Results and discussion 44
4.1. Effect of UHPH on microorganisms 44
4.2. Effect of UV-C on microorganisms 47
4.2.1. Effect of UV-C on microorganisms inoculated in PBS 47
4.2.2. Effect of UV-C on microorganisms inoculated in apple juices 49
4.3. Effect of combined UHPH and UV-C treatments on microorganisms 52
4.4. Physicochemical stabilisation in apple juices by UHPH-processing 54
5. Conclusions 58
Bibliography 60

Index of tables

Table 1. UHPH experiments performed on different microorganisms present in different food matrices. 8

Table 2. UV inactivation doses (mJ/cm^2) measured at 253.7 nm for various microbial groups 17

Table 3. UV-C effect on microorganisms in fruit juices. 18

Table 4. Absorption coefficient and light penetration at 253.7 nm of water and different liquid foods 23

Table 5. UV-C combined with different technologies in microorganisms inoculated into many types of matrices and the achieved effect of the combination of technologies. 24

Table 6. UV-C, thermal, chemical and freeze dry treatments applied to *B. subtilis* (wild type) in order to reduce 90% of the population 26

Table 7. Summary of UHPH experiments with equipment from Stansted FPG 12500 and Ypsicon model A machines, and the lethal effect on microbial spores inoculated into different types of matrices 45

Table 8. Phosphate-buffered saline inoculated with microbial spores and with modified interferences from the addition of caramel (PBSC) or from the addition of apple fibre (turbidity in NTU) treated by UV-C 49

Table 9. Clear and cloudy commercial juices were screened in their optical properties of α_{254} (cm^{-1}), turbidity (NTU), ºBrix and pH. The table also shows if the juices contained any type of additives. 50

Table 10. Cloudy apple juice's colour changes after UHPH treatments at 200 and 300 MPa, and different UV-C doses (14.3, 21.5 and 28.7 J/mL) in single or combined treatments 57

Index of figures

Figure 1. Representation of a sharp-angle homogenisation valve...................... 7

Figure 2. Ultraviolet light within the electromagnetic spectrum......................... 12

Figure 3. Annular-type UV-C reactor (Ypsicon). 15

Figure 4. Schematic of turbulent-flow UV-C reactor 16

Figure 5. Schematics of a Dean-flow UV-C reactor. 16

Figure 6. Distribution of particle sizes from apple fibre and UHPH-treated apple juices 55

Figure 7. Cloudy apple juice treated by UHPH at 200 and 300 MPa, or by UV-C at 28.7 J/mL, and compared to the non-treated product................................... 56

List of abbreviations

µL	Microlitre
µm	Micrometre
A	Absorbance
*a**	Colour angle of red to green
AIJN	International Fruit and Vegetable Juice Association
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
*b**	Colour angle of yellow to blue
CCUG	Culture Collection University Of Gothenburg
CECT	Type Culture Collection of Spain
Ci	Concentration of iodide
cm	Centimetre
d	Diameter
D	Equivalent annular diameter
DNA	Deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures
D_v	Dose in J/L
E	Dose in J/cm^2
Eq.	Equation
eV	Electronvolt
FDA	U.S. Food and Drug Administration
Fig.	Figure
FRAP	Ferric-ion Reducing Antioxidant Power
g	Gram
g	Gravity
GLM	Generalised linear model
h	Hour
HHP	High-hydrostatic pressure
HP	High pressure

HPH	High pressure homogenisation
I	Intensity
InT	Inlet temperature
J	Joule
Kg	Kilogram
KJ	Kilojoule
L	Litre
*L**	Angle of lightness
Log	Logarithm
LP	Low pressure
LPM	Low pressure mercury lamp
LPMHO	Low pressure high-output mercury lamps
m	Meter
M	Mole
min	Minute
mJ	Millijoule
mL	Millilitre
mm	Millimetre
mmol	Millimole
MPa	Megapascal
MPM	Medium pressure mercury lamp
mW	Milliwatt
NCMBI	National Collection of Industrial, Food and Marine Bacteria (U.K.)
NCYC	National Collection of Yeast Cultures
NET	Number of entrances in the tunnel
nm	Nanometre
NTU	Nephelometric turbidity unit
ºC	Celsius degree
ºF	Fahrenheit degree
P_{253nm}	Number of Joules per Einsteins of 253.7 nm photons

Pa	Pascal
PBS	Phosphate-buffered saline
PBSC	PBS with caramel
pl	Pathlength of the cuvette
PME	Pectin methylesterase
ppm	Parts per million
PPO	Polyphenol oxidase
Re	Reynolds number
RF	Radiofrequency
s	Second
t	Time
T	Temperature
UHPH	Ultra-high pressure homogenisation
UHT	Ultra-high temperature
USDA	United States Department of Agriculture
UV	Ultraviolet
UV-A	Long-wave ultraviolet radiation
UV-B	Middle-wave ultraviolet radiation
UV-C	Short -wave ultraviolet radiation
V	Velocity
VUV	Vacuum ultraviolet light
W	Watt
α_{254}	Absorption coefficient at 254 nm
ΔE	Colour difference
ε_{352nm}	Molar absorption coefficient of the triiodide at 352 nm
η	Dynamic viscosity
ρ	Density
$\emptyset$	Quantum yield

1. Introduction

As world's population keeps on growing, so it does the demand and consumption of foods including drinks. Since the year of 2002 this demand has followed an annual increase of about the 3.6%. By 2009 the production of fruit drinks, juices and nectars had reached the 6.3% of the total drinks' consumption, just below hot coffee and beer (Neves et al., 2011).

In 2016 the consumption of fruit juices and nectars in the European Union ascended to 9,299 million litres, and Spain was found to be number 4 among the top 5 countries with the largest juice consumption (AIJN European Fruit Juice Association, 2017).

It is clear that juice industry has to deal with big volume productions which later on would be divided in convenient recipients and distributed in markets in order to meet the demand. Therefore, there is a need within this process to assure that the products will remain shelf-stable, and moreover, safe to the consumer. For these purposes thermal technologies have been used for the treatment of different foods because they offer the advantage of greatly extending their shelf-life (Richardson, 2001). On the other hand, due to the presence of highly thermotolerant microorganisms and enzymes, foods must be treated with extreme temperatures in order to inactivate them, but at the same time, these treatments will adversely affect the organoleptic and nutritional properties of the product (Vicente and Machado, 2011).

It is important to maintain the organoleptic properties of foods the best as possible. The impact that they can have on their consumption is clear because people do not look for pure caloric sources such as sugar and fats, instead they find their cravings satisfied with a conjunction of odours, textures and flavours (Avena and Myers, 2015). No wonder why the slogan "from tree to bottle" is becoming a trend on internet web pages in order to give the consumer the assurance of buying a high-quality drink.

Moreover, society nowadays is gaining conscious of what they eat, not just because of the quality brought by their organoleptic properties, but also the

nutritional value that these foods can bring to their health and wellbeing (Lee et al., 2014).

In the search for obtaining food products with the newest high-quality standards emerging technologies are being developed and investigated in order to determine the applications that they can have in industry. The aim of these technologies, which work under different principles than heat, is the preservation of foods while maintaining their quality attributes (Butz and Tauscher, 2002).

This research presents two of these emerging technologies, namely ultra-high pressure homogenisation (UHPH) and ultraviolet type C radiation (UV-C) applied to different types of apple juices. It was investigated the effect that these technologies have on microbial spores potentially present in this product which could cause spoilage thereof, as well as the impact they can have on the nutritional and organoleptic properties of apple juice.

1.1 Thermal technologies used in food industry

Thermal treatments in food industry use thermal energy to submit foods to a treatment aggressive enough so as to eliminate any kind pathogen potentially present in them as well as detrimental enzymes in order to assure the innocuity of the product and extend their shelf-life. Numerous organisms around the globe like the FDA, USDA and others have set the lines for the minimum required temperatures and exposure times for the different types of products whereas it is from dairy or vegetable origin, and the minimum microbial reduction must be of 5 Log in any pasteurisation process (US FDA, 2020).

In liquid foods, thermal treatments are commonly done in a continuous flow processing, mainly divided in three types: 1) aseptically packed products that would render the product commercially sterile; 2) high-acid products like most juices that will be treated with the necessary pasteurisation temperatures and hold times to destroy spoiling microorganism. These products would be quickly cooled down in order to prevent the loss of volatile compounds, and filled into a pre-sterilised package in hygienic conditions; and 3) low-acid products that work under the same principle but will require the use ultra-high temperatures to destroy pathogens, that also represents shorter holding times. Independently from the type of processing, the heat source might come from direct (steam injection, steam infusion) or indirect heating (plate heat exchanger, tubular heat exchanger, scraped-surface heat exchanger) (Richardson,2001).

In the pasteurisation of juices, the FDA calls for a temperature of 160 ºF (71 ºC) for 3 seconds if the juice comes from citrus, non-apple juice or juice from concentrate, but 6 seconds at the same temperature for raw apple juice. Commercial sterilisation is usually achieved by ultra-high temperature processing (UHT) also called ultra-pasteurisation, in which higher temperatures over 135 ºC are held between 2 to 8 seconds (Richardson, 2001). UHT is effective in inactivating spores that otherwise will remain active after conventional pasteurisation treatments. Even, though, UHT is more commonly used in dairy products, it can also be applied to vegetable drinks including juices.

There are non-continuous processes in which heat is conveyed into foods can be by packaging them in containers or vessels and load them into a batch retort

system, which also allows for the use of pressures that make possible to reach sterilisation temperatures of 121 ºC. Some retorts operate with saturated steam for heating, some others are overpressure batch retorts which allow a better control of temperature and pressure with the purpose of minimizing pack damage. These last can operate by full water immersion, sprayed water, and others (Richardson, 2001).

There is still a concern of what a prolonged exposure to heat can do with the sensory properties and nutritional content of foods. Because of that, there are some other heat-transmitting technologies that were investigated and oriented to food production.

1.1.1. Ohmic heating

An electric current pass through the food and heat is internally produced as the food itself works as an electrical resistance and it warms up as a consequence of the movement of ions. Ohmic heating uses the effect of the electrical resistance within a conductive liquid or solid material converting electric energy directly to heat (Butz and Tauscher, 2002).

1.1.2. Microwave heating

It is based on the transfer of electromagnetic energy to foods. This energy acts mainly on food's water which is forced to move. This movement is extremely fast and oscillatory causing molecular friction, which in turn produces heat within the food itself. Moreover, microwaves also generates heat by the oscillatory movement of ionic charges toward oppositely charged areas leading to multiple collisions and disruption of hydrogen bonds with water (Vicente and Machado, 2011).

1.1.3. Infrared heating

Infrared radiation is a wavelength range within the electromagnetic spectrum and it is the major responsible for the heating effect of the sun. It can work at three categories based on the wavelength: near infrared (0.78 - 1.4 μm), middle infrared (1.4 - 3 μm) and far infrared (3 - 1000 μm) (Riadh et al, 2015).

1.1.4. Radio-frequency heating

It works under the same principle as microwave heating with the difference that free-space wavelength in the RF range is 20 - 360 times longer than that of commonly used microwave frequencies which allows it to penetrate and reach longer distances (Wang et al, 2012)

1.2. Non-thermal technologies used in food industry

1.2.1. High-hydrostatic pressure

High-hydrostatic pressure (HHP) works under the principle of submitting foods inside a confined vessel containing fluid (pressure-transmitting medium), usually water, which will be pressurised between 100 - 900 MPa, being the most common pressure range in industry the one between 400 - 700 MPa . This pressure would be applied isostatically (equally applied in all directions) allowing solid foods to retain their original shape. The pressure would be held for a given time and then released according to the purpose of the treatment, which is often enzyme and microbial inactivation due to denaturation of membrane proteins (San Martín et al., 2002).

In foods HHP is a batch process, and handling systems are usually baskets which are filled with packed foods in containers like bottles and bags that would be loaded into the HHP unit.

1.2.2. Ultra-high pressure-homogenisation (UHPH)

The first homogenisation valve was presented by Auguste Gaulin in the Paris World Fair in the year 1900 (Bevilacqua et al., 2007). From then onwards, it has been used and studied in food technology, pharmacy, and cosmetics among other areas for its capacity to reduce particle size of suspensions and to form emulsions. The different types of homogenisation vary according to the utilized pressures, a standard homogeniser works at a pressure between 20 to 60 MPa, the procedure is called high-pressure homogenisation (HPH) at pressures up to 150 to 200 MPa and its highest operational level so far corresponds to ultra-high-pressure homogenisation (UHPH) which can work up to 350 to 400 MPa. This technology has been in the spotlight for novel researches because it is not only capable of forming stable dispersions and emulsions, but also because it works

under a continuous process and it is able to inactivate enzymes and reduce the microbial load in liquid foods equivalent to that of thermal pasteurisation.

1.2.2.1. UHPH technological aspects

Homogenisers have a high-pressure valve (HP-valve) which consists in a needle and a sit with a design that allows for a narrow gap between them of a few micrometres width. Some homogenisers also have a low-pressure valve (LP-valve), this one may not be used in the homogenisation process depending on the ability of the HP-valve to disrupt particles or if there is low re-aggregation of particles which is usually observed after the first stage of standard homogenisation. The homogenisation performance depends on the characteristics in which these pieces were built (geometry of needle and seat, shape of the valve gap, the material they are made of) (Fig. 1) as well as the physicochemical properties of the liquid matrix that passes through this gap. The liquid food is submitted to high pressure for a brief time in the pressure intensifier, then it is pushed through the narrow gap which is the space in between the needle and seat of the valve, and then it occurs a pressure drop. After that point, there has been a number of physical phenomena or hurdles occurring such as cavitation, shear stress, friction and temperature increase, particle size reduction, etc. It is because of the combination of these hurdles that microbial and enzyme inactivation can be achieved at different levels depending on the shape of the needle and sit of the pressure valve. The temperature of the matrix after the valve rises up in a linear way at a rate of about 14 to 18 ºC per 100 MPa (Dumay et al., 2013) independently of the initial temperature, and this is attributed to shear effects and partial conversion of kinetic energy into heat (Chevalier-Lucia et al., 2011). Although microbial inactivation is usually attributed to these shear-induced temperature increments (Roig-Sagués et al., 2015), it is important for UHPH equipment to lower down these temperature elevations by the use heat-exchangers in order to avoid over-processing of heat-sensitive molecules.

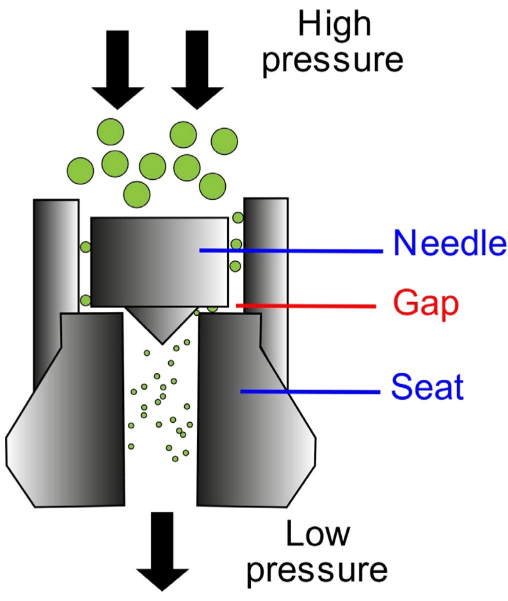


Figure 1. Representation of a sharp-angle homogenisation valve from Stansted based on the diagram shown by Dumay et al. (2013).

1.2.2.2. Effect of UHPH on microorganisms

Although it has been observed that UHPH treatments are able to reduce bacterial population in pumpable foods, the exact inactivation mechanism has not been fully elucidated. In many investigations it has been observed by microscopy that there is presence of cell debris as well as cell deformations after UHPH treatments. Hence most researches point out that there is a certain synergy between the temperature of the matrix at the inlet and the brief temperature rise when it reaches the valve where other phenomena like shear-stress, cavitation, turbulence and high pressure take place. Table 1 summarizes some research about UHPH treatments on different types of microorganisms in mostly food matrices.

Table 1. UHPH experiments performed on different microorganisms present in different food matrices.

UHPH Equipment	Matrix	Microrganism and strain	Vegetative or spore	Pressure (MPa)	Initial count CFU/mL (log10)	Inactivation CFU/mL (log10)	Inlet temp. (ºC)	Valve temp. (ºC)	Source
Stansted FPG 11300	Orange juice	*Alicyclobacillus acidoterrestris* CECT 7093	spore	300	~ 5.6	~ 0.35	20	93.14	Roig-Sagués et al. (2015)
		Alicyclobacillus acidoterrestris CECT 7093	spore	300	~ 5.6	UDL	80	130	
		Alicyclobacillus hesperidum CECT 5323	spore	300	~ 5.0	~ 0.1	20	93.14	
		Alicyclobacillus hesperidum CECT 5323	spore	300	~ 5.0	UDL	70	125	
Stansted FPG 11300	Milk	*Bacillus amyloliquefaciens* FAD82	spore	350	~ 6.0	3.5	80	>150	Dong et al. (2015)
Stansted FPG 12500N	Apple juice	*Aspergillus niger* CMI 17454, *Penicillium expansum* CMI 378779, *Byssoclamys fulva* ATCC 24474	spore	250	6 - 6.5	6 - 6.5	20	74 (at 300 MPa UHPH)	McKay (2009)
		Saccharomyces cerevisiae NCYC 1370/NCYC365	vegetative	200	~ 7.5	~ 7.5	20		
		Saccharomyces cerevisiae NCYC 1370/NCYC365	spore	200	~ 6.3 - 7.2	~ 0.5	20		
Stansted FPG 12500	Egg	*Salmonella enterica* serovar Senftenberg 775W	vegetative	250	~ 7.0	3.2	6	64.5	Velázquez-Estrada et al. (2008)
Stansted FPG 11300	Apple juice	Endogenous microbiota	non-stated	300	~ 4.3	~ 3.6	4	85.8	Suárez-Jacobo et al. (2010)

Table 1 (cont.)

UHPH Equipment	Matrix	Microrganism and strain	Vegetative or spore	Pressure (MPa)	Initial count CFU/mL (log10)	Inactivation CFU/mL (log10)	Inlet temp. (ºC)	Valve temp. (ºC)	Source
Stansted FPG 12500	Orange and grape juices	*Lysteria monocytogenes* CCUG 15526	vegetative	300	7	5.2 - 5.6	6	62.4	Vel et al. (2011)
		Salmonella enterica serovar Senftenberg 775W	vegetative	400	7	7	6	74.2	
Stansted nG 12500	Milk	Bacillus cereus (CECT 5144)	spore	300	6.47	6.47	85	139	Amador Espejo et al. (2014)
		Bacillus licheniformis (DSMZ 13)	spore	300	6.33	6.33	85	139	
		Bacillus sporothermodurans (DSMZ 10599)	spore	300	6.9	6.9	75	129.6	
		Bacillus coagulans (DSMZ 2356)	spore	300	6.57	6.57	75	129.6	
		Geobacillus stearothermophilus (CECT 47)	spore	300	-	5.26	85	139	
		Bacilus subtilis (CECT 4002)	spore	300	-	5.22	85	139	
Stansted FPG 11300	Almond beverage	Mesophilic aerobial	spore	200	4	4	75	114.3	Valencia-Flores et al. (2013)
		Bacilus cereus (endogenous)	-	200	3	3	75	114.3	
Stansted FPG 12500	Apple juice	E. coli K-12	vegetative	250	~ 7.6	~ 7.6	25	72	Kumar et al. (2009)

Table 1 (cont.)

UHPH Equipment	Matrix	Microrganism and strain	Vegetative or spore	Pressure (MPa)	Initial count CFU/mL (log10)	Inactivation CFU/mL (log10)	Inlet temp. (ºC)	Valve temp. (ºC)	Source
Stansted FPG 11300	Tigernut drink	Mesophilic bacteria	spore	300	2.53	0.3	40	116.3	Codina-Torrella et al. (2018)
Stansted FPG 12500	Skim milk (<0.2% fat)	Bacilus subtilis (CECT 4002)	spore	200	~ 6.0	≥0	60	-	Martinez-Garcia et al. (2019)
	Whole milk (3.5% fat)				~ 6.0	~ 0.8	60	-	

1.2.2.3. Effect of UHPH on pumpable foods

Being a homogenizer, the UHPH has been tested mainly in foods that need to be homogenized in order to prevent phase separation and to make them stable for longer periods. In fatty matrices such as dairy (milk, cream, etc.), soy and almond drinks, among others, UHPH can reduce the size of particles like fat globules even better than in conventional homogenisation. In some other matrices like pulpy fruit juices, UHPH can reduce the size of particles like fibre, and the activity of pectin methylesterase (PME) which helps to stabilize their level of cloudiness. (Velázquez-Estrada et al., 2012) found that UHPH treatments at 300 MPa and 20 ºC of inlet temperature are enough to inactivate PME in orange juice at the same level as a thermal treatment of 90 ºC for 1-2 min. (Saldo et al., 2009) reports that the content of hydroxymethyl furfural (HMF) in UHPH-treated apple juice (100-300 MPa, 4 and 20 ºC inlet temperature) was significantly lower than in the heat-treated one at 90 ºC for 4 min. HMF is normally used as an index in heat-treated juices in order to know whether that treatment was excessive or not. Experiments conducted by Velázquez-Estrada et al. (2019) in UHPH-treated orange juice report that treatments between 100 - 300 MPa and at 10 and 20 ºC of inlet temperature did not significantly change °Brix, reducing sugars, pH and non-enzymatic browning index when compared with those of fresh or pasteurised juice.

1.3. Ultraviolet radiation

Ultraviolet light comprehends the wavelength range in the electromagnetic field between 100 to 400 nm (Fig. 2). It can be categorized in UV-A (long wave) (315 to 400 nm) which is normally used in human skin tanning, UV-B (middle wave) (280 to 315 nm) that can cause skin damages like burning and even cancer, and UV-C (short wave) (200 to 280 nm) used mostly for germicidal processes. Under these ranges it can also be found vacuum ultraviolet light (VUV) (100 to 200 nm) which is absorbed by almost all substances and because of this it can only be transmitted in vacuum (Koutchma, 2009).

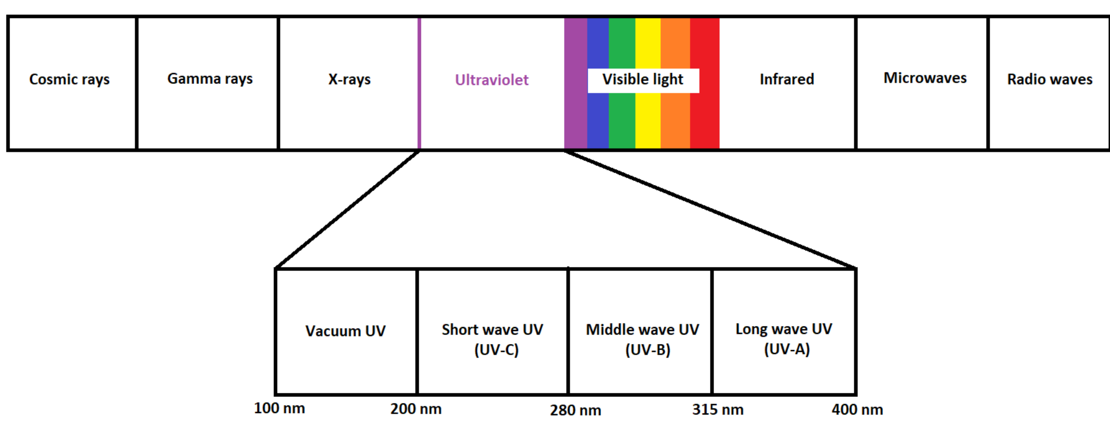


Figure 2. Ultraviolet light within the electromagnetic spectrum

Due to the germicidal effect in the UV-C region, it has been used for the decontamination of surfaces, air and water for the last decades. The germicidal-effect peak takes place in the range of 254 to 264 nm wavelength. For these purposes there are many types of UV-light sources that can be employed, from which mercury lamps are broadly utilized, typically three types: low pressure mercury lamps (LPM), low pressure high-output mercury lamps (LPMHO), and medium pressure mercury lamps (MPM) which are frequently chosen because of their good performance, quality and low cost. Their names are based on the vapor pressure of mercury when operating these lamps. They consist of a UV-transmitting envelope made from a sealed silica-gel tube sealed at both ends. There is an electrode located at each end of the envelope connected to the outside through a seal. The envelope is filled with mercury and an inert gas, usually argon with an ionization energy of 15.8 eV (Masschelein, 2002).

LPM lamps are operated at a nominal gas pressure between 100 to 1000 Pa with a carrier gas in excess at a proportion of 10 to 100. The optimum temperature for this pressure is of 40 ºC at the lamp wall. The linear total UV output of the discharge length appropriate for disinfection is in the range of 0.2 to 0.3 W (UV)/cm. Their emission spectrum is monochromatic, meaning that it is concentrated at a limited number of well-defined lines, these are at 253.7 and 185 nm, from which the line of 253.7 nm represents around the 85% of the total UV intensity and it is the one of interest for germicidal purpose (Koutchma, 2009). Contrary to that, MPM lamps are polychromatic and emit more powerful light between 250 to 600 nm which also touches some of the visible light wavelength range. Those lamps operate at temperatures of 400 to 800 ºC and pressures of 10^4 to 10^6 Pa. Although they are capable of reaching a higher penetration depth, government's regulatory organisms like U.S. FDA or Health Canada have not approved yet the use of MPM lamps, but only the LPM ones (Koutchma et al., 2016).

1.3.1. Types of UV-C reactors for pumpable matrices

The FDA has approved the use of ultraviolet radiation as an alternative to thermal pasteurisation for fresh juice products (US FDA, 2000). From this point onwards there has been a growing interest into designing UV-C reactors that can optimize the efficiency of that technology addressing to solve problems related with the physicochemical properties of the pumped foods like absorbance and viscosity among others that work as interferences between the UV light and the microorganisms. Those reactors are being designed in order to bypass such interferences so as to make all particles to get into contact with UV radiation long enough to inactivate harmful and spoiling microorganisms. The flow rate/pattern is crucial for the total applied UV dose. The equipment can be sorted according to the type of flow they can reach whether is laminar or turbulent flow, that can be known by means of the Reynolds number (Re) (Eq. 1) (Martinez-Garcia et al., 2019), If Re is higher than 2000 the flow will be considered as turbulent, a lower Re value would correspond to laminar flow (Koutchma et al., 2016).

$$Re = \frac{V \cdot d \cdot \rho}{\mu} \quad \text{Equation 1}$$

Where:

Re = Reynolds number

V = velocity of the fluid in m/s

d = diameter or the tube in m

ρ = density in Kg/m^3

μ = dynamic viscosity of the fluid Pa·s

1.3.1.1. Laminar flow UV-C reactors

UV-C reactors that work within the laminar-type of flow can come in different designs, one of them uses an extremely thin film of approximately 0.8 mm width to decrease the path length of UV light and avoid lack of penetration problems. Those reactors which work at laminar flow conditions are characterized by a parabolic velocity profile, which means that the highest velocity is achieved at the centre, that is twice as much the average velocity of the liquid. As a result, the processing conditions are non-uniform (Koutchma, 2008).

One of those designs is the CiderSure reactor (FPE inc, Macedon, NY). Eight LMP arc lamps are mounted inside a quartz sleeve at the centre of the reactor. Next to that quartz layer there is a small gap of 0.8 mm width where the liquid passes to get into contact with UV radiation. Another design is the Taylor-Couette flow UV reactor (Forney and Pierson, 2004), that type of reactor consists in two concentric cylinders from which the outer quartz cylinder contains the UV light source which is static, and an inner cylinder (called Taylor-Couette flow) that can rotate and consists of laminar vortices that fill the annular gap of several millimetres. Although it still works under laminar flow conditions, those vortices aim for a better mix and distribution of the pumped liquid in order to make all particles to get irradiated by the UV light source. One more UV reactor design is the UV-Therm (Ypsicon S.L., Barcelona, Spain) which is an annular-type reactor (Fig. 3). It is composed of a long LMP lamp covered by a quartz sleeve followed by an annular gap of 1 mm width. That design allows for the connection of many reactors in series so as to increase the dose and favour mixing of the matrix in order to expose as many particles as possible to the UV light source.

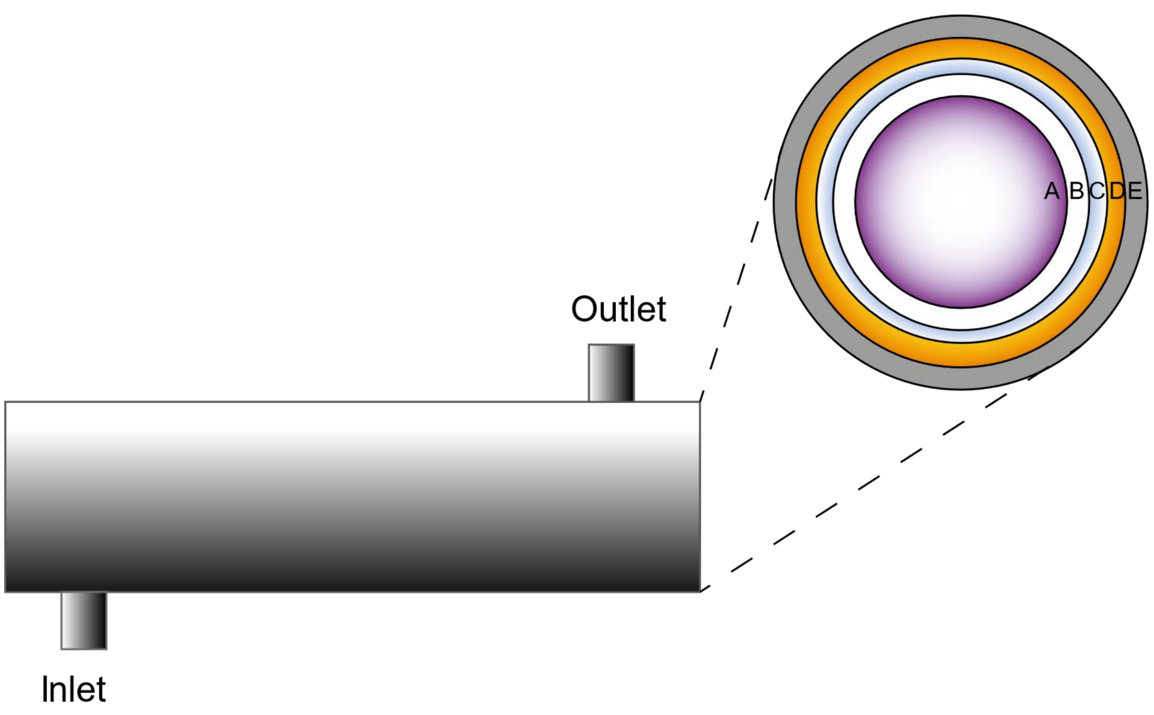


Figure 3. Annular-type (thin film) UV-C reactor (Ypsicon). The parts consist of A) UV lamp, B) empty space (air cooling), C) quartz sleeve, D) food matrix circulation area (concentric space) (1 mm), E) water-cooling/heating system.

1.3.1.2. Turbulent flow UV-C reactors

Those types of reactors increase turbulence in order to bring all the liquid into contact with the UV light during the treatment in a more homogenous way than in laminar flow-type reactors due to a better mixing (Fig. 4). The disadvantage of this approach is that as turbulence increase, pressure drops across the reactor, and the required high flows to achieve turbulent flow lead to shorter residence times and consequently to possible complications in scale-up equipment. An example of turbulent UV reactor is the Aquaionics (Hanovia Ltd, Slough, England, UK), it is composed by a stainless-steel chamber containing UV emitting low pressure arc tubes, each one mounted in a quartz sleeve and fitted inside the chamber. The liquid is passed though that chamber touching the quartz sleeves on all sides (Koutchma, 2009)

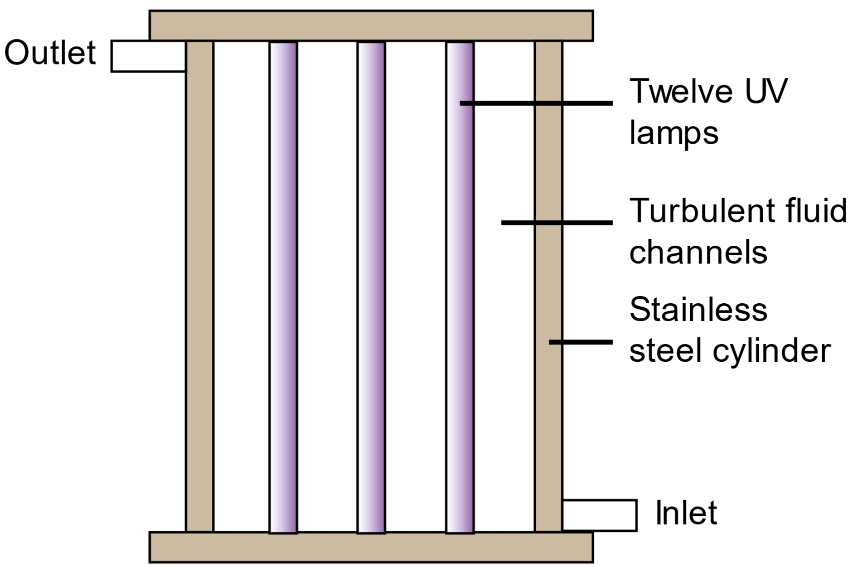


Figure 4. Schematic of turbulent-flow UV-C reactor adapted from Koutchma (2008).

1.3.1.3. Dean-flow UV-C reactors

Those reactors (Fig. 5) are composed of a coiled Teflon tube with UV lamps and reflectors located inside and outside the coiled tube, with this approach there is a better UV irradiance of the flowing liquid as well as its uniformity. The coiled tube promotes additional turbulence but also a secondary flow effect called Dean effect which promotes a more uniform velocity and residence time distribution (Koutchma, 2009).

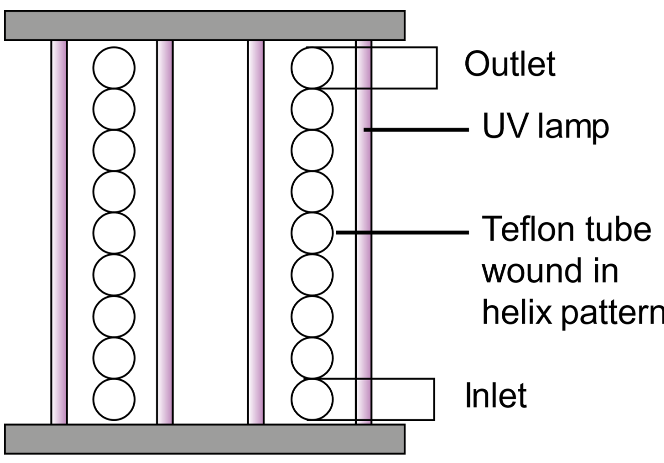


Figure 5. Schematics of a Dean-flow UV-C reactor adapted from Koutchma (2008).

1.3.2. Effect of UV-C on microorganisms

Many studies point out that UV-C can inactivate microorganisms by penetrating through the outer membranes of cells occasioning them tremendous DNA damage.

The absorption peak of DNA is located at around 260 nm, a wavelength found within the UV-C range (Fig. 2). UV-C affects genetic material by the formation of pyrimidine dimers preventing the microorganism from undertaking DNA transcription and replication leading to cell death (Choudhary and Bandla, 2012).

However, UV light sensitivity of microorganisms varies accordingly, this is a key factor that affects the efficacy of the treatment of liquid foods and might be due to different factors: cell wall structure and thickness, composition, UV-absorbing proteins, or even different structures of nucleic acids. The effectiveness of the treatment might also be affected by the capability of microorganisms to repair UV damage (Koutchma, 2009).

In thermal treatments, the different microorganisms have a *D* value which marks the exposure time required to obtain 1 Log reduction at a given temperature. Similarly, in UV-C treatments the *D* value will represent the necessary dose in mJ/cm^2 at 253.7 nm to irradiate the microorganism in order to obtain 1 Log reduction as seen in Table 2.

Table 2. UV inactivation doses (mJ/cm^2) measured at 253.7 nm for various microbial groups from (Koutchma, 2009).

Microbial group	***D* value (mJ/cm^2)**
Cocci and micrococci	1.5 to 20
Enteral bacteria	2 to 8
Yeast	2.3 to 8
Spore formers	4 to 30
Enteric viruses	5 to 30
Fungi	30 to 300
Protozoa	60 to 120
Algae	300 to 600

UV-C has already been tested on different microorganisms whether they were already present in foods naturally or inoculated in many types of matrices in order to validate these treatments applicated by the different types of UV reactors known to date (Table 3).

Table 3. UV-C effect on microorganisms in fruit juices. Modified from Shah et al. (2016).

Juice	Microflora	Reactor/flow type	UV-C dosage	Log reduction	Reference
Mango nectar	Aerobic plate count	Annular	45 J/cm^{-2}	2.7	Guerrero-Beltrán & Barbosa-Cánovas (2006)
Pineapple	Aerobic plate count	Thin film	10.76 mJ/cm^{-2}	1.9	Shamsudin et al. (2014)
	Yeast and mold		10.76 mJ/cm^{-2}	1.4	
	Salmonella typhimurium	Dean flow	0.000154 L/s (flow)	3	Mansor et al. (2014)
Starfruit	Aerobic plate count	Petri plate	NA	1.3 (total inac.)	Bhat et al. (2011)
Lemon-melon	*E. coli* K12	Annular	0.44-2.86 mJ/cm^{-2}	0.06-6	Kaya et al. (2015)
Apple cider	*E. coli* O157:H7	Taylor couette/turbulent	4.45, 6.67, 13.34 mJ/cm^{-2}	2.85-4.76	Orlowska et al. (2014)
	Aerobic plate count	Thin film	14 mJ/cm^{-2}	1.8	Tandon et al. (2003)
	Yeast and mold		14 mJ/cm^{-2}	1.4	
	E. coli O157:H7	Annular	8.77-35.11 mJ/cm^{-2}	>5	Donahue et al. (2005)
	E. coli K12	Annular	19.4 mJ/cm^{-2}	<2	Koutchma & Parisi (2004)
	E. coli O157:H7	Thin film	14 mJ/cm^{-2}	7.2	Basaran et al. (2004)
	E. coli O157:H7	Thin film	0.1 mJ/cm^{-2}	5.4	Wright et al. (2000)
	E. coli O157:H7	Thin film	34 mJ/cm^{-2}	4.7	Geveke (2005)
Apple	*Listeria innocua*	Annular	2.7 mJ/cm^{-2}	4.8-5.8	Caminiti et al. (2012)
	E. coli	Dean flow	7.7 kJ/L	6	Müller et al. (2011)
	Saccharomyces cerevisiae		9.6 kJ/L	4	
	Lactobacillus plantarum		3.9 kJ/L	>5 (total inac.)	
	Alicyclobacillus acidoterrestris		9.6 kJ/L	4	
	E. coli K12	Annular	24.9 mJ/cm^{-2}	>5	Koutchma & Parisi (2004)
	E. coli O157:H7	Petri plate	5.25, 75 mJ/cm^{-2}	1.95	Yin et al. (2015)
	Alicyclobacillus acidoterrestris	Petri plate	0.38-1.31 mW/cm^{-2} (Irradiance)	2.3	Baysal et al. (2013)

Table 3 (cont.)

Juice	Microflora	Reactor/flow type	UV-C dosage	Log reduction	Reference
Apple	*E. coli* O157:H7	Petri plate	NA	2.76	Gabriel (2012)
	E. coli STCC 4201	Annular	2.66 mJ/cm^{-2}	1.23	Gayán et al. (2011)
	E. coli STCC 471		2.66 mJ/cm^{-2}	1.64	
	E. coli STCC 27325		2.66 mJ/cm^{-2}	2.36	
	E. coli O157:H7		2.66 mJ/cm^{-2}	4.01	
	E. coli ATCC 25922		2.66 mJ/cm^{-2}	6.22	
	E. coli	Thin film	6-24 mJ/cm^{-2}	6	Lu et al. (2010)
	Lactobacillus brevis		6-24 mJ/cm^{-2}	5.75	
	Saccharomyces cerevisiae		6-24 mJ/cm^{-2}	4	
	E. coli	Thin film	NA	>5	Ukuku & Geveke (2010)
	Aerobic plate count	Turbulent flow	230 J/L	3.5 (total inac.)	Keyser et al. (2008)
	Yeast and mold		230 J/L	3 (total inac.)	
	E. coli K12		1377 J/L	>7	
	E. coli O157:H7	Petri plate	300 mJ/cm^{-2}	4.5	Ngadi et al. (2003)
Apple-cranberry	*Pichia fermentans*	Annular	5.3 mJ/cm^{-2}	<2	Palgan et al. (2011)
	E. coli		5.3 mJ/cm^{-2}	6	
Grape	*Saccharomyces cerevisiae*		138 mJ/cm^{-2}	5	Kaya et al. (2015)
	Yeasts		280 mJ/cm^{-2}	3	
	Lactic acid bacteria		280 mJ/cm^{-2}	4.3	
Grape (white)	*E. coli* K12		0.9 mL/s	5.2	Unluturk & Atilgan (2015)
	Alicyclobacillus acidoterrestris	Petri plate	0.38-1.31 mW/cm^{-2} (Irradiance)	5.8	Baysal et al. (2013)
	Brettanomyces bruxellensis	Collimated beam	1377 J/L	>5	Fredericks et al. (2011)
	Saccharomyces cerevisiae		3672 J/L	>5	
Grapefruit	*E. coli*	Thin film	19 mJ/cm^{-2}	5.1	Geveke & Torres (2012)
	Saccharomyces cerevisiae		14 mJ/cm^{-2}	6	

Table 3 (cont.)

Juice	Microflora	Reactor/flow type	UV-C dosage	Log reduction	Reference
Grape (red)	*Brettanomyces bruxellensis*	Collimated beam	3672 J/L	2	Fredericks et al. (2011)
	Saccharomyces cerevisiae		3672 J/L	>5	
	Lactobacillus plantarum		3672 J/L	>5	
Pomegranate	*E. coli*	Dean flow	62.4 J/mL	6.2	Pala & Toklucu (2011)
	Aerobic plate count		62.4 J/mL	1.8	
	Yeast and mold		62.4 J/mL	1.5	
	Yeast and mold		NA	4	
Watermelon	Aerobic plate count	Dean flow	2.7-37.5 J/mL	1.5	Feng et al. (2013)
Passion fruit	Aerobic plate count	Annular	NA	0.53	Guevara et al. (2012)
	Yeast and mold		NA	Total inactivation	
Guava nectar	Aerobic plate count	Annular	NA	0.51	Guevara et al. (2012)
	Yeast and mold		NA	1.36	
Guava-pineapple	Yeast and mold	Turbulent flow	918 J/L	4.5 (total inac.)	Keyser et al. (2008)
	Aerobic plate count		1377 J/L	3.3	
Orange	*Lactobacillus plantarum*	Dean flow	9.6 kJ/L	5	Müller et al. (2011)
	E. coli O157:H7	Dean flow	36.1 kJ/L	5.7	Pala and Toklucu (2013)
	Aerobic plate count	Turbulent flow	1607 J/L	<1	Keyser et al. (2008)
	Yeast and mold		1607 J/L	<1	
	E. coli O157:H7	Petri plate	2.2 J/cm^{-2}	>5	Oteiza et al. (2010)

1.3.3. Effect of UV-C on foods

It is important to understand how this technology can change some of the sensory and nutritional properties of foods. So far, the different authors have not reported important changes in juices treated by UV light regarding pH, soluble solids and turbidity. Overall flavour and colour neither seem to be affected in juices, though apparently foods with higher *L** colour value, which represents lightness, have higher absorbance at 253.7 nm, which acts as an interference. (Choi & Nielsen, 2005) report that the sensory characteristics of UV-C-treated apple cider are not significantly different than the untreated product, and that is better accepted than the pasteurised one. On the other hand, it has been found the production of odd flavours and odours in dairy products. This occurs because of photodegradation of organic molecules in foods, especially amino acids with aromatic compounds, due to photochemical reactions (Dumay et al., 2013). Two conditions must be met in order for a photochemical reaction to take place: 1) photons must be absorbed to start the reaction, and 2) they should have enough energy to promote a reaction to break or to form a bond. Theoretically, UV light at 253.7 nm has a radiant energy able to affect the following bonds if absorbed: O-H, C-C, C-H, C-N, H-N and S-S (Koutchma et al., 2016; Koutchma, 2009).

Nutrients in foods greatly differ from one another, as well as their sensitivity to UV-lights. Some of the photosensitive nutrients that can be affected by UV are: vitamin A, carotenes, cyanocobalamin (vitamin B12), vitamin D, folic acid, vitamin K, riboflavin (vitamin B2), tocopherols (vitamin E) and tryptophan. In the case of fatty acids, unsaturated residues in oils, solid fats and phospholipids can also be affected by irradiation. That happens because in the presence of oxygen, radiation can accelerate oxidation and reactions like the production of free radicals, the formation of hydrogen peroxides and the destruction of antioxidant compounds like carboxylic acids (Lima et al., 2018; Koutchma et al., 2016). This last issue represents no concern for irradiated fruit juices because of their extremely low content of fatty acids. Proteins can have their amino acid chains altered in the presence of water due to electron transfer; this might accelerate protein denaturation by altering the secondary and tertiary structures, though this denaturation is less intense than in a thermal process. Guerrero-Beltrán & Barbosa-Cánovas (2006) found that the

activity of polyphenol oxidase in mango nectar was reduced down to a 19 % after 30 min of UV-C with a final dose of 825 mJ/cm^2. On the other hand, Tran & Farid (2004) achieved only 5 % inactivation of pectin methylesterase (PME), and Torkamani & Niakousari (2011) an 8 % of PME-inactivation by applying both a UV light treatment of 73.8 mJ/cm^2. Gayán et al. (2012) report a reduction of PME activity of 64 % in orange juice after an UV-C light treatment of 23.72 J/mL at 55 °C and 3.6 min of total time, but in that case, they attributed PME inactivation to the heat used in the combined treatment. The inactivation of these enzymes is of vital importance to obtain shelf-stable products.

1.3.4. Interferences from foods against UV penetration

The main purpose for using UV-C light in foods is effectively reducing their microbial load, which is given due to DNA's absorption peak. Therefore, it is very important for UV-C light to be able to pass through the food matrix, and reach and penetrate microorganism in order to affect their genetic material. The different components in foods also determine their optical properties like turbidity and absorption ranges. For this reason, the measurement of their absorbance coefficient at 254 nm (α_{254}) and turbidity can work as an indicator of how much these components can absorb, block or scatter UV light and act as an interference (Table 4). In fruit juices α_{254} is strongly influenced by the content of ascorbic acid, even at low concentrations (Fan & Geveke, 2007). The presence of pigments (absorptivity) and suspended matter (turbidity) also affect the penetration of UV light into the food matrix. In the case of milk and other turbid and opaque foods, UV light cannot penetrate deeply, hence thin film reactors are commonly employed to address this problem (Choudhary and Bandla, 2012)

Table 4. Absorption coefficient and light penetration at 253.7 nm of water and different liquid foods. Adapted from Koutchma (2009).

Substance	Absorption coefficient (cm^{-1})	Penetration for 90% absorption (cm)
Water	0.01	100
Clear apple juice	15	0.067
Apple cider (cloudy)	40	0.025
Orange juice	100	0.01
Liquid sucrose	4.5	0.022
Beer	16	0.063
Coca-cola	31	0.032
Milk (raw)	290	0.003
Egg white	104	0.001
Wine, Sherry	9	0.111

1.3.5. UV-C combined treatments with other technologies

In order to address the problems of low microbial inactivation due to interferences in food matrices that block the activity of UV light, many technologies have been tested in combination with UV-C in the search for a synergistic or additive effect between them in order to increase the lethality rates of microorganisms. Table 5 shows some of the successful combinations found in literature to date and the type of effect that the authors explain that an interaction between UV-C and other technology together have on microorganisms in certain foods or matrices. So far, most combinations with UV-C have addressed vegetative bacteria and yeasts with promising results. Yet, there is much more to investigate in the case of microorganisms in their most resistant forms, which are spores.

Table 5. UV-C combined with different technologies in microorganisms inoculated into many types of matrices and the achieved effect of the combination of technologies.

Complimentary technology	Microorganism	Matrix	Effect	Source
Heat (45 and 50 ºC)	*Escherichia coli* ATCC 35218 *Pseudomonas fluorescence* ATCC 49838	Carrot juice	Synergistic	García-Carrillo et al. (2017)
Ultrasound	*Escherichia coli* ATCC 35218	Orange juice	Additive	Char et al. (2010)
Pulsed electric field	*Escherichia coli* ATCC 23472	Apple juice	Additive	Gachovska et al. (2008)
Laser and microwaves or heat	*Escherichia coli* DH5α *Listeria monocytogenes* R479a *Shewanella putrefaciens* NCIMB 1732 *Pseudomonas fragi* NCIMB 1353 *Micrococcus luteus*	Physiological saline	Synergistic	Maktabi et al. (2011)
Heat (50 to 60 ºC)	*Escherichia coli* (many strains)	Orange juice	Synergistic	Gayán et al. (2012)

1.4. Heat-, pressure- or UV-resistant microbial spores potentially present in fruit juices

1.4.1. *Alicyclobacillus acidoterrestris*

The genus *Alicyclobacillus* was first recognized in 1992 by Wisotzjsey et al. (1992) with the characteristic of containing ω-cyclohexane fatty acids in their membrane. *A. acidoterrestris* is a Gram-positive, aerobic, acidophile and thermophile spore-forming bacteria commonly found in soil and in food products that come into contact with it. It can grow at temperatures of up to 60 ºC and pH between 2 - 5 (Walker and Phillips, 2008). Its spores can germinate at pH < 4, and their D_{90}-value ranges between 16 – 23 min (Smit et al., 2011) which is greater than any pasteurisation treatment for juice processing. Researchers suggest that the high resistance of *Alicyclobacillus* against acidic pH conditions and high temperatures is given to the presence of ω-cyclohexane fatty acids.

A. acidoterrestris was identified as responsible for the contamination and spoilage of a whole batch of shelf-stable apple juice in Germany 1984 (Cerny et al., 1984). Furthermore, it has been implicated in many other cases where different types of fruit products were spoiled by this microorganism such as iced tea with berry juice (Duong and Jensen, 2000), carbonated fruit drink (Pettipher and Osmundson, 2000) and canned tomatoes (Chang & Kang, 2004). Even though *A. acidoterrestris* represents no health risks, when it grows in a product it is able to produce off-flavours and odours because it produces guaiacol, without generating any visible gas or any other signs of product spoilage. For these reasons, it has become of great concern to manufacturers and it's suggested as a possible target microorganism when designing pasteurisation processes for low pH food products.

The interest in *Alicyclobacillus* as a microbe with high spoilage potential arouse after the report of Cerny et al. (1984). There, *A. acidoterrestris* was implicated as the causative microbe in a large-scale spoilage incident in Germany, affecting what it was supposed to be shelf-stable, aseptically packaged apple juice. Subsequently, spoilage incidents attributed to *Alicyclobacillus* species were reported worldwide in various fruit juices (Splittstoesser et al., 1994; Yamazaki et al., 1996; Jensen, 2000; Matsubara et al., 2002), fruit juice blends (Splittstoesser et al., 1994; Jensen and Whitfield, 2003; Goto et al., 2003), carbonated fruit juice drinks (Pettipher and Osmundson, 2000; Gouws et al., 2005), fruit pulps (Gouws et al., 2005), lemonade and isotonic water (Yamazaki et al., 1996), iced tea (Duong and Jensen, 2000), and canned diced tomatoes (Walls and Chuyate, 1998).

1.4.2. *Bacillus subtilis*

Bacillus subtilis is an aerobic Gram-positive rod-shaped bacterium able to form endospores. This genus was first proposed by Ferdinand Cohn in 1872 and it has become of a prominent role in the development of microbiology since then, being nowadays an important source in the production of industrial enzymes, fine biochemicals, antibiotics and insecticides (Harwood, 2007). This genus is mostly comprised by apathogenic species found active in soil and associated water sources.

B.subtilis spores are known because of its UV-C resistance, this makes them to be commonly used as a bioassay microorganism in water treatment (Koutchma, 2009). They're not only resistant to radiation, but also to many types of heat-based treatments and to a wide variety of chemicals like acids, bases, oxidizing agents, alkylating agents, aldehydes and organic solvents (Table 6) (Setlow, 2006). For these reasons, it is commonly taken as a model microorganism to investigate novel antimicrobial treatments.

Table 6. UV-C, thermal, chemical and freeze dry treatments applied to *B. subtilis* (wild type) in order to reduce 90% of the population. Data was obtained with experiments performed at 23 ºC unless indicated otherwise. Adapted from Setlow (2006).

Treatment	**UV-C dose, exposure time and number of freeze dryings to reduce 90% of *B. subtilis* population**	
	Wild-type cells (growing)	**Wild-type (spores)**
UV-C radiation (254 nm) (KJ/m^2)	36	330
Wet heat (90 ºC) (min)	<0.1	18
Dry heat (90 ºC) (min)	-	18
Dry heat (120 ºC) (min)	5*	-
H2O2 (15% at 23 ºC) (min)	<0.2	50
Formaldehyde (25 g/L) (min)	<0.1	22
Nitrous adic (100 mmol/L) (min)	<0.2	100
NaOCl (50 mg/L, pH 7) (min)	<0.1	5
Freeze dryings (times)	<1	>20

1.4.3. *Geobacillus stearothermophilus*

G. stearothermophilus is an aerobic Gram-positive spore-forming bacterium whose spores are amongst the most heat-resistant ones known until today. Vegetative cells can grow and cause spoilage at temperatures between 37-70 ºC (Wells-Bennik et al., 2019). It has been reported to cause spoilage of canned liquid foods like coffee during storage in automatic vending machines (Watanabe et al., 2003). Because of its high resistance to heat, this microorganism is used for the valildation of industrial

sterilization of many food products. It is also taken as a surrogate for *Clostridium botulinum* spores, one of the most heat-resistant pathogens known to date that can be present in packaged foods, because they have similar death kinetics, more specifically the *Z* value, and their typical required time for inactivation at 121.1 ºC of 4 and 3 min respectively (Richardson, 2001; Ahn et al., 2015; Holdsworth & Simpson, 2016). According to (Wells-Bennik et al., 2019) and their analysis of 18 different *G. stearothermophilus*, there are strains whose spores have *D* value of 2 min or longer at 125 ºC.

1.4.4. *Aspergillus niger*

A. niger is the most common species of fungi of the genus *Aspergillus* frequently found in diverse environments such as soil, indoors and foods. It is the best-characterized conidiospores-forming fungus. One of its main characteristics is its blackness product of the combination of brown and green pigments like melanin. These characteristics make *A. niger* a highly resistant microorganism against UV-C and pulsed light treatments. Because of this, *A. niger* conidiospores are commonly found as bioindicators in testing the quality and decontamination levels of devices at automated packaging lines (Esbelin et al., 2013).

1.4.5. *Talaromyces macrosporus*

T. macrosporus is a fungus able to produce ascospores with the characteristics of being heat- and pressure-resistant. They can also tolerate desiccation and some chemicals like ethanol (Kikoku, 2003). Moreover, these ascospores mostly remain in a dormant state (>95%) for even months and even years, potentially able to contaminate fruits (Kikoku, 2003; Reyns et al., 2003). This fungus is important for food science because they can cause spoilage of pasteurised products for it can withstand temperatures of 85 ºC in its spore state (Dijksterhuis and Teunissen, 2004). Once these ascospores contaminate foods and these are submitted to a pasteurisation process, this heat-shock can make spore to break from their dormant state, germinate and grow. Experiments conducted by Reyns et al. (2003) demonstrated that *T. macrosporus* ascospores can also break dormancy after high-hydrostatic pressure treatments up to 700 MPa at 20 ºC for 15 s to 60 min, with the finding that the highest assayed pressures and longer exposure times also made

these ascospores more vulnerable to heat (80 ºC 30 min). The aspect of its colonies in solid medium is first a mould of yellow colour, but once they spread and mature, the medium turns into a dark orange/brown colour, the same as the spore suspension even after being filtered through glass-wool and washed many times. This is because of the presence of pigments in these ascospores which suggests a possible resistance against UV-C treatments, but prior to this work there was no record of any experiments testing this possibility.

1.4.6. *Neosartorya spinosa*

N. spinosa is a filamentous fungus from the genus *Aspergillus* which able to produce ascospores. It is often found and isolated from fruit sources like spoiled strawberries and cherries. The most visible difference with the commonly known *A. niger* is that *N. spinosa* lacks of dark-coloured pigmentation, but in the other hand it produces white and cotton-like colonies on solid media, being its ascospores suspension of a pale white colour once filtered through glass-wool and washed several times. *N. spinosa* ascospores and from other species of *Neosartorya* have proven to be heat-resistant, even temperatures around 70 ºC can trigger spore germination. They are as well resistant against other treatments such as HHP, pulsed electric fields and thermosonication (Evelyn et al., 2016; Raso et al., 1998). These characteristics make them of importance in food industry, because their ascospores can survive and germinate after conventional pasteurisation treatments, grow and consequently spoil fruit products that were supposed to be shelf-stable. Moreover *Neosartorya fischeri* (anamorph *Aspergillus fischerianus*) can produce the mycotoxins Terrain, Fumitremorgin A and B and Verruculogen, making it of public health concern (Evelyn et al., 2016).

2. Aim of this dissertation and sampling protocol

2.1. Aim of this dissertation

The objective of these experiments was to elucidate the effects that UHPH and UV-C treatments applied separately and combined have on the inactivation of spores of microorganisms with a known resistance against different types of stresses, that can be present in apple juice, an acidic-pH matrix. Many types of apple juices and suspensions that imitate some of the interferences that could oppose against the lethal effect of these technologies were assayed. Changes in some of the physicochemical and quality properties of treated apple juices were also investigated.

2.2. Specific objectives

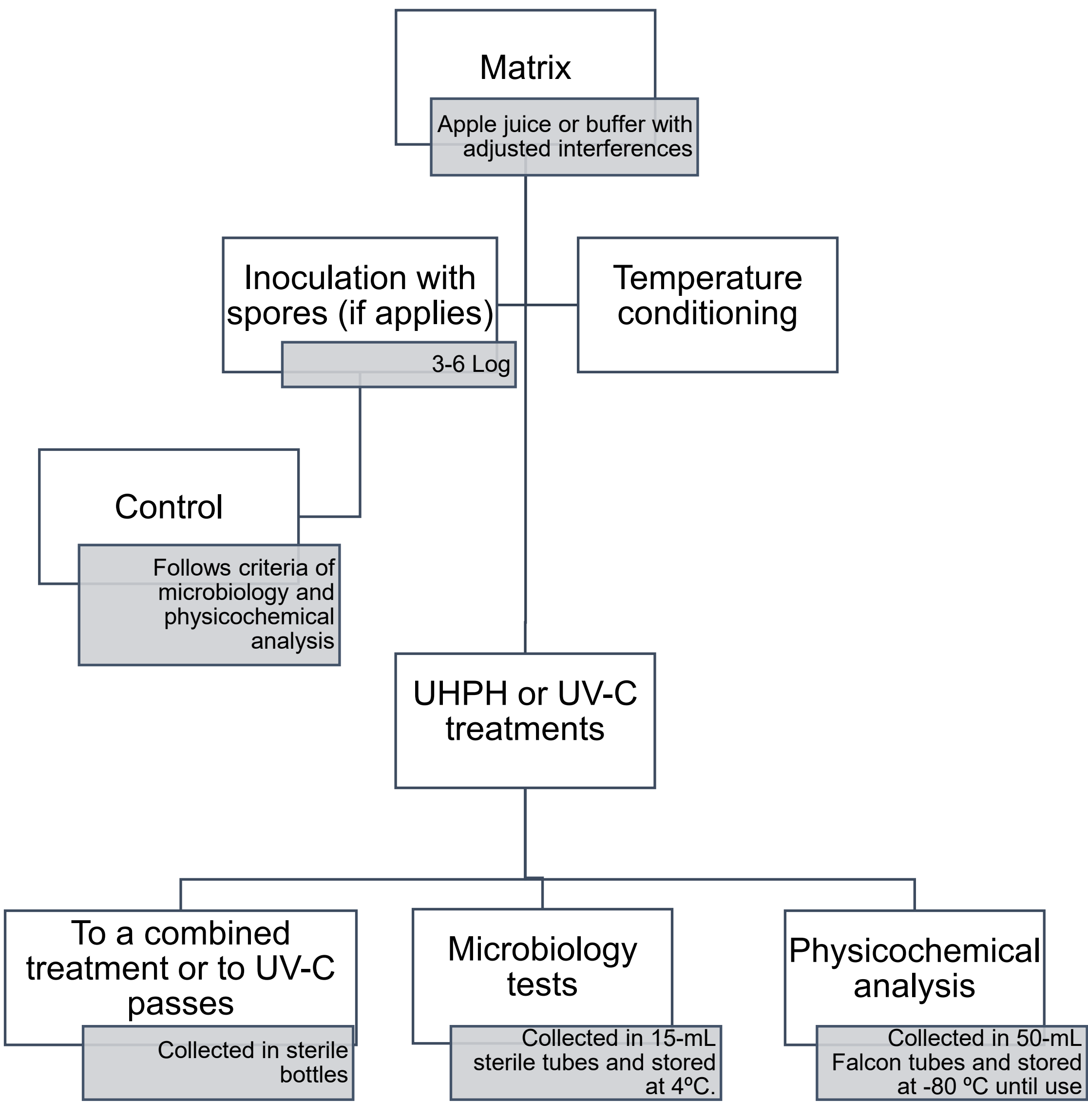
1) Evaluate the lethal effect that UHPH and UV-C have on different microbial spores inoculated in apple juice for the validation of the treatments and/or due to economical interests, and the type of complementarity that those technologies combined could have for that purpose.

2) Evaluate the resistance that microbial spores could oppose against UHPH and UV-C treatments due to their own characteristics.

3) Evaluate the degree in which interferences of the matrices due to their optical properties (absorption coefficient at 254 nm and turbidity) could oppose against the effectiveness of UV-C treatments.

4) Evaluate the degree in which UHPH and UV-C could affect the physicochemical properties, antioxidant capacity, stability and quality aspects of apple juice, including the acceptability of treated product.

2.3. Sampling protocol



3. Materials and methods

3.1. UHPH equipment

Two different UHPH machines were utilized in these experiments: 1) the Stansted FPG 12500 model (Stansted Fluid Power LTD., Essex, United Kingdom) with a flow rate capacity of 15 L/h and a pressure valve consisting in a combination of a needle and a seat made of zirconium with a cutting angle of 60 ± 0.5° in the needle and 45 ± 0.5° in the seat, which operated at a maximum pressure of 300 MPa, and 2) the Ypsicon Model A (Ypsicon S. L., Barcelona, Spain) of 60 L/h capacity with needle and seat model Ultra Shear Valve (Ypsicon S. L.) which operated at a maximum pressure of 300 MPa.

3.1.1. Cleaning and disinfection of the UHPH equipment

Cleaning and disinfection of the UHPH inner circuit makes use of the heating after the pressure valve in order to rise the temperature between 140 - 150 ºC. Before using, water from the network is heated by an external cauldron at 80 ºC approximately with the UHPH machine set at 300 MPa, that water passes through all the inner UHPH system all the way to the sampling outlet, from which it came in the form of water steam during 30 min. After treatments the procedure is repeated to rinse residues from the matrix without pressure and for 15 min. The circuit is treated with a solution of 15 L of 3% NaOH which recirculated at 80 ºC for 15 min. Finally, the system was rinsed with water from the network during 15 min at 20 ºC.

3.2. UV-C equipment

The UV-C reactor used was the UV-Therm model of Ypsicon (Ypsicon S.L.). This was of concentric ring type with a 70-mL capacity, and measures 76.5-cm long and 1-mm width (thin film). This reactor is composed by a feeding tank connected to a UV-C lamp (55 W) (LAB81055 NNI 150/76 XL, UV- Consulting Peschl España, Geldo, Spain) coated with a quartz tube. The irradiance was measured with an UVM-CP handheld radiometer and a calibrated sensor (90,155, UV-Consulting Peschl, Mainz, Germany) at a wavelength of 254 nm. The operational temperature was controlled by a heat conditioner. Treatments that implied to perform several passes throughout the circuit were done by varying the pumping speed of the

peristaltic pump of the UV-C reactor. The final UV-C dose received by the sample was calculated from the total amount of time that sample remained in the circuit exposed to the UV-C radiation from the lamp.

3.2.1. UV-C Dose calculation

It was calculated as described by Equation 2

$$E = l \cdot t$$

Where:

E = dose given in $\frac{J}{cm^2}$

I = the intensity that the matrix receives in $\frac{W}{cm^2}$

t = time that the matrix had spent exposed to UV light in s

3.2.2. Measurement of UV-C lamp's irradiance

3.2.2.1. Radiometry

Irradiance in mW/cm^2 was calculated by a radiometer (UVM-CP) and a calibrated sensor 90155 (UV-Consulting Peschl®, Mainz, Germany).

3.2.2.2. Actinometry

This procedure allows to calculate the UV-C radiation that a substance, in this case the actinometer, is receiving during its pass through the system by a photochemical reaction. The actinometer that was used corresponded to a potassium iodide/iodate one. This substance is optically transparent at wavelengths higher than 330, but irradiation provokes the linear production of triiodide, which can be read by spectrophotometry at 352 nm wavelength. Its preparation was done according to Rahn (1997) with 0.6 M potassium iodide and 0.1 M potassium iodate in a 0.01 M sodium borate (Panreac Química SLN, Castellar del Vallès, España) buffer with pH adjusted to 9.25, with 0.6 M potassium iodide (Panreac) and 0.1 M potassium iodate (Panreac). Samples were diluted in distilled water and read by spectrophotometer

Nanophotometer Pearl (IMPLEN, Munich, Germany) at 352 nm wavelength. The dose was then calculated using Equation 3 (Linden & Mofidi, 2004).

$$D_v = \frac{(A_{352nm})(P_{253nm})}{(pl)(\emptyset)(\varepsilon_{352nm})}$$

Where:

D_v = dose in J/L

A_{352nm} = absorbance at 352 nm

P_{253nm} = number of Joules per Einsteins of 253.7 nm photons ($4.716X10^5$ J $einst^{-1}$)

pl = pathlength of the cuvette

∅ = quantum yield (effects per photon in mol·$einst^{-1}$)

$$\emptyset = 0.73\big(1 + 0.23(Ci - 0.577)\big)\big(1 + 0.02(T - 20.7ºC)\big)$$

Ci = concentration of iodide = (A_{300nm}/1.061) = 0.7738

T = mean temperature (ºC)

ε_{352nm} = molar absorption coefficient of the triiodide at 352 nm (27,600 L/mol·cm)

3.2.3. Reynolds number calculation

Reynolds number (Re) was calculated with the equation $Re = \rho Vd/\mu$. Density (ρ) at 20 °C was measured with a density hydrometer (HYDR- 100-001) (Labbox, Vilassar de Dalt, Spain), *V* is fluid's velocity in m/s, *d* is the equivalent diameter inside the UV-C reactor (Ozbayoglu & Omurlu, 2006) of 0.001632 m, and dynamic viscosity (*μ*) in Pa·s was measured at 20 °C with a Haake RheoStress1 rheometer (Thermo Fisher Scientific, Inc., Karlsruhe, Germany).

3.2.4. Cleaning and disinfection of the UV-C equipment

Before any treatment, a solution of 0.1% sodium hypochlorite was recirculated for 10 min at 20 ºC. After that the equipment was rinsed with 10 L of decalcified water to eliminate residues. Finally, sterile water was recirculated during 10 - 15 min with both UV-C lamps turned on, and a sample of that water was taken from the reactor's

outlet as a blank in a 15-mL sterile tube before the treatment and plated on plate count agar (Oxoid, Basingstoke, UK) and in any of the pertinent medium according to the experiment. After treatments the circuit was recirculated with an alkaline soap solution Capture-VC16 (Diversey Spain, Viladecans, Spain) for 15 min at 60 ºC in order to remove residues from the matrix. Then that soap was rinsed with 10 L of water, and the circuit was once more disinfected with sodium hypochlorite for 10 min at 20 ºC. 10 L of water were pumped to rinse sodium hypochlorite.

3.3. Microorganisms, spore suspension preparation and plating media

3.3.1. *Alicyclobacillus acidoterrestris*

A. acidoterrestris CECT 7094 was obtained from the Spanish Type Culture Collection (CECT, University of Valencia, Valencia, Spain). *A. acidoterrestris* spores were prepared as explained by (Chang & Kang, 2004), the lyophile was recovered in K broth and incubated at 43 ºC for 48 h. That stock culture was refrigerated at 4 ºC. Sporulation was induced by transferring 1 mL of the stock culture to a 250-mL Roux bottle with 50 mL of potato dextrose agar (PDF) (Oxoid), and incubated at 43 ºC from 7 to 15 days until reaching 80% sporulation or higher according to phase-contrast microscopy screening. Spores were collected by pouring 30 mL of sterile distilled water into each bottle and the surface was scraped with a Digralsky stick, this suspension was collected inside 50-mL Falcon tubes and centrifuged at 10,000 *g* for 20 min at 4 ºC using a Sigma 4K15 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), the supernatant was discarded and the resulting pellet was washed times with 30 mL of sterile water and centrifuged with the same settings, this procedure was done four times. The pellet was resuspended in 40 mL of sterile distilled water and heated at 80 ºC for 20 min in order to inactivate vegetative cells. The suspension was cooled down in iced water and kept at 4 ºC until use.

Survival spores after treatments were plated and counted on SK agar (Chang & Kang, 2005) composed of: meat peptone 5 g (Oxoid), yeast extract 2.5 g (Oxoid), glucose 1 g (Panreac), Bacteriological Agar 15 g (Oxoid), Tween 80 1 mL (Scharlab, S.L., Sentmenat, Spain), and 1 L of distilled water. After autoclaving 2 mL of sterile

CaCl 10% (Panreac) and tartaric acid (Sigma-Aldrich, St. Louis, USA) 10% (adjust pH to 4) were added to the preparation. After inoculation, plates were incubated at 43 ºC for 1 – 2 days.

3.3.2. *Bacillus subtilis*

The strain *B. subtilis* CECT 4002, obtained from the Spanish Type Culture Collection (CECT), was sporulated based on the procedure UNE EN ISO 13704:2002 (Martinez-Garcia et al., 2019) The lyophile was recovered in glucose-tryptone broth: 2.5 g of yeast extract (Oxoid), 5 g of tryptone (Oxoid),1 g of glucose (Sigma-Aldrich), and 1 L of distilled water, its pH was adjusted to 7.2. It was incubated at 30 ºC for 24 h, 2 mL of the stock culture were transferred into Roux bottles containing yeast extract agar: 10 g of meat extract (Oxoid), 2 g of yeast extract (Oxoid), 15 g of agar (Oxoid), 0.04 g of MnSO4·H2O (Merck, Darmstadt, Germany), and 1 L of distilled water. These bottles were incubated at 30 ºC for a maximum of 30 days to induce sporulation. Spores were collected by pouring 20 mL of sterile distilled water into each bottle and the surface was scraped with a Digralsky stick, this suspension was collected inside 50-mL Falcon tubes and centrifuged at 10,000 *g* for 20 min at 4 ºC using a Sigma 4K15 centrifuge (Sigma Laborzentrifugen GmbH), the supernatant was discarded and the pellet was washed times with 15 mL of sterile water and centrifuged under the same settings, this procedure was done four times and finally resuspended in 30 mL of sterile distilled water. The suspensions were heated at 75 ºC for 10 min in order to inactivate vegetative cells. Suspensions were cooled down in iced water and kept at 4 ºC until use.

Survival spores after treatments were plated on glucose-yeast agar: glucose (Panreac) 20 g, yeast extract (Oxoid) 5 g, bacteriological agar (Oxoid) 12 g, distilled water 1 L; or on triptone-soya agar (Oxoid). Samples were plated in a double layer in order to prevent excessive growth, and incubated at 30 ºC for 2 days.

3.3.3. *Geobacillus stearothermophilus*

The strain *G. stearothermophilus* CECT 47, obtained from the Spanish Type Culture Collection (CECT), was sporulated on meat yeast agar following the European Standard 13704 (2002). It was incubated at 30 ºC until it reached 80% sporulation (8 – 10 days) or higher according to phase-contrast microscopy screening. The

recovery of the spores was done as also explained in 3.3.2. Vegetative spores were inactivated by heating the spore suspensions at 80 ºC for 10 min. They were cooled down in an ice bath and kept at 4 ºC until use.

Survival spores after treatments were plated on glucose-yeast agar and incubated at 45 ºC for three days.

3.3.4. *Aspergillus niger*

The strain *A. niger* CECT 2574, obtained from the Type Culture Collection of Spain (CECT), was rehydrated in 9 mL of Tryptic Soy Broth (Oxoid) and incubated at 30 ºC for 24 h. This cultured broth was used to inoculate Petri dishes with potato dextrose agar (Oxoid) and put in incubation at 30 ºC for 7 days. After incubation, 3 mL of nutrient broth (Oxoid) were added into the Petri dishes and the surface was scratched with a Digralsky spreader. One mL of the resulting suspension was transferred into 9 mL of nutrient broth and incubated at 30 ºC for 24 h. Two mL of that culture were used to inoculate Roux flasks containing potato dextrose agar and incubated at 30 ºC until the maximum sporulation rate was reached, as an indicator when the colour of the culture turned black. Then, spores were recovered with 10 mL of sterile distilled water with Tween 80 at 0.05 % (v/v) with the aid of a Digralsky spreader. The obtained suspension was filtered in a sterile Büchner funnel with a pore size of 45 µm (Labbox). All spore suspensions were collected in sterile conditions and stored at 4 ºC until use.

Survival spores after treatments were plated on plate count agar and incubated at 30 ºC for three days.

3.3.5. *Talaromyces macrosporus*

T. macrosporus CBS 130.89 was supplied from Westerdijk Fungal Biodiversity Institute (Netherlands) as a freeze-dried culture. Cultures were revived in malt-peptone broth composed of malt extract 1.0% (Biolife, Milano, Italy) and peptone water 1.5% (Oxoid) during 4 hours at 20 ºC, Broth was inoculated into Petri dishes with potato dextrose agar (Oxoid) and incubated for 3 weeks at 25 ºC. Plates were flooded with 10 mL of sterile water with 0.05% of tween 80 (Scharlab) and the surface was scratched with a Digralsky spreader and filtered over a Buchner funnel

(40-60 µm pore diameter) (Labbox). Ascospore suspensions were diluted with malt-peptone broth in a 1:1 volume ratio and inoculated into Roux bottles containing potato dextrose agar, they were incubated during 5 - 7 weeks at 25 ºC. Ascospores were recovered in 10 mL of water with 0.05% of Tween 80 by scratching the surface of the culture with an inoculation loop and a Digralsky spreader. The suspensions were collected in 50-mL Falcon tubes, vortexed with sterile glass beads (2 mm diameter) to break down clumps until homogenous (2 – 3 min) (Chapman et al., 2007), sonicated in a Branson 2510 sonicator (Branson Ultrasonics, Danbury, USA) 3 times during 2 min in order to disrupt asci and release the ascospores. Suspensions were filtered over a sterile Buchner funnel packed with glass wool (Dijksterhuis et al., 2002). Ascospore suspensions were centrifuged at 13,751 g for 15 min at 4 ºC and washed with sterile water 4 times. The resulting pellets were suspended in 10 mL of water and counted by haemocytometer (Bürker) (Brand, Wertheim, Germany). Suspensions were kept at 4 ºC until uses for up to 2 weeks, and cell integrity was observed by microscope with trypan-blue staining (Gibco, Grand Island, USA) before every experiment.

Survival spores after treatments needed a heat-shock of 10 min at 85 ºC (Dijksterhuis & Teunissen, 2004) in a water bath in order to germinate, then they were cooled down on the water stream. Samples were plated on malt extract agar (Oxoid) before and after heat-shock and incubated at 25 ºC for 3 days.

3.3.6. *Neosartorya spinosa*

N. spinosa CBS 586.90 was obtained as freeze-dried culture from the Westerdijk Fungal Biodiversity Institute. The revival procedure and spore suspension preparation were followed as explained in 3.3.5.

Heat-shock was done in a water bath for 1 hour at 70 ºC (Splittstoesser et al., 1993) to promote germination, then they were cooled down on the water stream. Samples were plated on malt extract agar (Oxoid) before and after heat-shock and incubated at 25 ºC for 3 days.

3.4. Matrices

3.4.1. Phosphate-buffered saline (PBS) with caramel

PBS (Panreac) was added with caramel (Grupo Carinsa, Sant Quirze del Vallès, Spain) to adjust its absorbance coefficient at 254 nanometres wavelength (α_{254}) to the values of 7, 26, 170 and 220 cm^{-1} following the method explained by Murakami & Schickedanz, (2000) using quartz cuvettes of 0.5- and 1-cm depth. Absorbance was measured at 254 nm with a Ultorospec 2100 pro spectrophotometer (GE Healthcare, United Kingdom).

3.4.2. PBS with apple fibre

PBS (Panreac) was added with apple fibre (The Hut Group, Manchester, U. K.) in different known concentrations and they were autoclaved at 121.5 ºC for 15 min. After that the turbidity was measured with a turbidimeter Hach 2100Q (Hach Lange Spain, L'Hospitalet de Llobregat, Spain) at 20 ºC. The different turbidity values and concentrations were plotted in Microsoft Excel® 2013 and showed a linear behaviour, which allowed to extrapolate concentrations in order to calculate the desired final turbidity of the sample.

3.4.3. Clarified apple juice

Clarified apple juice, untreated (Golden delicious var.) and Ultra High Temperature (UHT) treated, were purchased from local providers or extracted from locally-purchased apples (Golden delicious var.). Juices were characterized in every experiment as needed.

Cloudy apple juice was clarified mechanically or preceded by an enzymatic treatment. The extracted juice was first centrifuged in a centrifuge model Eppendorf 5804R (Eppendorf Ibérica, San Sebastián de los Reyes, Spain) in capsules of 250-mL capacity at 3100 *g* per 10 min, and then it was filtered through Whatman #4 by vacuum. Clarification involving an enzymatic treatment was done by adding 100 µL/L of Pectinex® Ultra Clear (polygalacturonase) (Novozymes, Bagsværd, Denmark) and left at 6 ºC overnight. Centrifugation and filtration were followed after the enzymatic treatment as described above without vacuum.

3.4.4. Cloudy apple juice

Cloudy apple juice (Golden delicious var.) was obtained from local producers with or without 300 ppm of ascorbic acid added at the moment of extraction according to producer. Juices were characterized in every experiment as needed.

3.5. Physicochemical analysis

3.5.1. pH and ºBrix

They were measured with the undiluted sample at 20 ºC. pH was measured with a a pH-meter pH básico-20 (Crison, Barcelona, Spain), and ºBrix by a Spectronic Instruments refractometer (Rochester, N.Y., USA).

3.5.2. Absorbance

It was measured at 20 ºC by spectrophotometer NanoPhotometer Pearl (IMPLEN) and a quartz cuvette (Fisher Scientific Inc.) of 10-mm depth for readings within the ultraviolet spectrum, and in disposable plastic cuvettes of 10-mm depth for readings within the visible spectrum. Samples were diluted with distilled water as necessary to fit into the range of reading (0 - 2) according to the desired wavelength.

3.5.3. Turbidity

It was measured at 20 ºC by turbidimeter Hach 2100Q (Hach Lange Spain). with the sample as diluted as necessary with distilled water to fit into the range of reading (0 - 1000).

3.5.4. Colour

Colour was measured with a colorimeter (MiniScan XETM, Hunter Associates Laboratory Inc., Reston, USA.) in the CIELAB colour space which comprehends *L** (lightness), *a** (red to green) and *b** (yellow to blue) coordinates. The total colour difference (ΔE^*) between the raw juice and treated samples was calculated following Eq. 4 (Velázquez-Estrada et al, 2019):

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}. \text{ Equation 4}$$

Colour reproduction was done by putting the value of the coordinates in the online tool colorizer.org.

3.5.5. Browning

This essay was followed as explained by Meydav et al. (1977). Samples were filtered by syringe filters of 0.45 μm pore diameter (Labbox) and mixed with ethanol 1:1 ratio. Samples were read by spectrophotometer (IMPLEN) and the results were reported as absorbance at 420 nm (A_{420}).

3.5.6. Particle size distribution

it was measured by a laser diffraction particle size analyzer Mastersizer Micropulus 2.15 (Malvern Instruments, Inc., Works, United Kingdom). Samples were diluted in distilled water until optimal obscuration level for the laser (5 - 15%). An optical model based on Mie scattering theory by spherical particles was applied using the following conditions: refractive index for the dispersed phase = 1.471; refractive index of dispersion phase (water) = 1.334. The size distribution was characterized by the mean diameters D[3, 2] and D[4, 3].

3.6. Enzymatic activity

3.6.1. Polyphenol oxidase (PPO)

That assay was performed adapting the methodology of Cano et al. (1997) using 37.5 μL of juice filtered by syringe filters (0.45-μm pore diameter) (Labbox) and 1.5 mL of 0.7 M catechol (Sigma Aldrich) in phosphates buffer (pH 6.5). The absorbance of samples was read at 420 nanometres wavelength (A_{420}) by spectrophotometer (IMPLEN); absorbance changes (ΔA_{420}) were recorded before and after incubation at 20 ºC for 20 min; ΔA_{420} of the treated samples were compared with that of the untreated juice which represented a 100% PPO activity, and results were expressed as % PPO residual activity.

3.6.2. Pectin methylesterase (PME)

Residual activity of PME was done by adapting the method of Carbonell et al. (2013) using a solution of 1% citrus pectin (Sigma Aldrich) and 0.2 M of NaCl (Sharlau, Sentmenat, España). pH was adjusted to 8.0 with NaOH 4N (Panreac). The employed volumes for each test were of 20 mL of pectin solution and 5 mL of sample. The mix was titrated by 0.1N of NaOH (Panreac) by an automatic titrator

Titrando 842 (Metrohm, Heri- sau, Switzerland) until it reached a pH of 7.8. pH changes were recorded during 30 min at 20 ºC and ΔpH/Δt of the treated samples were compared with the untreated juice which represented a 100% PME activity. Results were expressed as % PME residual activity.

3.7. Antioxidants analysis

3.7.1. Total polyphenols

They were measured based on the method of Singleton et al. (1998). The filtered sample was diluted with distilled water in equal volumes. Samples consisted of a mix of 100 µL of sample or gallic acid standard (Sigma Aldrich), 0.75 mL of 0.2N Folin-Ciocalteau (Sigma Aldrich), and 0.75 mL of 6% sodium carbonate anhydrous (Panreac). Samples were read by spectrophotometer (IMPLEN) at 760 nm. Results were expressed as gallic acid equivalent in mg/L.

3.7.2. Antioxidant capacity

For antioxidant capacity assays on apple juice, samples were filtered by syringe filters (Labbox), and it was also employed Trolox (Sigma Aldrich) at different concentrations (50 - 1000 µM) as the equivalent standard. Assays were performed by two methods: (1) FRAP (Ferric-ion Reducing Antioxidant Power) method was based on the method explained by Benzie & Strain (1996) with the apple juice sample diluted with water in a 1:1 proportion. The mix consisted on 90 µL of diluted sample or standard, 270 µL of distilled water and 2.7 mL of FRAP reagent pre-warmed at 37 ºC. The mix samples were stored at 37 ºC for 30 min and read by spectrophotometer (IMPLEN) at 593 nm wavelength. (2) DPPH method (radical scavenging activity) was based on the method explained by Bondet, et al. (1997). The mix consisted in 80 µL of sample and 2.92 mL of DPPH reagent (Sigma Aldrich). Samples were incubated at 25ºC for 1 hour and read by spectrophotometer (IMPLEN) at 515 nm wavelength. Results were expressed in Trolox equivalent (µM).

3.7.3. Partial characterization of phenolic compounds by HPLC

The aglycon content of the phenolic compounds was quantified by doing a hydrolytic excision of polyphenol glycosides following the method of Taga et al. (1984) with modifications proposed by López-Hernández & Ortega-Villarreal (2014): 5 mL of

sample were dissolved in 5 mL of methanol with HCL (6N). Samples were tightly closed and heated at 100 ºC for 45 min and cooled down to 25 ºC. Samples were filtered over Whatman 1 and extracted with 2 ml of diethyl-ether three times. The aqueous fraction was discarded and the organic part was evaporated to dryness. The residue was resuspended in 1 mL of methanol 70% HPLC grade.

HPLC (high-performance liquid chromatography) analysis was done in a Thermo Scientific Spectra System provided with a UV/visible detector fixed in 350 nm and a column Purospher Star RP-C18 (250 mm x 4.6 mm x 5 µm) (Merck). It was used the technic described by Hempel & Böhm (1996) with some modifications in the elution gradient and in the pH of the mobile phase. The standards were gallic acid, caffeic acid, chlorogenic acid, myricetin, apigenin, kaempferol, and quercetin (Sigma-Aldrich). The mobile phase consisted in a gradient of water-acetic acid (98:2) and acetonitrile HPLC grade, with a flux of 1 mL/min and a runtime of 17 min. Ten µl of each one of the samples and standards were filtered by Millipore 0.45 µm and injected for analysis. Both, hydrolysed and non-hydrolysed samples were analysed with the purpose of finding whether the compounds of interest were found in a conjugated state or not.

3.8. Statistical analysis

Averages, standard deviations and analysis of variance (ANOVA) were done by using the generalised linear models (GLM) via software (Statistica ver 7, StatSoft, Inc) with a number of 6 independent samples (n = 6). Tukey or Games-Howell tests were employed for comparisons between samples means. Differences were considered as significant when $p < 0.05$.

3.9. Inactivation kinetics of microorganisms

Survival data of microorganisms were adjusted to a linear/non-linear model by using GInaFiT software add-in for Microsoft® Excel (Geeraerd et al., 2005). The most suitable model was chosen according to the lowest root mean square error and the highest R^2 coefficient.

3.10. Sensory evaluation

Sensory evaluation was carried according to ISO 8587:2006 which describes a method that allows to sort a series of samples in rank order based on the intensity of a single attribute or an overall (ISO 8587:2006).

4. Results and discussion

4.1. Effect of UHPH on microorganisms

The effect of UHPH on microorganisms varies depending on many factors. The main targets of this technology are their outer cell layers, so it could be expected that one of the greatest limitations would be the spore state that microorganisms can present like some rod-shaped bacteria and moulds because that provides them with a high resistance against many types of stresses. In that regard it could be observed that bacterial spores were the most resistant ones among all the tested microbial spores (Table 7). The high resistance of spores against UHPH treatments could be explained due to their structure than grants them protection through the many covering layers and consituents that vegetative cells don't posses (Black et al., 2009). The best results when innactivating bacterial spores came with a combination of the highest assayed UHPH pressure of 300 MPa and the use of higher inlet temperatures. *A. acidoterrestris* and *B. subtilis* spores inoculated into apple juice could be reduced in almost 5 Log when 300-MPa UHPH were applied at an inlet temperature of 80 ºC. Those results were in consonance with those of Roig-Sagués et al. (2015) who obtained more than 5 Log inactivation of *A. acidoterrestris* and *Alicyclobacillus hesperidum* after 300-MPa UHPH at 80 ºC InT in apple juice. Other experiments with *B. subtilis* and other bacterial spores report that it is only at high InT (75 – 85 ºC) when 300-MPa UHPH become effective in inactivating them (Amador-Espejo et al., 2014; Dong et al., 2015). Although the inactivation mechanisms by UHPH have not been fully elucitaded, it is suspected that the short increase of temperature at the pressure valve along with the rest of the mechanical forces are the causes behind cell disruption (Dumay et al., 2013). Similar to that observation, Lee et al. (2002) reports that *A. acidoterrestris* spores are better inactivated with pressure (HHP) when it is applied at higher temperatures. From all the assayed microbial spores, the only one who did not present a high resistance to UHPH was *A. niger*, which was inactivated at the range of 5 Log after 300-MPa UHPH at 20 ºC of InT. Those results were in accordance with the experiments perfomed by McKay (2009) with *A. niger* and other phylamentous fungi in which the concentration of conidiospores was even higher, with about 6 Log inactivation after utilizing pressures between 250 – 300 MPa at 20 ºC of InT. Different from those

results were obtained when ascospores of *T. macrosporus* and *N. spinosa* were assayed, those fungal spores are known for their resistance to heat and pressure as some previous research point out (Dijksterhuis & Teunissen, 2004; McKay, 2009; Reyns et al., 2003). In the case of *T. macrosporus* spores it is suspected that its high content of threhalose protects membranes and proteins by preventing them from denaturation (Dijksterhuis et al., 2002).

Table 7. Summary of UHPH experiments with equipment from Stansted FPG 12500 and Ypsicon model A machines, and the lethal effect on microbial spores inoculated into different types of matrices. UHPH was assayed varying pressure and inlet temperature (InT).

UHPH Equipment	Matrix	Microrganism	Pressure (MPa)	InT (ºC)	Inactivation spores/mL (log10)	Source
Stansted	PBS	*A. acidoterrestris*	100	20	0.07 ± 0.05	Paper 1
			200		0.2 ± 0.2	
			300		0.46 ± 0.23	
Stansted	Clear apple juice		300	20	0.23 ± 0.15	Paper 3
				40	0.63 ± 0.31	
				60	1.83 ± 0.25	
				80	4.80 ± 0.26	
Stansted	Clear apple juice with chia extract		200	20	0.71 ± 0.11	Paper 6
Ypsicon	Cloudy apple juice		200	20	0.29 ± 0.04	Paper 3
			300	20	0.77 ± 0.09	
Stansted	Cloudy apple juice with ascorbic acid		200	20	0.24 ± 0.28	Paper 5
Stansted	PBS	*B. subtilis*	100	20	0.16 ± 0.23	Paper 1
			200	20	0.29 ± 0.2	
			300	20	0.58 ± 0.23	
				50	2.37 ± 0.5	
				70	5.53 ± 0.25	
Ypsicon	Cloudy apple juice		200	20	0.03 ± 0.1	Paper 3
			300	20	0.02 ± 0.06	

Table 7 (cont.)

UHPH Equipment	Matrix	Microrganism	Pressure (MPa)	InT (ºC)	Inactivation spores/mL (log10)	Source
Stansted	PBS	*G. stearothermophilus*	100	20	0.24 ± 0.08	Paper 1
			200		0.41 ± 0.25	
			300		0.68 ± 0.24	
Stansted	PBS	*A. niger*	100	20	1.77 ± 0.4	Paper 1
			200		3.93 ± 0.55	
			300		5.1 ± 0	
Stansted	Clear apple juice	*T. macrosporus*	100	20	0.02 ± 0.85	Paper 2
			200		0 ± 0.87	
Stansted	Clear apple juice	*N. spinosa*	100	20	0.01 ± 0.03	Paper 2
			200		0.22 ± 0.1	

High inactivation rates were observed in indigenous microbiota of apple juices when treated even at 200-MPa UHPH and at room temperature (20 ºC). It would be expected to have all kinds of microorganisms in a fruit matrix, not only spores but mostly vegetative cells, because sporulation is triggered as a mean of cell protection in the presence of a harsh environment and in scarcity of the nutrients and energy required for cell growth, which is not the case when they are present in a food matrix, and neither because not all microorganisms are able to sporulate. The presence of spores in fruit products could be mainly attributed to contamination from soil (Bevilacqua et al., 2007), therefore there are always chances to find spores contaminating these products. Other experiments with UHPH in food matrices (Codina-Torrella et al., 2018; Donsì et al., 2009; Patrignani et al., 2019; Suárez-Jacobo et al., 2010; Valencia-Flores et al., 2013; Velázquez-Estrada et al., 2012) are in accordance with the results in Paper 6, in which it could be seen that UHPH treatments at 200 MPa were able to reduce the quantity of aerobic mesophiles comparing it with the inicial load, and the level of inactivation increased with higher inlet temperatures, not being detectable on plates from treatments at 75 ºC-InT. The microorganisms which were vulnerable the most against these treatments were faecal coliforms, moulds and yeasts and lactobacilli, for they were not detected on plates right after UHPH treatments under those conditions. UHPH can inactivate vegetative cells due to cell disruption, but vulnerability against this technology

among microorganisms, even being all vegetative cells, can depend on their membrane composition (Vachon et al., 2012). A follow-up on the shelf-life of apple juice with chia extract (Paper 6) revealed that it could be preserved at 5 ºC for the recorded storage time which was of 30 days, but presenting microbial growth from survival cells towards the second week of storage for a consequent drop down in the count of microorganisms on plate at day 30. However, there was no detection of any odd smell or changes in the aspect of those treated samples even after 2 months of storage. A similar behaviour was observed by Suárez-Jacobo et al. (2010) on aerobic mesophiles in apple juice treated at 200-MPa UHPH and stored at 4 and 20 ºC.

4.2. Effect of UV-C on microorganisms

4.2.1. Effect of UV-C on microorganisms inoculated in PBS

UV-C was highly effective in inactivating all types of microorganisms when inoculated in PBS. The only microorganisms that opposed a higher resistance against UV-C were *A. niger* and *B. subtilis*. *A. niger* is known for its dark-coloured pigmentation (melanin) which is able to absorb UV-C radiation (Anderson et al., 2000), that represented a natural interference against UV-C by blocking its penetration and making it harder to reach the genetic material for spore-inactivation purposes; and *B. subtilis* due to the presence of proteins that protect DNA and for its mechanisms of DNA repair (Setlow, 2006). When interferences were added to PBS the energy expenditure had to increase in order to keep the same inactivation rates (Table 8). The main reasons for that were the presence of interferences from two kinds, absorption coefficient at 254 nm wavelength (α_{254}) from soluble and coloured compounds (caramel), and turbidity from suspended solid matter (apple fibre). Suspended solid matter can also influence α_{254} that absorbs light preventing it from penetrating further into the matrix, but it mainly contributes to the phenomena of light scattering and reflection (Gayán et al., 2012). From these experiments it could be observed that as the interference value increased, the inactivation rates decreased. Prove to that were the different experiments with *B. subtilis*, when treated in PBS alone, the minimum tested dose to achieve more than 5 Log was of 7.2 J/mL, while a higher dose of 14.3 J/mL could reach an average of 4.03 and 0.97 Log inactivation in PBSC (phosphate-buffered saline with caramel) with α_{254} values

of 7 and 26 cm^{-1} respectively. The inactivation pattern that was observed with *B. subtilis* in PBSC with the increasing α_{254} values was exponential with tail (Paper 1), that meant that at a certain point an increase in the α_{254} value will not represent any drastic changes in spore inactivation, probably because that the spores that were being inactivated were those ones in closest contact with the UV-C source, being that one unable to penetrate furthermore into the matrix. When the interference in PBS was fibre it could also be observed that a successful inactivation of *B. subtilis* and *A. acidoterrestris* spores depended on the use of higher doses than in PBS alone, but once again it could be observed that the inactivation of bacterial spores was also related to the microorganism *per se*. *A. acidoterrestris* spores could only be detected in samples with the highest assayed turbidity value (3000 NTU) and the lowest assayed UV-C dose (14.3 J/mL), while for the same treatments conditions, *B. subtilis* spores were always detected after treatments (Table 8). Those results were in consonance with the ones obtained in PBS alone. Other authors have also tested the required UV-C doses emitting at 254 nm to achieve 100% inactivation of many types of microorganisms and a compilation of these results can be found in the review of Guerrero-Beltrán & Barbosa-Cánovas (2004), in them it can be seen that the minimum required dose varies according to the microorganism with doses ranging from 25 to 4400 J/m^2.

Table 8. Phosphate-buffered saline inoculated with microbial spores and with modified interferences from the addition of caramel (PBSC) or from the addition of apple fibre (turbidity in NTU). UV-C treatments were done in a single pass and the dose was expressed in J/mL. Inactivation rates were expressed in Log_{10} with the corresponding standard deviation value when survival spores were detected on plates. Otherwise the inactivation value corresponded to the highest assayed spore concentration in the experiments.

Matrix	Microorganism	Dose (J/mL)	Inactivation (log10)	Source
PBS	*A. acidoterrestris*	1.8	5.1	Paper 1
	B. subtilis	7.2	5.3	
	G. stearothermophilus	1.8	5.2	
	A. niger	21.5	4.1	
	T. macrosporus	1.8	5	Paper 2
	N. spinosa	1.8	5.2	
PBSC (7 cm^{-1})	*B. subtilis*	14.3	4.03 ± 0.2	Paper 1
PBSC (26 cm^{-1})			0.97 ± 0.2	
PBS apple fibre 2000 NTU	*B. subtilis*	28.7	3.95 ± 0.1	Paper 5
PBS apple fibre 2500 NTU			2.45 ± 0.1	
PBS apple fibre 3000 NTU			2.8 ± 0.1	
PBS apple fibre 3000 NTU	*A. acidoterrestris*	14.3	3.66 ± 0.1	
		21.5	5.25	

4.2.2. Effect of UV-C on microorganisms inoculated in apple juices

In food matrices, all of the aforementioned interferences come together as part of the product's own properties or characteristics. In fruit juices, the presence of coloured compounds, soluble antioxidants like ascorbic acid and suspended matter like fibre will always play an important role in the optical properties of the product, that at the same time affects the approach in which the UV-C treatment should be carried out and its final outcome. The apple juices assayed in this research presented different types and degrees of interferences. A clarified apple juice would present interferences mostly related to the absorption coefficient, even more if that juice was added with ascorbic acid at the time of extraction in order to prevent it from polyphenol oxidation. A cloudy apple juice would not only have that value increased but also the one of turbidity. Table 9 gathers some of the optical properties, ºBrix and pH of many types of apple juices that can be purchased in the area of Barcelona, Spain. Due to the differences between juices in their optical properties it could be expected that a certain UV-C treatment might be able to

successfully treat some types of juices while others would need higher doses to achieve the same results. There was a wide amount of results according to the type of juice used in these experiments as matrix. Usually, clarified apple juices presented no much problem in inactivating *A. acidoterrestris* spores (Papers 3 and 6) and others like *T. macrosporus* and *N. spinosa* (Paper 2). Although those apple juices came from different productions, the characteristics they all had in common was they went through a clarification process and the lack of common additives like ascorbic or citric acids. *A. acidoterrestris* spores could be inactivated in more than 5 Log after a dose of 21.5 J/mL at any of the assayed InT (20, 40 and 60 ºC) being the treatment given at 60 ºC the one with a slight inactivation increase compared to the others. A synergistic or additive effect between moderate inlet temperatures (40, 45, 50 ºC) and UV-C was also observed by García Carrillo el al., (2017) according to the microorganism. They tested *Escherichia coli*, *Saccharomyces cerevisiae* and *Pseudomonas fluorescence*, all vegetative cells in carrot-orange juice blend. Similar results were obtained by Gayán et al. (2012) with *E. coli* in orange juice, they reported a maximum synergistic effect at 13.55 J/mL treated at 55 ºC.

Table 9. Clear and cloudy commercial juices were screened in their optical properties of α_{254} (cm^{-1}), turbidity (NTU), ºBrix and pH. The table also shows if the juices contained any type of additives.

Apple juice brand	Type	α_{254} (cm^{-1})	Turbidity (NTU)	ºBrix	pH	Additives
Pink Lady Cold Press	Clear	8.67	0.37	3.9	3.9	Ascorbic acid
Tropicana	Cloudy	103.87	2720	10	3.51	Ascorbic acid
Carrefour	Clear	4.99	1.09	7	3.14	Citric acid, water, sugar
Disfruta	Clear	9.39	0.52	4.5	3.23	Ascorbic acid, citric acid, water, sugar
Don Simón	Clear	18.12	0.56	10	3.45	Antioxidants
Lambda	Clear	12.56	0.85	10	3.83	No additives
Zumosol	Cloudy	106.47	3910	11	3.58	No additives

Inactivation rates of *A. acidoterrestris* treated at 20 ºC decreased in cloudy apple juice (Paper 3) most likely because of its increased turbidity value from 0.56 to 2357 NTU. The reductions rate dropped down to 57 - 60 % comparing it with results with clear apple juice in treatments with the same UV-C dose (21.5 and 14.3 J/mL

respectively). Although it is well known that turbidity could be one of the most limiting factors when applying UV-C treatments for sterilisation purposes, studies comparing the results in clarified juices against their cloudy counterparts are scarce in literature. One of those studies was conducted by Kaya & Unluturk (2016) with clear and cloudy grape juice treated in a collimated beam UV-C reactor at 254 nm. They evaluated the lethal effect on *S. cerevisiae*, yeast, moulds and lactic-acid bacteria, and in their conclusions, they also state that clear grape juice was more easily treated than the cloudy one. Although these experiments with apple and grape juices where performed in UV-C equipment of different designs (thin film and collimated beam respectively), the results when facing higher levels of interferences were similar, and there are chances that the same phenomena could be observed in all types of UV-C reactors. In order to overcome those problems in a thin-film reactor, like the one used in these experiments, a same final UV-C dose was applied in different recirculation approaches in order to try to expose as much volume of the matrix as possible. Experiments in PBSC inoculated with *B. subtilis* spore demonstrated that even at low doses (7.2 J/mL) an increase in the number of passes (3 and 4 passes) of the matrix through the reactor also increased the inactivation of these spores significantly compared with a single pass (Paper 1). Similar to those results were the ones with ascospores, *T. macrosporus* and *N. spinosa* inoculated in clear apple juice (Paper 2). In *T. macrosporus*, which was more resistant to UV-C than *N. spinosa*, for every assayed dose (7.2, 14.3 and 21.5 J/mL) the inactivation was significantly higher in treatments with greater number passes (1, 2 and 3 passes). *N. spinosa* also showed an increase in its inactivation with greater number of passes up to the dose of 14.3 J/mL, because 21.5 J/mL were enough to inactivate these spores to the point of not being detected on plate. The use of many passes has been documented as usual in the treatment of fruit juices or matrices by the different types of UV-C reactors (Koutchma et al., 2016). Martinez-Garcia et al. (2019) conducted experiments with milk, which is a matrix of high absorption coefficient at 254 nm and turbidity, inoculated with *B. subtilis* spores. In their work they introduce the concept of NET (number of entries to the tunnel), which is the number of passes that the matrix goes in and out the reactor for a given UV-C dose, with the purpose of increasing the time that the spores remain in the effective distance from the UV-C source. That approach is different from UV-C treatments in

a single pass, because in that case the pump speed varies in order for the sample to stay within the lamp area time enough to be irradiated until it receives the desired dose. In recirculation treatments, the pump is set to a fixed speed in which the matrix passes through the system in a closed circuit, and the dose depends on the time it is being left in recirculation. They observed that the most the NET value increased so did the inactivation of *B. subtilis* spores as well. The same approach was utilized in the experiments with cloudy apple juice and ascorbic acid (300 ppm) (Paper 5) which showed the highest interferences in its optical properties among all the tested apple juices. This juice was inoculated with spores of *A. acidoterrestris* and results showed that UV-C doses in a single pass could not reach further into the matrix and for that reason the inactivation rates were very much low, not even the highest assayed dose of 125 J/mL it could achieve 1 Log inactivation. A different outcome was observed when the same doses were applied with the pump speed set at 500 rpm (64.4 mL/s), after 50 J/mL the line of 2 Log inactivation was surpassed, and more than 5 Log after 125 J/mL.

4.3. Effect of combined UHPH and UV-C treatments on microorganisms

It was noticeable that none of these technologies were perfect in terms of inactivating the broad range of microorganisms in food matrices. UHPH because it had to be combined with mild temperatures at 300 MPa in order to be able to inactivate many of the tested microbial spores, and UV-C because depending on the type of food matrix, its optical properties could represent interferences that will not allow it to reach through. If the two technologies were combined, then an additive or synergistic effect could possibly be observed in inactivating microorganisms, because the effect of UHPH is not affected by the optical properties of foods, and UV-C was more efficient in inactivating bacterial and some fungal spores. For those reasons UHPH and UV-C were tested together in some of the experiments.

The combination of those technologies has been tested so far by our group. In order to test the effect that the combination of technologies could have in microorganisms there were different assays in PBSC inoculated with *B. subtilis* spores and treated in the order UHPH – UV-C and *vice versa* (Paper 1). From those experiments it was observed an additive effect between the two technologies in the inactivation rates

of *B. subtilis.* The type of effect is additive if the combination of technologies achieves inactivation rates equal to the sum of the results obtained by the same technologies applicated separately. There were no statistical differences in the order in which the treatments were applied. The maximum inactivation rates of about 1.53 Log were obtained with the UV-C dose of 14.3 J/mL and 200-MPa UHPH (70 ºC InT). A different outcome was observed when treating ascospores of *T. macrosporus* and *N. spinosa* in clear apple juice. The inactivation rates in combined treatments of either 100 and 200 MPa of UHPH at an InT of 20 ºC and combined with UV-C (7.2, 14.3 and 21.5 J/mL) (Paper 2) were significantly higher than in those treatments applied separately. In that case, the observed effect was synergistic rather than additive, because in a synergistic effect the observed results is observable higher than the sum of the inactivation rates of the technologies applied separately. UHPH was apparently ineffective in inactivating these ascospores, there was only a small reduction of *N. spinosa* spores (0.3 Log) treated at 200 MPa, but that did not happen to *T. macrosporus*. Due to those results it could look like UHPH did not affect the aforementioned ascospores, but scanning electron microscopy could give a closer insight to the possible reason of why a combination between UHPH and UV-C in that direction presented a synergistic effect, and that could be attributed to a sublethal damage from UHPH as it can be seen in Paper 2. Samples of spores treated at 200-MPa UHPH were compared against those ones from UV-C treatment at 21.5 J/mL and the untreated ones, and it could be observed that there were changes in the outer structure of spores only after UHPH treatments. That could be expected because UHPH technology focuses on the damage occasioned by physical phenomena such as pressure changes, sheer stress, cavitation and others, while UV-C damages the genetic material of cells if able to reach it. UHPH sublethal damage could have left spores better exposed to UV-C activity, and consequently, leading to higher inactivation rates than in UV-C-only treatments. Similar results were observed by (Reyns et al, 2003) submitting *T. macrosporus* spores to high hydrostatic pressure (600 MPa, 15 s, 20 ºC), their results from scanning electron microscopy suggest that there was a change in spore permeability after pressure treatments and spores collapsed when exposed to air. Those changes in permeability could have made possible to UV-C to penetrate better into the cell and be able to inactivate them.

A combination of UHPH and UV-C was also applied in cloudy apple juice inoculated with spores of *A. acidoterrestris* (Paper 3). Once again, the type of effect that was observed between those two technologies was additive. That effect could be better seen in treatments at 200 MPa and the doses of 14.3 and 21.5 J/mL, but not in the combined treatments at 28.7 J/mL which was the highest assayed dose. In that case a single UV-C treatment could inactivate those spores better than in combination with UHPH, most likely due to the changes in particle size after UHPH treatments which resulted in different diameter and distribution according to particle size tests (Paper 4) and that also changed the turbidity value of the juice. Different inactivation rates could be expected in matrices with different optical properties like turbidity.

4.4. Physicochemical stabilisation in apple juices by UHPH-processing

Treatments in apple juices brought different types of changes, and some of them contributed to the physicochemical stability of the product because they modified particle size and the activity of enzymes like polyphenol oxidase (PPO) and pectin methylesterase (PME) which have an impact in the sensory characteristics and stability of the product. Those changes were mainly attributed to the effect that UHPH had on apple juice, principally the one with apple fibre, while UV-C did not significantly affect those parameters, or they were hardly noticeable in the case of colour and enzymes inactivation.

Experiments with cloudy apple juice treated by UHPH (Ypsicon) at 200 and 300 MPa (InT 20ºC) demonstrated that particle (fibre) size was reduced in all occasions in which that technology was utilized. Particle size measurement from the different UHPH machines (Stansted and Ypsicon) showed that there was a difference in the result at a same pressure, 200 MPa and 20 ºC InT (Fig. 6). In both results there was an important volume of particles observed at the diameter size between 6 - 7 µm, that also marked the highest distribution peak in Stansted's 200-MPa treatment, but the main differences came at larger particle sizes, Ypsicon's 200-MPa had a higher frequency of particles in between 20 - 30 µm, and Stansted's 200-MPa had a second important peak at the size between 200 - 300 µm diameter. That could have happened mostly because of the differences between the designs of the needles and sits at the pressure valves, as well as the angle that is formed in their gap. The pressure of 300 MPa could only be reached by Ypsicon's equipment, but from the

comparison in between that pressure and 200 MPa from the same machine, results suggested that the higher the pressure, the more particle size could be reduced. Still the graph-line of 300 MPa was polymodal which could represent aggregation of particles that were detected as if they were bigger particles as Paper 4 explains. Velázquez-Estrada et al. (2019) also reports a shrinking of particle size of a partially filtered orange juice after UHPH (Stansted) treatments at 100, 200 and 300 MPa. There were not many differences in the size and distribution of particles between the different utilized pressures, but different to our results they obtained a monomodal curve with peaks between 70 - 100 µm diameter. Similar results were obtained by Donsì et al. (2009) with sieved apple juice (Annurca var.) submitted to UHPH at 200 and 300 MPa and particle sizes around 20 - 40 µm diameter. Those differences could be attributed to the different types of matrices and equipment.

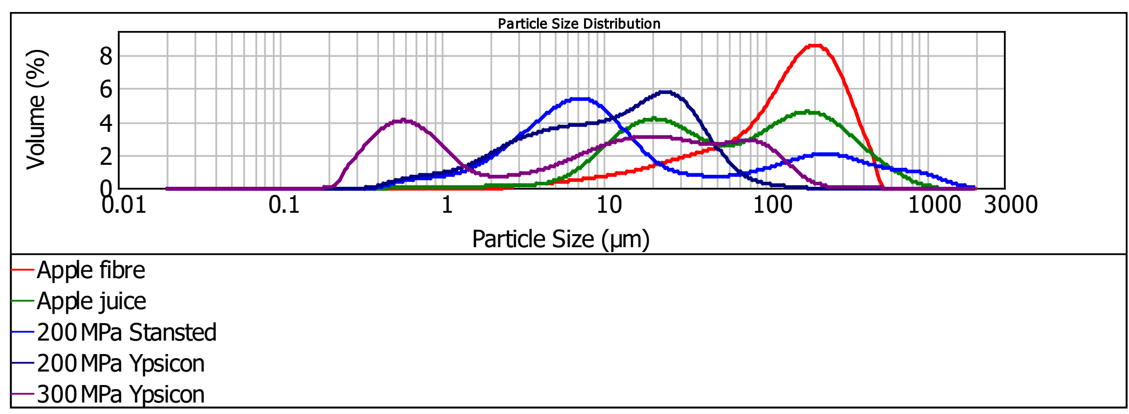


Figure 6. The distribution of particle size of: apple fibre (red), cloudy apple juice (green), and cloudy apple juice treated by different UHPH equipment and pressures, 200 MPa by Stansted (blue) and Ypsicon (dark blue) machines, and 300 MPa (purple) by Ypsicon. All juices were treated at 20 ºC InT.

The effects of that particle shrinking was much noticeable to eyesight. When samples were left unshaken for one day (Figure 7), particles in processed juice by UHPH, especially those treated at 300 MPa, remained more disperse contributing to the homogeneity of the juice, while those ones from the control and UV-C treatment went to the bottom of the tube. The diminished particle size could make them less likely to form big aggregates and settle down which contributes to the stability of the product (Velázquez-Estrada et al., 2019). Some differences were observed in the colour of the samples after treatments (Table 10), Although there were significant changes in all UV-C-treated samples compared with the control, there were no significant changes in combined treatments after UV-C exposure

when compared with the UHPH-treated juice alone. The treatment with most differences in colour and particle shrinking compared with the non-treated juice was the one of 300-MPa UHPH. Those changes could have been due to the modification in its turbidity or due to the inactivation of PPO which could not be detected after those treatments as explained in Paper 4, and PME remained at an average residual activity of 24.62 %. On the other hand, 200-MPa treatment seemed to have released more enzymes into the matrix without inactivating them, because PPO activity increased up to an average of 1.87 times higher than in raw juice. Contrary to that, in the same treatment PME could be detected in a lower activity than in raw juice which was an average of 56.21 % residual PME activity.

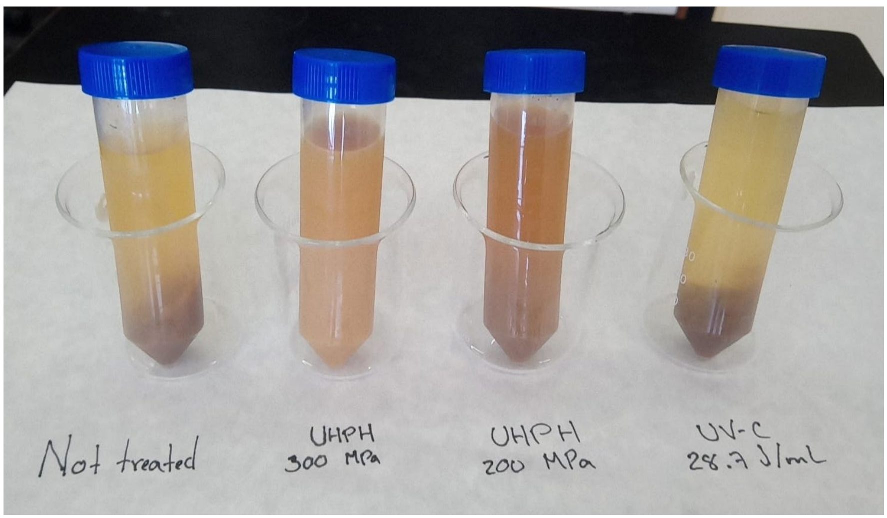


Figure 7. Cloudy apple juice treated by UHPH at 200 and 300 MPa, or by UV-C at 28.7 J/mL, and compared to the non-treated product. Samples were left without shaking during 24 h at 4 ºC. All processed samples were done at an inlet temperature of 20 ºC.

Table 10. Cloudy apple juice's colour changes after UHPH treatments at 200 and 300 MPa, and different UV-C doses (14.3, 21.5 and 28.7 J/mL) in single or combined treatments. *ΔE* represents the total colour change compared with the untreated control with their respective standard error values. Different letters mark significant differences ($p < 0.05$) among sample means according with Tukey test. Colour reproduction by software is also shown.

Treatment		ΔE	Colour
UHPH	**UV-C (J/mL)**		
0 MPa	**0**	0^{a}	
	14.3	$2.24^{b} \pm 0.36$	
	21.5	$2.44^{b} \pm 0.75$	
	28.7	$2.15^{b} \pm 0.66$	
200 MPa	**0**	$6.64^{c} \pm 1$	
	14.3	$6.54^{c} \pm 0.3$	
	21.5	$6.75^{c} \pm 0.34$	
	28.7	$6.76^{c} \pm 0.48$	
300 MPa	**0**	$21.12^{d} \pm 0.15$	
	14.3	$21.51^{d} \pm 0.23$	
	21.5	$21.91^{d} \pm 0.59$	
	28.7	22.22^{d} 0.95	

5. Conclusions

1) UHPH and UV-C are technologies that can complement each other for the inactivation of a wide spectrum of microorganisms in apple juice.

2) Resistance against UHPH and UV-C depends on the characteristics of the microorganism and it can vary amongst them for the same treatment. UHPH can act better on vegetative cells and in *A. niger* spores, and UV-C acts better on low-pigmented microorganism.

3) There is a synergistic effect between UHPH and UV-C when treating ascospores of *T. macrosporus* and *N. spinosa.* Sublethal damage caused by UHPH can increase the effectivity of UV-C treatments in inactivating those ascospores in apple juice.

4) There is an additive effect between UHPH and UV-C when treating bacterial spores in apple juice.

5) Lethality rates by UHPH increases with higher InT between 70 and 80 ºC, and it is independent from the optical properties (α_{254} and turbidity) of the matrix.

6) Lethality rates by UV-C in a thin-film reactor with these characteristics increases with higher doses and passes throughout the system. And it is affected by the increase of the values of the optical properties of the matrix.

7) UHPH changes the particle size of the matter suspended in cloudy apple juice, but those changes depend on the pressure and the characteristics of the pressure valve.

8) 300-MPa UHPH can stabilise the cloudiness of an apple juice with fibre. Not only by shrinking particle size, but it also inactivates PPO making it undetectable, and it greatly reduces the activity of PME. Those characteristics contribute to the physical stability of the product.

9) UHPH and UV-C treatments make perceptible changes in the sensory characteristics of apple juice, but most of changes are not too far from the preference score that had in the untreated product.

10) UHPH applied at room temperature (~20 ºC) can homogenise chia seed extract into clarified apple juice and combined with UV-C treatments can help to reduce the microbial load, even if spores of *A. acidoterrestris* were present, and improve the stability of the product.

11) UHPH and UV-C treatments can preserve many of the phenolic compounds in apple juice with chia extract.

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Paper 1

“Inactivation study of *Bacillus subtilis*, *Geobacillus stearothermophilus*, *Alicyclobacillus acidoterrestris* and *Aspergillus niger* spores under ultra-high pressure homogenization, UV-C light and their combination”

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Paper 2

“Inactivation of ascospores of *Talaromyces macrosporus* and *Neosartorya spinosa* by UV-C, UHPH and their combination in clarified apple juice”

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Paper 3

"Effect of single and combined UV-C and ultra-high pressure homogenisation treatments on inactivation of *Alicyclobacillus acidoterrestris* spores in apple juice"

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Paper 4

Combined effects of ultra-high pressure homogenization and short-wave ultraviolet radiation on the properties of cloudy apple juice

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Abstract

This work addresses the physicochemical, enzymatic and sensory changes in cloudy apple juice treated by ultra-high pressure homogenisation (UHPH) and short-wave ultraviolet radiation (UV-C) applied at 20 ºC. Those technologies were applied in single and combined treatments at different UHPH pressures (200-300 MPa) and UV-C doses (14.3-27.8 J/mL). UV-C treatments could not effectively inactivate enzymes, but treatments at 300-MPa UHPH reduced pectin methylesterase activity to a 24.6%, and polyphenol oxidase activity was not detected. Those samples presented a higher antioxidant capacity (283% measured by FRAP, and 286.4% by DPPH) than in non-treated juice, and after a combination with 28.7 J/mL of UV-C the polyphenols content augmented to 277.6%. Sensory evaluation revealed that

UHPH at 300 MPa and UV-C at 21.5 J/mL significantly changed perceptible odour and overall flavour of cloudy apple juice, while treatments at 200 MPa didn't produce any significant changes in the different parameters. The results obtained in this study give a promising perspective of what a combination of both technologies can bring about in terms of obtaining stabilized fruit juices with improved antioxidant activity and polyphenol availability.

Keywords: UHPH, UV-C, apple juice, physicochemical properties, sensory evaluation.

1. Introduction

Apple juices in markets are most likely processed by heat-technologies like thermal pasteurisation in order to prevent them from deterioration by enzymes such as polyphenol oxidase (PPO) and pectin methylesterase (PME) (Krapfenbaue et al., 2006; Suárez-Jacobo et al., 2012) or from the growth of microorganisms naturally present in fruits (Suárez-Jacobo, Gervilla, Guamis, Roig-Sagués & Saldo, 2010). Thermal technologies can address these problems, but they have a negative impact in some of the characteristics of juices like changes in their organoleptic properties (colour, aroma, flavour) (Carbonell, Navarro, Izquierdo & Sentandreu, 2013) as well as in their vitamin content (Velázquez-Estrada et al, 2013).

There are non-thermal technologies for liquid-food processing currently on investigation. Ultra-high pressure homogenization (UHPH) works by forcing a liquid through a narrow gap in a pressure intensifier (Dumay et al., 2013) and it has been proved to inactivate microbial cells by a conjunction of stresses (turbulence, cavitation, shear stress) that provoke different types of damages on the cell wall of microorganisms, and proved to inactivate enzymes like polyphenol oxidase and

pectin methylesterase in apple juice (Suárez-Jacobo et al., 2012) while preserving most of the antioxidant activity and phenolic components. However, it is ineffective against bacterial spores when the treated matrix is at low inlet temperatures (20 - 50 ºC) (Reverter-Carrión et al., 2018)

Short wavelength ultraviolet radiation (UV-C) is another emerging technology currently under study. It can penetrate microorganisms, including bacterial spores (Reverter-Carrión et al., 2018), and form pyrimidine dimers in DNA preventing it from replicating and consequently causing death of the cell (Sauceda-Gálvez et al., 2019). UV-C also spares most of the original characteristics of fruit juices and vitamin content (Koutchma, 2009). Unfortunately, UV-C is ineffective in inactivating PME (Torkamani & Niakousari, 2011) unless juices are irradiated for longer periods at high doses (Falguera, Pagán & Ibarz, 2011).

Recent investigations point out that UHPH and UV-C technologies can be used together in order to inactivate fungal and bacterial spores, UV-C can inactivate pressure-tolerant spores, while UHPH can inactivate some microorganisms that are resistant to radiation or make them more vulnerable to it (Reverter-Carrión et al., 2018; Sauceda-Gálvez et al., 2019). In them it was observed that there was an additive or synergic effect. But there is no much information about the impact that both technologies can have on the physicochemical properties of foods when used together.

The objective of this research was to elucidate the effect that a combination of different pressures of UHPH and UV-C doses have on physicochemical properties of a cloudy apple juice, as well as on its naturally present detrimental enzymes, and moreover, how each technology affects the sensory attributes of this product.

2. Materials and methods

2.1 UHPH treatments

UHPH treatments were done in a UHPH homogenizer (model A) (Ypsicon S. L., Barcelona, Spain) of 60 L/h capacity with needle and seat model Ultra Shear Valve (Ypsicon S.L., Barcelona, Spain). The homogenisation pressures for these experiments were of 200 and 300 MPa. The inlet temperature of the process was of 24 ± 2 °C and the volume of juice was 13 L. The residence time at the valve was of 0.7 s, the temperature at the valve at 200 MPa was of 77 ± 1 ºC, and of 107 ± 1.5 ºC at 300 MPa. Outlet temperature was of 32 ± 2 ºC. The juice was immediately cooled down to 20 ºC by water bath before the combined treatments with UV-C. Samples for analysis were frozen at -80 ºC in 50-mL Falcon tubes. Samples for particle size and sensory analysis were refrigerated at 4 ºC up to 24 hours.

2.2 UV-C treatments

UV-C treatments were done in a 70 mL capacity thin film (1 mm) concentric-type reactor (UV-Therm, Ypsicon) that consisted in a feeding tank connected to a UV-C lamp (55W) (LAB81055 NNI 150/76 XL, UV- Consulting Peschl España, Geldo, Spain) coated with a quartz tube and a measured irradiance of 31 mW/cm^2 at a wavelength of 254 nm by an UVM-CP handheld radiometer and a calibrated sensor (90155, UV-Consulting Peschl, Mainz, Germany). The apple juice was pumped through the reactor at different speeds to be irradiated in the doses of 14.3, 21.5 and 28.7 J/mL. The temperature of the whole process was of 20 ± 0.5 °C. Samples were frozen at -80 ºC in 50 mL falcon tubes. Samples for particle size and sensory analysis were refrigerated at 4 ºC up to 24 hours.

2.3 Apple juice physicochemical characterization

Raw fresh-pressed cloudy apple juice (Golden delicious var.) was provided by a local producer. The juice was characterized in different physicochemical parameters before and after treatments.

2.3.1. Absorption coefficient

Absoption coefficient at 254 nm wavelength (α_{254}) was measured at 20 °C by spectrophotometer NanoPhotometer Pearl (IMPLEN GmbH, München, Germany) and a quartz cuvette (Fisher Scientific, Hanover, Illinois, USA) of 10 mm depth, and it was reported as cm^{-1}.

2.3.2. Turbidity, pH and ºBrix

Turbidity was measured in nephelometric turbidity units (NTU) with a turbidimeter Hach 2100Q (Hach Lange Spain, L'Hospitalet de Llobregat, Spain).

pH was measured with a pH-meter pH básico-20 (Crison, Barcelona, Spain), and ºBrix by a Spectronic Instruments refractometer (Rochester, N.Y., USA) at 20 °C.

2.3.3. Colour

Colour was measured by a colorimeter (MiniScan XETM, Hunter Associates Laboratory Inc., Reston, USA.), using the CIELAB colour space which comprises L^* (lightness), a^* (red to green) and b^* (yellow to blue) coordinates. The total colour difference (ΔE^*) between the raw juice and treated samples was calculated following the formula (Velázquez-Estrada, Hernández-Herrero, Guamis-López & Roig-Sagués, 2019):

$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$.

2.3.4. Particle size distribution

Particle size distribution was measured by a Mastersizer Micropulus 2.15 (Malvern Instruments, Inc., Works, United Kingdom), a laser diffraction particle size analyzer. Samples were diluted in distilled water until optimal obscuration level for the laser (5 – 15%). An optical model based on Mie scattering theory by spherical particles was applied using the following conditions: refractive index for the dispersed phase = 1.471; refractive index of dispersion phase (water) = 1.334. The size distribution was characterized by the mean diameters D[3, 2] and D[4, 3].

2.4 Enzymatic activity

Residual activity of polyphenol oxidase (PPO) was done as explained by Cano, Hernandez & Ancos (1997) using 37.5 µL of juice filtered by syringe filters (0.45-µm pore diameter) (Labbox, Vilassar de Dalt, Spain) and 1.5 mL of 0.7 M catechol (Sigma Aldrich, St. Louis, USA) in phosphates buffer (pH 6.5). Samples' absorbance was read at 420 nanometres (A_{420}) wavelength by spectrophotometer (IMPLEN); absorbance changes (ΔA_{420}) were recorded before and after incubation at 20 ºC for 20 min; ΔA_{420} of the treated samples were compared with the untreated juice (100% PPO activity).

Residual activity of pectin methylesterase (PME) was done by adapting the method of Carbonell et al. (2013) using a solution of 1% citrus pectin (Sigma Aldrich) and 0.2 M of NaCl (Sharlau, Sentmenat, España). pH was adjusted to 8.0 with NaOH 4N (Panreac Química SLN, Castellar del Vallès, España). The volumes used for each test were of 20 mL of pectin solution and 5 mL of sample. This mix was titrated by 0.1N of NaOH (Panreac) by an automatic titrator Titrando 842 (Metrohm, Herisau, Switzerland) until this mix reached a pH of 7.8. pH changes were recorded

during 30 min at 20 ºC and ΔpH/Δt of the treated samples were compared with the untreated juice (100% PME activity).

2.5 Non-enzymatic browning

This essay was performed according to Meydav et al. (1977). Samples were filtered by syringe filters (Labbox) and mixed with ethanol 1:1 ratio. Samples were read by spectrophotometer (IMPLEN, GmbH, München, Germany) and results were reported as A_{420}.

2.6 Antioxidant capacity

Antioxidant capacity was performed via two methods with apple juice filtered by syringe filters (Labbox), and Trolox (Sigma Aldrich) at different concentrations (50-1000 µM) as the equivalent standard. FRAP (Ferric-ion Reducing Antioxidant Power) method was based on the method explained by Benzie & Strain (1996) with the filtered juice diluted with water in a 1:1 proportion. The mix consisted on 90 µL of sample or standard, 270 µL of distilled water and 2.7 mL of FRAP reagent pre-warmed at 37 ºC. Samples were stored at 37 ºC for 30 min and read by spectrophotometer (IMPLEN) at 593 nm wavelength. DPPH method (radical scavenging activity) was based on as explained by Bondet, Brand-Williams & Berset (1997). The mix consisted in 80 µL of sample and 2.92 mL of DPPH reagent (Sigma Aldrich). Samples were stored at 25ºC for 1 hour and read by spectrophotometer (IMPLEN) at 515 nm wavelength. Results were expressed in Trolox equivalent (µM).

2.7 Total polyphenols

These were measured based on the method of Singleton, Orthofer & Lamuela-Raventós (1998). The filtered sample was diluted with distilled water in a 1:1 proportion. The mix consisted on 100 µl of sample or gallic acid standard (Sigma Aldrich), 0.75 mL of 0.2N Folin-Ciocalteau (Sigma Aldrich), and 0.75 mL of 6% sodium carbonate anhydrous (Panreac). Samples were read by spectrophotometer (IMPLEN) at 760 nm. Results were expressed as gallic acid equivalent in mg/L.

2.8 Sensory evaluation

Sensory evaluation was carried according to ISO 8587:2006 which describes a method that allows to sort a series of samples in rank order based on the intensity of a single attribute or an overall (ISO 8587:2006). This test can determine differences between the samples, but not the degree of difference. In order to find which parameters had significant differences among the samples, F_{test} had to be obtained through the formula:

$$F_{test} = \left(\frac{12}{j \cdot p(p+1)}\right)({R_1}^2 + {R_2}^2 + {R_3}^2 + {R_4}^2) - 3j(p+1)$$

Where: j = number of judges; p = number of different samples; $R1$ – $R4$ = total score of the sample. For a test of these characteristics if $F_{test} > 7.81$ it means that there were significant differences among the samples.

A group of 21 tasters (judges) from CIRTTA-UAB scored samples coming from the untreated juice, UHPH treated juice at 200 and 300 MPa, and UV-C treated juice at 21.5 J/mL.

2.9 Statistic analysis

Experiments were performed three times and from each experiment duplicate samples were analysed (n = 6). Averages, standard deviations and analysis of variance (ANOVA) were done by using the linear models (GLM) via software (Statistica ver 7, StatSoft, Inc). Tukey test was employed for comparisons between samples means. Differences were considered as significant when $p < 0.05$.

3. Results and discussion

3.1 Samples characterization and changes after treatments

Treatments by UHPH greatly changed α_{254} in cloudy apple juice reaching the maximum values after 300 MPa (Table 1). This might have happened due to the fragmentation of particles and from the liberation of soluble solids as it could be seen in Table 1 that there was an increase in turbidity and ºBrix in comparison with the untreated control. Contrary to this, ºBrix faced a reduction after 200-MPa UHPH. Velázquez-Estrada et al. (2019) also noticed a similar behaviour in filtered orange juice after UHPH treatments at different pressures (100 – 300 MPa), though they observed an increase in ºBrix after 100 MPa, and a decrease after 300 MPa, which is in contrast with our results, but this could be due to the properties of the matrices used in both experiments. Many of the changes found here could be observed presumably because of the high content of suspended particles in cloudy apple juice being affected by the different UHPH treatments (Fig. 1 and Fig. 2).

Average pH values slightly augmented after UHPH treatments (Table 1). Experiments on clear apple juice by Suárez-Jacobo et al. (2011) show that there was a small increase in pH after 300 MPa. On the other hand, Velázquez-Estrada

et al. (2019) reported no significant changes in filtered orange juice's pH treated by UHPH (100 – 300 MPa).

The increase in turbidity is in consonance with the results of Velázquez-Estrada et al. (2019) who observed higher cloudiness in orange juice after UHPH treatments (100 - 300 MPa). The highest turbidity values were seen in the treatments of UHPH at 200 MPa, and these were even higher than in samples of 300 MPa. This could be because turbidity is a relative measure of light scattering which is more related to the optical effects of suspended matter than to mass concentration (Davies-Colley & Smith, 2001). From these results it could be said that particles in apple juice changed after UHPH treatments in different ways according to the pressure. This reasoning could be supported by particle size-results (Fig. 1).

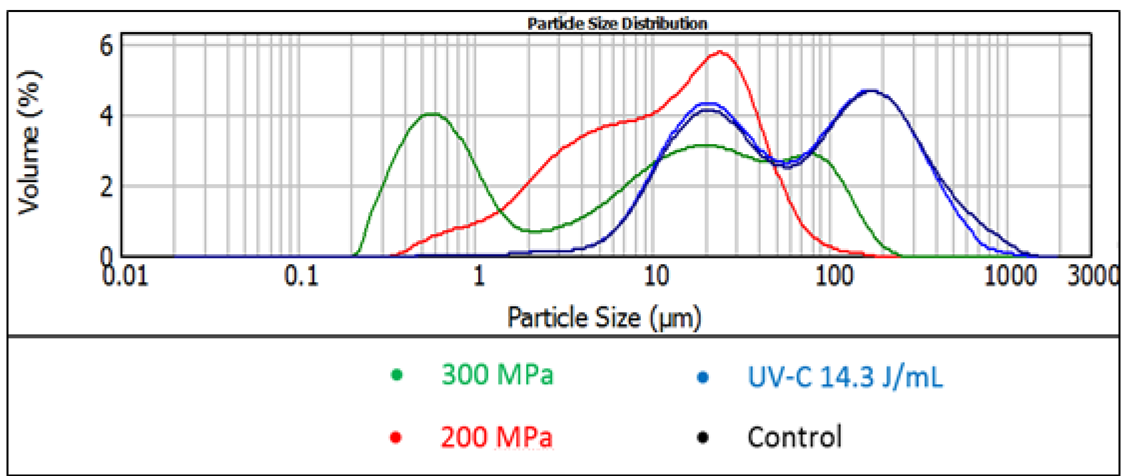

Figure 1. Particle size distribution of cloudy apple juice before (black line) and after treatments by UHPH at 200 (red line) and 300 (green line) MPa, or by UV-C at 14.3 J/mL (blue line). Particle size was measured in µm compared with the volume they occupied in the sample.

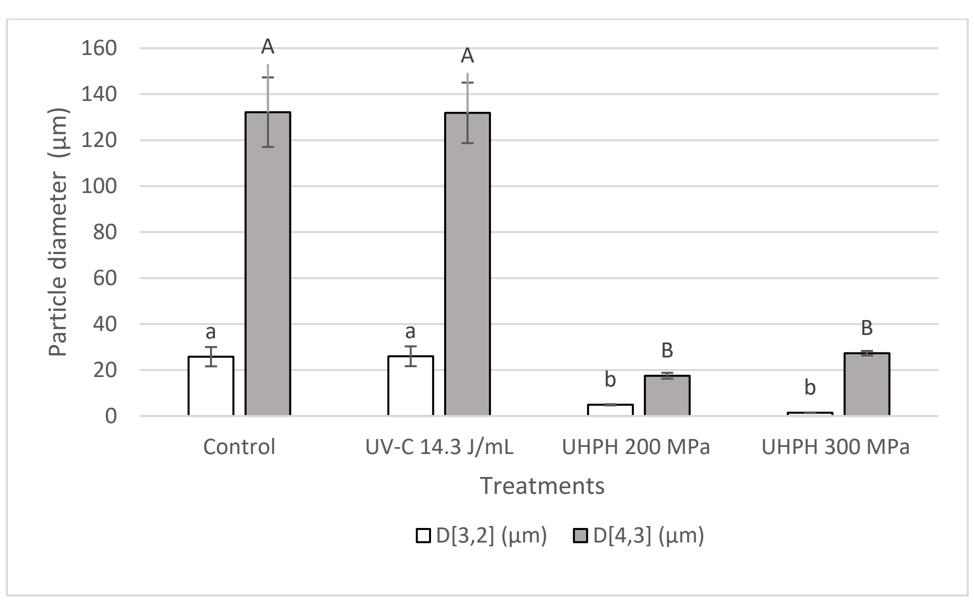

Figure 2. Particle diameters from cloudy apple juice treated by UHPH at 200 and 300 MPa or by UV-C at 14.3 J/mL. Sauter diameter (surface-weighed mean diameter) D[3, 2] (white bars) is influenced by smaller particles, and D[4, 3] (volume-weighed mean diameter) (grey bars) is usually affected by bigger particles. Lowercase letters mark significant differences ($p < 0.05$) between sample means of D[3, 2], and capital letters mark significant differences ($p < 0.05$) between sample means of D[4, 3] both obtained by Tukey test. Error bars mark the standard deviation of the samples.

According to data in Fig.1, there were differences between samples treated by UHPH at 200 and 300 MPa, where the size and distribution of particles changed differently after the two assayed pressures resulting in smaller particle sizes than in juices non-treated by UHPH. The increase of turbidity of UHPH-processed samples (Table 1) might have been influenced by this phenomenon. Particles from samples treated at 300 MPa present an important size distribution in the range of smaller than 2 µm diameter. These results can be contrasted with those of Donsì, Esposito, Lenza, Senatore & Ferrari (2009) in UHPH-treated apple juice (Annurca var.) in which treatments at 300 MPa diminished particle size with a larger distribution of particles measuring 11-µm diameter, they observed product destabilization during storage at 37 ºC. Cloud stability is much influenced by the presence of particles smaller than 2 µm diameter (Betoret E., Betoret N., Carbonell & Fito., 2009). Larger particles, like those ones from the control and UV-C samples, tend to settle down by gravity (Velázquez-Estrada et al., 2019). After UHPH treatments, D[3, 2] and D[4,

3] values decreased significantly ($p < 0.05$) (Fig. 2). High D[3, 2] values relate to the potential of interaction between particles and higher chances to form aggregates, while high values of D[4, 3] indicate that particles are heavier and tend to settle down during storage (Velázquez-Estrada et al., 2019).

Table 1. Characterization of cloudy apple juice samples from Control and UHPH treatments at 200 or 300 MPa. The measured parameters are separated by columns and from left to right: absorption coefficient at 254 nm (α_{254}), Turbidity (NTU), pH and ºBrix. Lowercase letters for each parameter mark significant differences ($p < 0.05$) between samples means obtained by Tukey test.

Sample UHPH	α_{254}	NTU	pH	ºBrix
0 MPa	11.54^{c} ± 0.3	2356.61^{c} ± 12.85	3.91 ± 0.02	11.9
200 MPa	49.27^{b} ± 0.44	3307.06^{b} ± 3.19	3.92 ± 0.02	11.2
300 MPa	61.93^{a} ± 0.69	2641.89^{a} ± 1.77	3.94 ± 0.01	12

From these results it could be expected that UHPH-processed apple juice at 300 MPa would remain more cloud-stable than in 200-MPa or non-UHPH treated samples.

There were no significant changes from UV-C treatments in any of the previous parameters, and consequently these data were not shown in tables or figures.

3.2 Residual enzyme activity

Polyphenol oxidase in cloudy apple juice increased its activity 87% after 200-MPa UHPH (Fig. 3) compared with the untreated control. Previous experiments on clarified apple juice (Saldo, Suárez-Jacobo, Gervilla, Guamis & Roig-Sagués, 2009) report that PPO seems not to be inactivated after UHPH treatments of 100 – 300 MPa. Some other authors observed higher PPO activity after high-pressure treatments (Cano et al., 1997), and it has also been stated that any damage to cells can result in the release of enzymes (Chisari, Barbagallo & Spagna, 2007). On the other hand, there was no PPO activity detected in juices processed at 300-MPa

UHPH (Fig. 3), that could have happened due to the loss of the enzyme's native structure because of the intensity of the mechanical forces at the pressure valve (Bot et al., 2018), and because of the temperature reached there (107 ± 1.5 ºC) when the juice was passing through, that temperature was considerably higher than in the treatment at 200 MPa (77 ± 1 ºC). PPO activity in 200-MPa treated juice decreased after UV-C treatments. A reduction of PPO activity by UV-C has been reported as well by other authors in clarified apple juice (Falguera et al., 2011). Interestingly, PPO activity seemed to remain stable after UV-C single treatments, probably because of the possible damage to cells provoked by hydro-dynamical stresses and the release of enzymes into the matrix as explained by Orlowska, Koutchma, Kostrzynska, Tang & Defelice (2014) who observed an increase in PPO activity in raw cloudy apple juice after a UV-C treatment of 44.5 mJ/cm^2 in a Taylor-Couette reactor.

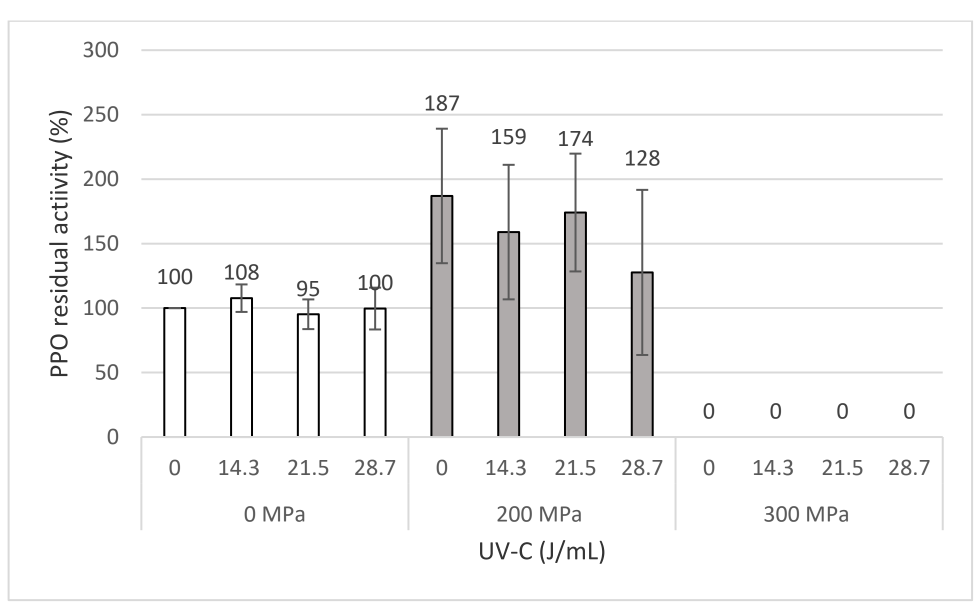

Figure 3. Residual activity of polyphenol oxidase in cloudy apple juice treated by UHPH at 200 or 300 MPa or by UV-C at 28.7 J/mL. Treatments were also combined in the order UHPH → UV-C. Error bars mark the standard deviation of the samples.

UHPH treatments at 200 and 300 MPa considerably inactivated PME (Fig. 4), with a residual PME activity (RPME) of 56.2 % after a 200 MPa treatment, but yielding the best results at 300 MPa with a RPME activity of 24.6%. These results can be compared with those from a similar experiment reported by Velázquez-Estrada, Hernández-Herrero, Guamis-López & Roig-Sagués (2012) with filtered orange juice treated by UHPH which explains that PME can be better inactivated as the pressure increases, but there is also a contrast in between their results and those obtained here with apple juice. In their experiments they observed that after a treatment of 200 or 300 MPa the RPME activity was less than 10%. This could be attributed to the different matrices used in both experiments and the ratios between thermolabile and thermostable types of PME that can differ even among varieties of the same fruit (Krapfenbauer, Kinner, Gössinger, Schönlechner & Berghofer, 2006).

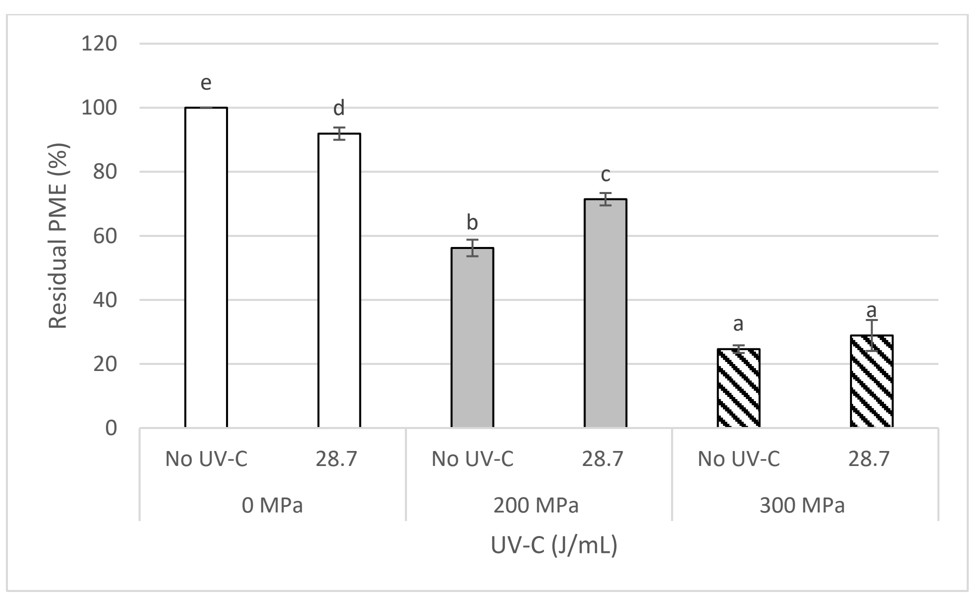

Figure 4. Residual activity of pectin methylesterase in cloudy apple juice treated by UHPH at 200 and 300 MPa or by UV-C at 28.7 J/mL. Treatments were also combined in the order UHPH → UV-C. Lowercase letters mark significant differences (p < 0.05) between samples means obtained by Tukey test. Error bars mark the standard deviation of the samples.

In UV-C treatments PME activity was slightly reduced by 28.7 J/mL of UV-C with an average of 91.9% RPME activity (Fig. 4). Other experiments with apple juice (Starking var.) show that after 40 min of exposure to a UV-C lamp PME was completely inactivated (Falguera et al., 2011), but in their experiments PME content in juice was already low because the clarification treatment (centrifugation) separated the juice from the pulp that was discarded along with most of the enzymes (PME) already acting on it. Experiments with other fruit juices like orange juice that passed through a thin film reactor showed a low PME inactivation after a treatment of 73.8 mJ/cm^2 with a RPME activity of 92% (Torkamani & Niakousari, 2011).

UV-C (28.7 J/mL) applied after a UHPH treatment of 200 MPa did not contribute to a greater inactivation of PME. In fact, there was a significant increase (p < 0.05) of PME activity. Its residual activity after a single UHPH treatment of 200 MPa was of 56.2%, but the same sample exposed to UV-C had an increase in RMPE activity back to 71.4% (Fig. 4). There were no significant differences with the results coming from the treatment of UHPH at 300 MPa and with its combination with UV-C.

3.3 Colour and non-enzymatic browning

These experiments demonstrated that UV-C and UHPH treatments caused significant changes ($p < 0.05$) in apple juice's colour parameters, principally in the *L** value (Table 2) which represents the lightness. This value increased more with UHPH treatments (at both pressures) than with UV-C. Velázquez-Estrada et al. (2019) also reported an increase of lightness in orange juice after UHPH treatments, which is positively correlated to cloudiness, a decrease of particle size and a consequent increase of light diffraction. An increment of the *L** value after UV-C exposure of centrifuged apple juice (Golden delicious var.) was also observed by Falguera et al. (2011). UHPH treatments also decreased *a** value meaning that the product was less red than the original one and the samples treated only by UV-C. The *b** value significantly changed only after 300 MPa of UHPH treatment. Compared with the untreated juice, overall colour change (ΔE) was significantly different in all treatments and it became higher in the samples treated by UHPH, being the combined treatment of 300 MPa with 28.7 J/mL of UV-C (Table 2) the one which changed the most, probably due to browning changes and particle size and distribution. UV-C increased ΔE slightly after UHPH treatments, but these changes were not significant.

Table 2. Colour measurements in CIELAB colour-space system where L^* = lightness; a^* = green to red; b^* = blue to yellow; ΔE = Total colour change. Cloudy apple juice was treated by UHPH at 200 and 300 MPa, or by different UV-C doses (14.3, 21.5 and 28.7 J/mL). Both technologies were also combined in the order UHPH → UV-C. Lowercase letters for each value mark significant differences ($p < 0.05$) between samples means obtained by Tukey test.

Treatment		L^*	a^*	b^*	ΔE
UHPH	**UV-C (J/mL)**				
0 MPa	**0**	$25.13^{a} \pm 0.66$	$13.5^{a} \pm 0.73$	26.59 ± 1.26	0^{a}
	14.3	$27.01^{b} \pm 0.57$	$14.51^{a} \pm 0.18$	26.71 ± 0.59	$2.24^{b} \pm 0.36$
	21.5	$26.62^{b} \pm 0.36$	$13.74^{a} \pm 0.54$	26.29 ± 2.11	$2.44^{b} \pm 0.75$
	28.7	$27^{b} \pm 0.67$	$13.45^{a} \pm 0.73$	26.41 ± 1.06	$2.15^{b} \pm 0.66$
200 MPa	**0**	$31^{c} \pm 0.58$	$11.19^{b} \pm 0.3$	25.74 ± 2.24	$6.64^{c} \pm 1$
	14.3	$31.03^{c} \pm 0.11$	$11.22^{b} \pm 0.31$	25.61 ± 1.48	$6.54^{c} \pm 0.3$
	21.5	$31.43^{c} \pm 0.27$	$11.37^{b} \pm 0.67$	26.14 ± 1	$6.75^{c} \pm 0.34$
	28.7	$31.49^{c} \pm 0.39$	$12.16^{b} \pm 0.74$	25.4 ± 1.48	$6.76^{c} \pm 0.48$
300 MPa	**0**	$45.05^{d} \pm 0.12$	$8.41^{c} \pm 0.17$	$31.38^{a} \pm 0.7$	$21.12^{d} \pm 0.15$
	14.3	$45.34^{d} \pm 0.24$	$7.74^{c} \pm 0.9$	$30.99^{a} \pm 1.13$	$21.51^{d} \pm 0.23$
	21.5	$45.84^{d} \pm 0.8$	$7.68^{c} \pm 0.96$	$30.36^{a} \pm 1.63$	$21.91^{d} \pm 0.59$
	28.7	$45.95^{d} \pm 1.08$	$7.35^{c} \pm 0.86$	$31.09^{a} \pm 1.28$	22.22^{d} 0.95

Apple juice's browning index after 300 MPa of UHPH and combinations with UV-C presented a lower value than in the untreated juice (Fig. 5), the evolution of browning is related to an increase of a^* because of the appearance of melanoidins (Ibarz, Pagán, Panadés & Garza, 2005), and probably UHPH was capable to degrade these compounds. These results were in consonance with those of Saldo et al. (2009) who observed a reduction of a^* value after submitting clarified apple juice to a treatment of 300 MPa of UHPH. The other significant difference found was on the treatment of UV-C at 14.3 J/mL, but browning index kept increasing with higher doses.

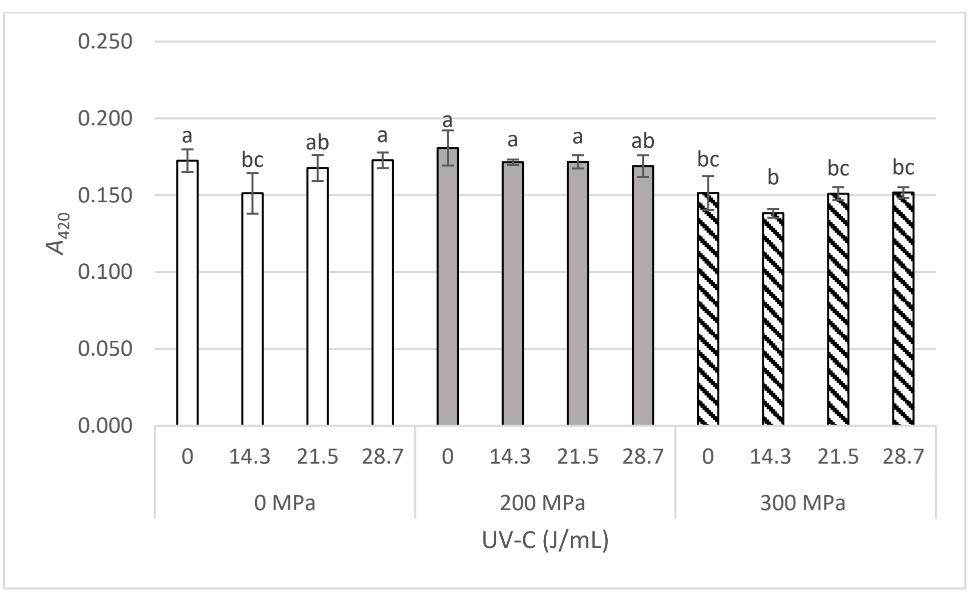

Figure 5. Browning index measured at A_{420} of cloudy apple juice treated by UHPH at 200 and 300 MPa, or by different UV-C doses (14.3, 21.5 and 28.7 J/mL). Both technologies were also combined in the order UHPH → UV-C. Lowercase letters mark significant differences ($p < 0.05$) between samples means obtained by Tukey test. Error bars mark the standard deviation of the samples.

3.4 Antioxidant capacity

Fig. 6 shows that antioxidant capacity measured by FRAP and DPPH assays, as well as polyphenols content increased considerably in samples treated by UHPH at 300 MPa compared with the untreated control (a 283% when measured by FRAP, and a 286.5% by DPPH). This might have happened due to the disruption of suspended particles (cells) and the liberation of antioxidant compounds. Similar experiments carried out with clear apple juice (Suárez-Jacobo et al., 2011) demonstrated that UHPH (100 – 300 MPa) did not affect the antioxidant capacity in this juice, though there were no differences between the treated samples and the control. UV-C treatments seemed to slightly increase the availability of polyphenols according to FRAP assay, being the samples treated by 21.5 and 28.7 J/mL statistically different than the untreated juice. There were no differences detected by DPPH method in the aforementioned UV-C treatments. The antioxidant activity in apples is mostly given because of its content in polyphenols (Suárez-Jacobo et al., 2011). Samples processed by 200 MPa of UHPH did not show any improvement in

polyphenols content despite their reduction in particle size (Fig. 1 and Fig. 2). This probably happened because polyphenols were affected by PPO which was not inactivated but even made more available into the matrix by a 200 MPa treatment (Fig. 3). On the other hand, UV-C treatments in raw juice and in the juice treated at 300 MPa seemed to lower down the total polyphenol content with a dose of 14.3 J/mL, but it increased after 21.5 and 28.7 J/mL treatments (Fig. 6). Samples treated by UHPH at 300 MPa and combined with UV-C showed that there was a decrease in the antioxidant capacity with higher doses when analysed by FRAP assay, but differences were not significant according to DPPH assay. These antioxidant capacity-measuring methods work under different principles, FRAP assay measures the capacity to reduce ferric-ions, and DPPH assay measures the capacity to scavenge free radicals. It is recommendable to use more than one type of antioxidant assays so as to have a better elucidation of the total antioxidant activity of the sample (Velázquez-Estrada et al., 2013).

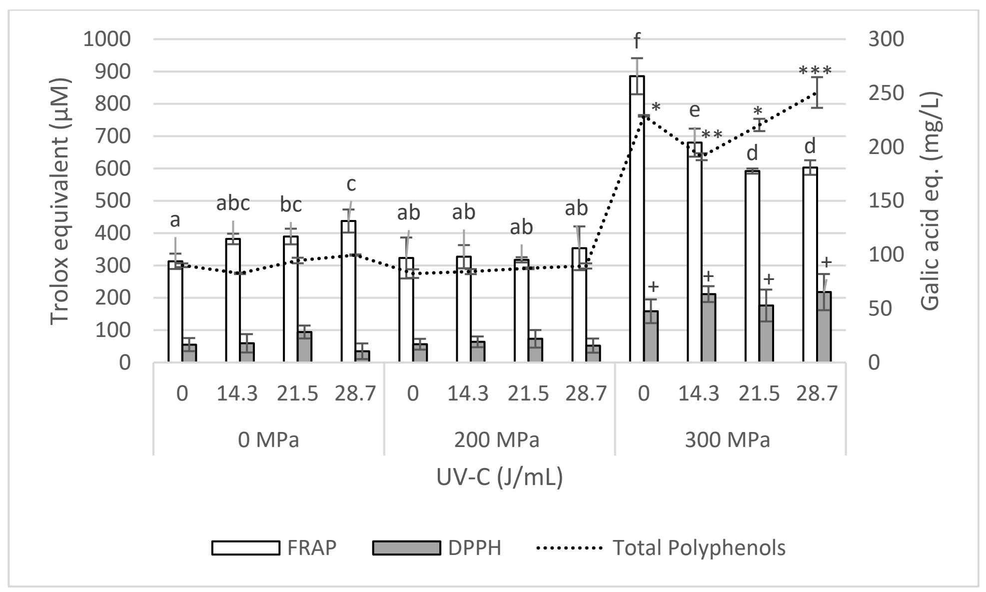

Figure 6. Antioxidant capacity and total polyphenols of cloudy apple juice treated by UHPH at 200 and 300 MPa, or by different UV-C doses (14.3, 21.5 and 28.7 J/mL). Both technologies were also combined in the order UHPH → UV-C. Antioxidant capacity was measured by FRAP (white bars) and DPPH (grey bars) methods, the results were expressed as trolox equivalent (µM). Antioxidant capacity was compared with the content of total polyphenols (dotted line) expressed as galic acid equivalent (mg/mL). Significant differences ($p < 0.05$) between samples means obtained by Tukey test divided according to the test, lowercase letters for differences in FRAP; plus symbol (+) for differences in DPPH compared with the untreated control; and asterisks (*) for differences compared with the untreated control and between the samples treated by 300 MPa of UHPH and the different combinations with UV-C. Error bars mark the standard deviation of the samples.

3.5 Sensory evaluation

The results of the test of preference are shown in Fig. 7. Samples were scored by the judges according to their preference. If the minimal significant difference (MDS) between two samples in a test of this magnitude is higher than 16.4, it could be said that samples were significantly different. This was observed in the parameters of odour and overall flavour and results were detailed in Table 3. Raw juice was better preferred than the treated ones at most parameters, and it was significantly better in flavour and odour compared with juices after UV-C (21.5 J/mL) and a pressure of 300 MPa because de difference between scores surpassed MDS value (Table 3). Even though there were significant changes in overall flavour, the treated samples were all similar to the raw juice in the other parameters related to taste (sweetness, sourness, freshness and texture). The treated juice that resulted the most similar to a raw product was the one treated by 200 MPa of UHPH, though it scored the least

on the parameter of overall aspects most likely because of its appearance due to the distribution of particles (Fig. 1) and colour (Table 2) together.

Figure 7. Results of the sensory evaluation test. Samples were scored in order of preference.

Table 3. Significant differences between samples in the sensory parameters of “odour” and “overall flavour”. For a test of this magnitude the minimal significant difference (MDS) between the samples’ scores had to be higher than 16.4. Samples were coded as A (raw juice), B (UV-C 21.5 J/mL), C (UHPH 200 MPa) and D (UHPH 300 MPa).

Odour MSD > 16.4		**Overall flavour MSD > 16.4**	
A-B	17.5	**A-B**	28.5
A-D	26.5	**A-D**	23
C-D	20.5	**B-C**	18

Conclusions

UHPH treatments at 300 MPa were the best treatment for processing cloudy apple juice from the physicochemical and enzymatic standpoint. It enhanced the antioxidant activity of juice and when combined with higher doses of UV-C there was an increment in the total content of polyphenols. This treatment could greatly reduce the activity of PME, and PPO activity was not detected. That along with the cloud stability from the reduction of particle size could improve apple juice stability overall. Although this survey focuses on the effect that these technologies have on the physicochemical, enzymatic and organoleptic properties of cloudy apple juice, some other research done by our group point out that UV-C could be a key factor in the preservation of apple juice from the microbial standpoint in inactivating bacterial and some fungal spores at low temperatures, which is something that UHPH is ineffective at. For these reasons it could be said that UHPH and UV-C are technologies that can complement each other when combined and applied to liquid foods in order to bring physiochemically and microbially stable foods with a low impact in their organoleptic properties and acceptation while preserving, or even making more available the polyphenol content and antioxidant capacity in fruit juices.

Acknowledgments

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Paper 5

Short wave ultraviolet light (UV-C) effectiveness on bacterial spores inoculated in turbid suspensions and in cloudy apple juice.

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Abstract

The effect of turbidity as an interference factor against UV-C for sporicidal purposes was analysed by using apple fibre suspensions in phosphate-buffered saline solution (PBS) with turbidity values of 2000, 2500 and 3000 NTU, and using spores of *Bacillus subtilis* and *Alicyclobacillus acidoterrestris*. While higher UV-C doses increased the inactivation rates of spores, higher turbidity values had a negative impact on them, a dose of 28.7 J/mL achieved inactivation rates of *B. subtilis* spores of 3.96 Log in a 2000-NTU suspension compared with 2.81 Log in the one of 3000 NTU. Spores of *B. subtilis* were more UV-C-resistant than *A. acidoterrestris*. Cloudy apple juice inoculated with *A. acidoterrestris* spores was processed by UV-C at different doses and in a single pass or with recirculation of the matrix through the reactor. Inactivation increased with recirculation surpassing 5 Log after 125 J/mL compared with 0.13 Log inactivation from the single-pass treatment. UV-C treatments with recirculation affected the optical properties (absorption coefficient at 254 nm and turbidity) of juice and increased browning as doses became higher.

1. Introduction

In recent years short-wave ultraviolet light (UV-C) has been tested as an alternative to thermal treatments in food industry, not only for the microbiological decontamination of surfaces, but also for the reduction of the microbial load of liquid foods (de Souza and Fernández., 2011). Among the microorganisms potentially present in these foods, bacterial spores are much resistant to temperatures used for common pasteurisation processes and to pressure-based technologies such as high-hydrostatic pressure and ultra-high-pressure homogenisation (San Martín et al., 2002; Reverter-Carrión et al., 2018; Sauceda-Gálvez et al., 2020). Once these spores contaminate foods and survive pasteurisation treatments, they germinate and grow even at low pH spoiling the final product meant to be shelf-stable (Bevilacqua et al., 2007). This has been the case in apple juice batches contaminated with spores of *Alicyclobacillus acidoterrestris* (Lee et al., 2002), which is harmless to humans and represents no health hazard. This bacterium might not produce any visible signs of deterioration like gas or higher turbidity before an excessive cell concentration, but it spoils the product with the production of odd flavours and odours with the synthesis of guaiacol, making it not suitable for their consumption, and naturally, one of the target microorganisms in novel technologies for juice processing (Chang and Kang., 2004; Walker and Phillips., 2008).

UV-C has been proved to successfully inactivate microorganisms in their vegetative of spore state by penetrating through their membranes and being absorbed by their DNA with the maximum absorption peak in the wavelength range between 254-264 nm, forming pyrimidine dimers which blocks DNA transcription and replication processes leading to cell death (Choudhary and Bandla., 2012). Experiments performed in phosphate-buffered saline (PBS) which opposes no interference against UV-C (254 nm), demonstrate that a wide range of bacterial and fungal spores are susceptible to be inactivated by this technology (Reverter-Carrión et al., 2018; Sauceda-Gálvez et al., 2019). The main problem that UV-C when treating food matrices is the interferences naturally present in them which would not allow light to penetrate through the matrix and reach microorganisms for their inactivation. The most common interferences found in foods are those which act as a shield to

microorganisms by blocking the pass of UV-C light. To address these problems, many continuous-flow UV-C reactors have been designed up to date, and one of those approaches are the thin-film reactors that shortens the distance in which UV-C has to travel through so as to be able to reach as many cells as possible to inactivate them (Koutchma et al., 2004). The purpose of this work was to evaluate the effectiveness of a thin-film UV-C reactor in inactivating bacterial spores inoculated in suspensions of different turbidity values as interference. For that purpose, spores of *B. subtilis* and of *A. acidoterrestris* were chosen since the first is commonly used as a model microorganism for the study of the characteristics of endospores (Harwood, 2007), and the second for being a target microorganism in the fruit juice industry due to their resistance to many types of stresses and low pH values (Chang & Kang, 2004). The inactivation kinetics of *A. acidoterrestris* in cloudy apple juice of high absorption coefficient with the addition of ascorbic acid by UV-C applied in a single pass or in recirculation were also elucidated.

2. Materials and methods

2.1. Preparation of the spore suspensions

Bacillus subtilis CECT 4002 and *Alicyclobacillus acidoterrestris* CECT 7094 were supplied from the Spanish Type Culture Collection (CECT, University of Valencia, Valencia, Spain). *B. subtilis* spores were obtained with a modified procedure based on the UNE EN ISO 13704:2002 as explained by (Martinez-Garcia et al., 2019). The lyophile was rehydrated in glucose-tryptone broth consisting in 2.5 g of yeast extract (Oxoid, Basingstoke, UK), 5 g of tryptone (Oxoid) and 1 g of glucose (Sigma-Aldrich, St. Louis, USA) per litre of distilled water, its pH was adjusted to 7.2. It was incubated at 30 ºC for 24 h, 2 mL of this stock culture were transferred into Roux bottles containing yeast extract agar (MYA) which consisted in 10 g of meat extract (Oxoid), 2 g of yeast extract (Oxoid), 15 g of agar (Oxoid) and 0.04 g of $MnSO4·H2O$ (Merck, Darmstadt, Germany) per litre of distilled water. These bottles were incubated at 30 ºC for a maximum of 30 days to induce sporulation. Spores were collected by pouring 20 mL of sterile distilled water into each bottle and the surface was scraped with a Digralsky stick, this suspension was collected inside 50-mL Falcon tubes and centrifuged at 10,000 *g* for 20 min at 4 ºC using a Sigma 4K15 centrifuge (Sigma

Laborzentrifugen GmbH, Osterode am Harz, Germany), the supernatant was discarded and the resulting pellet was washed times with 15 mL of sterile water and centrifuged with the same settings, this procedure was done four times to be finally resuspended in 30 mL of sterile distilled water. The suspensions were heated at 75 ºC for 10 minutes in order to inactivate vegetative cells.

A. acidoterrestris spores were recovered in K broth as explained by (Chang & Kang, 2004) by incubating them at 43 ºC for 48 h. This stock culture was refrigerated at 4 ºC. Sporulation was induced by transferring 1 mL of the stock culture to 250-mL Roux bottles containing 50 mL of potato dextrose agar (PDA) (Oxoid, Basingstoke, UK), and incubated at 43 ºC from 7 to 15 days until reaching 80% sporulation or higher. Spores were collected by pouring 30 mL of sterile distilled water into each bottle and the surface was scraped with a Digralsky stick, this suspension was collected inside 50-mL Falcon tubes and centrifuged at 10,000 *g* for 20 min at 4 ºC, the supernatant was discarded and the resulting pellet was washed with 30 mL of sterile water and centrifuged with the same settings, this procedure was done four times to be finally resuspended in 40 mL of sterile distilled water and heated at 80 ºC for 20 minutes in order to inactivate vegetative cell.

Spore preparations were placed in an ice bath after heat treatment for 10 minutes and stored at 4 ºC until used.

2.2. Matrices preparation

Phosphate-buffered saline (PBS) (Panreac Química S. L. U., Castellar del Vallès, Spain) with a pH of 7.4 was added with apple fibre (PBS-fibre) (The Hut Group, Manchester, U. K.) until reaching the turbidity values of 2000, 2500 and 3000 NTU (Nephelometric Turbidity Unit). Apple juice (Golden delicious var.) was obtained from a local producer. The cold-pressed apple juice was added with 300 ppm of ascorbic at the moment of its extraction according to the producer. The matrices were inoculated with *B. subtilis* spores (final concentration ~5.5 Log CFU/mL) and *A. acidoterrestris* spores (final concentration ~4.5 to 5.3 Log CFU/mL) right before UV-C treatments.

2.3. UV-C treatments

These treatments were done in a thin-film concentric-ring type UV-C reactor (UV-Therm, Ypsicon S.L., Barcelona, Spain) of 70-mL capacity, with dimensions of 76.5-cm long and 1-mm width. This reactor has a feeding tank connected to a UV-C lamp (55 W) (LAB81055 NNI 150/76 XL, UV- Consulting Peschl España, Geldo, Spain) coated with quartz. The lamp's irradiance was measured by actinometry with a potassium iodide/iodate solution as explained by the method of (Rahn, 1997). PBS suspensions were irradiated in a single pass through the reactor with the doses of 14.3, 21.5 and 28.7 J/mL. Apple juice was irradiated with the doses of 25, 50, 75, 100 and 125 J/mL for microbiological analysis in a single pass and in recirculation through the reactor at a pumping speed of 0.708 m/s. In the same way, samples were irradiated at the doses of 50, 100, 150 and 200 J/mL for physicochemical analysis. The time needed for an irradiation and the number of entrances was calculated according to Martinez-Garcia et al. (2019) which are found in Table 1. Samples destined for microbiological analysis were refrigerated at 4 ºC, and samples destinates for physicochemical analysis were deposited in 50-mL Falcon tubes and stored at -80 ºC.

2.3.1. Reynolds number calculation in UV-C treatments

Reynolds number (*Re*) was calculated with Equation 1:

$$Re = \rho V d/\mu.$$

Where V is fluid's velocity in m/s, d is the equivalent diameter of 0.001632 m (Ozbayoglu & Omurlu, 2006). Dynamic viscosity (μ) was measured at 20 ºC with a Haake RheoStress1 rheometer (Thermo Fisher Scientific, Inc., Karlsruhe, Germany) with a value for apple juice of 4.13 ± 0.07 x 10^{-3} Pa · s. Density (ρ) at 20 ºC was obtained with a density hydrometer (HYDR-100-001) (Labbox, Vilassar de Dalt, Spain) being of 1.052 g/mL (1052 Kg/m^3) in apple juice,

2.4. Microbiological analysis

Serial dilutions of the samples were done in sterile peptone water (Oxoid) and plated on petri dishes. Samples with *B. subtilis* were plated on tryptone-soya agar (Oxoid) using a double layer in order to prevent excessive colony growth. Plates were incubated at 30 ºC for 2 days. *A. acidoterrestris* samples were plated on SK agar (Chang and Kang, 2005) and they were incubated at 43 ºC for 3 days. Spore inactivation was calculated as the difference between the logarithmic counts of the control and the treated sample ($Log_{10}N_0$-$Log_{10}N$).

2.5. Physicochemical analysis

2.5.1. Optical properties

Absorption coefficient (α_{254}) was measured using a spectrophotometer NanoPhotometer Pearl (IMPLEN GmbH, München, Germany) and a quartz cuvette with a 1 cm pathlength. Turbidity was measured with a turbidimeter Hach 2100Q (Hach Lange Spain, L'Hospitalet de Llobregat, Spain). Samples were read at 20 ºC.

2.5.2. Browning index

It was determined following the method explained by (Meydav et al., 1977). Samples were filtered with syringe filters of 0.45 µm pore diameter (Labbox) and mixed with ethanol in equal proportions. Samples' absorbance was read at 420 nm (A_{420}) by spectrophotometer and results were expressed as A_{420}.

2.6. Statistical analysis

Statistics and analysis of variance (ANOVA) were done by using the linear models (GLM) via software (Statistica ver 7, StatSoft, Inc). Tukey test was used for making comparisons between samples means. Differences were considered as significative when $p < 0.05$.

2.7. Inactivation kinetics

Survival data of *A. acidoterrestris* spores in apple juice was adjusted to a non linear model by using GInaFiT software add-in for Microsoft® Excel. The most suitable model was chosen according to the root mean square error and the R^2 coefficient. The inactivation model that better adjusted to that data was Geeraerd model with shoulder and tail (Geeraerd et al., 2000) which is represented in Equation 2:

$$Log_{10}(N) = Log_{10}[\left(10^{Log_{10}(N_0)} - 10^{Log_{10}(N_{res})}\right) * e^{(-k_{max}*t)} * e^{(k_{max}*Sl)})]/[1 + \left(e^{(k_{max}*Sl)} - 1\right) * e^{(-k_{max}*t)})] + 10^{log_{10}(N_{res})}$$

Where:

N_0 = starting concentration of spores

N_{res} = number of survival subpopulations of resistant bacteria

t = time (in this adaptation it would be UV-C dose with recirculation)

k_{max} = inactivation rate = 0.15 ± 0.01

Sl = shoulder length = 15.64 ± 3.64

3. Results and discussion

3.1. UV-C effectiveness in the different turbidity suspensions

As it can be observed in Figure 1, the inactivation of *B. subtilis* spores was inversely proportional to the turbidity value of the PBS-fibre suspension, and in the same turbidity values, inactivation increased with the applied dose. The highest inactivation (3.96 Log) was obtained in the suspension of the least turbidity (2000 NTU) and processed with the highest UV-C dose (28.7 J/mL). A similar behaviour was observed in a previous experiment (Reverter-Carrión et al., 2018) where caramel was added to PBS to modify its absorbance coefficient, showing that even slight α_{254} increments can drastically reduce *B. subtilis* spores inactivation. Vulnerability of fungal ascospores to UV-C is higher when there are no interferences

in the matrix, as was also observed in a previous experiment with plain PBS (Sauceda-Gálvez et al., 2019). In this experiment *A. acidoterrestris* could not be detected after UV-C treatments under these conditions, therefore data was omitted from figures and the highest inactivation rate for these experiments in PSC-fibre was of 4.58 ± 0.09 Log. These results can also be contrasted with those of Reverter-Carrión et al. (2018) in which *B. subtilis* spores show a higher UV-C-tolerance compared with other *bacillus* spores including *A. acidoterrestris* in plain PBS.

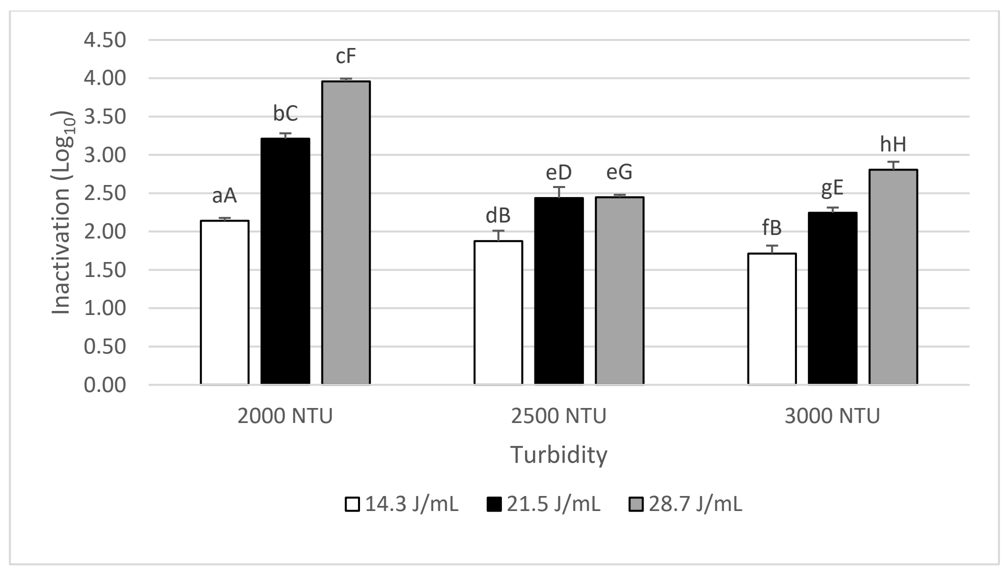

Figure 1. Inactivation of *B. subtilis* spores in PBS-fibre with different turbidity values modified with apple juice fibre. These suspensions were treated by UV-C at the doses of 14.3, 21.5 and 28.7 J/mL. Different lowercase letters represent significant differences ($p < 0.05$) between samples means of the same turbidity value but different UV-C dose; capital letters represent significant differences between sample means of samples treated with the same UV-C dose but of different turbidity value.

3.2. *A. acidoterrestris* inactivation in cloudy apple juice with ascorbic acid.

Cloudy apple juice with ascorbic acid presented high values of interferences coming from turbidity and α_{254}. Previous experiments with apple juice (Sauceda-Gálvez et al., 2020) located cloudy apple juice's α_{254} at 11.54 cm^{-1}, while in experiments with the same type of apple juice but with added ascorbic acid, the value of α_{254} was of 152.1 cm^{-1} (Figure 4). It is known that ascorbic acid has its absorption peak within the deep UV spectra between 243.5 and 265.5 nm (Berg, 2015), then it is reasonable that the addition of ascorbic acid would represent an immediate interference against UV-C, though it is a common practice in fruit juice production. The approach of a thin-film UV-C reactor is to reduce the depth that radiation has to travel through so as to reach as many cells as possible and inactivate them, being

most likely to be affected the closest ones facing the UV-C source. Those reactors work under laminar flow conditions, which means that liquids travel throughout the system in a layered way, if the properties of the matrix don't allow UV-C to reach further, then the deeper "layers" won't get reached and affected by UV light. As an attempt to increase the uniformity of particles to get into a closer proximity to the UV-C source, the matrix was re-circulated with the peristaltic pump set at 500 rpm (speed of 0.708 m/s), which would process an estimated volume of juice of 64.4 mL/s (Martinez-Garcia et al., 2019) which yields a dose of 0.42 J/mL every pass. The result from these experiments can be seen in Figure 2 where the different doses were applied in different approaches, in a single pass and in recirculation until reaching the desired dose. Results of *A. acidoterrestris* showed that lethality by UV-C from this equipment is ineffective in a matrix with those characteristics if treated by a single pass and there were no significant differences between the doses, but different results were observed in treatments with recirculation where an inactivation of more than 5 Log was observed in the treatment at 125 J/mL, in which no cells were detected growing on plates. Similar results were observed by Martinez-Garcia et al. (2019) in treating *B. subtilis* spores by using the same approach on whole and skim milk, they report higher inactivation rates as the re-circulation and doses augmented surpassing 4 Log after 100 J/mL which is significantly higher than their results with the same dose in a single pass. That could have happened because re-circulations allow the sample to mix and re-enter the circuit, and there were higher chances for the whole matrix and spores to get into a closer distance with the UV-C source. Reynolds number inside the UV-C reactor was also considerably higher in samples treated with recirculation ($Re = 290.8$), although that type of flow still corresponded to the range of laminar flow ($Re < 2100$), it might have propitiated a better exposure of the fluid layers that were immediately closer to the outermost one. Another factor that could have contributed to a better inactivation of *A. acidoterrestris* spores could be that higher UV-C doses decreased the α_{254} value of the samples. Comparing results between the experiments with PBS-fibre and juice, it could be presumed that the most limiting factor of the matrix in a UV-C treatment could be the α_{254} value more than the turbidity. The data gathered from UV-C experiments with recirculation was utilized for obtaining an inactivation model. Several inactivation models were assayed, from which it was selected the one with

the highest R^2 (0.9772) and the lowest RMSE (0.3293), being that the Geeraerd shoulder tail model (Geeraerd et al., 2000) (Figure 4), with an equation (Equation 2) to calculate the inactivation kinetics of *A. acidoterrestris* spores in this type of apple juice. Experiments conducted by Martinez-Garcia et al. (2019) on *B. subtilis* spores inoculated into milk and treated by UV-C working under the same settings show a different inactivation kinetics model, Weibull with tail (Albert and Mafart, 2005). A tail in the model represents a slow-down or a less exponential inactivation of spores after reaching certain dose in a treatment. In our experiments it was observed not only a tail, but also a shoulder which represented the opposite of a tail, meaning a slow spore inactivation at the beginning of the treatment followed up by an exponential behaviour. The analysis of the inactivation kinetics allowed to obtain the 4D value of 76.25 J/mL, that was the estimated dose required to inactivate 4 Log of *A. acidoterrestris* spores in apple juice under these treatment conditions.

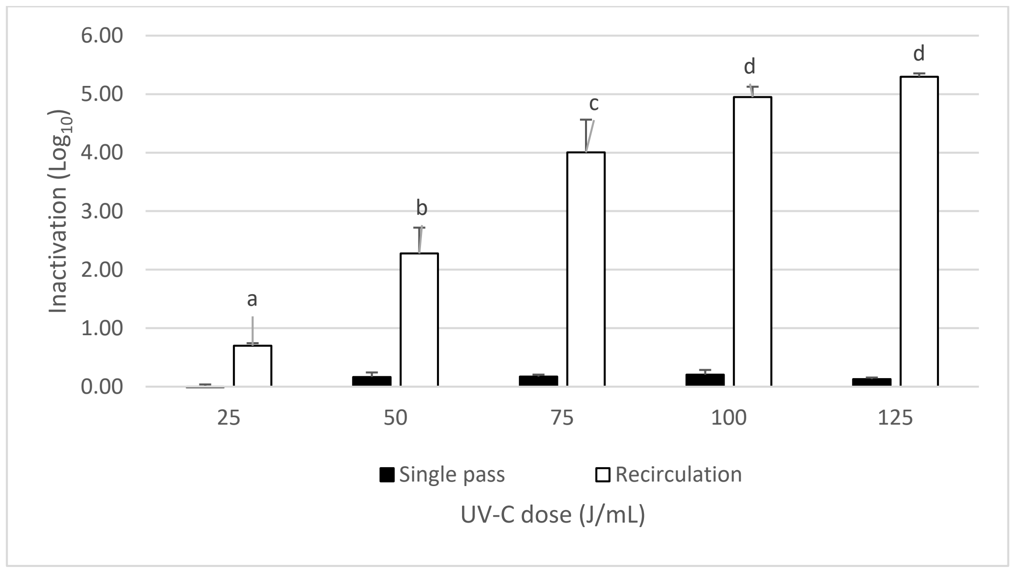

Figure 2. Inactivation of *A. acidoterrestris* inoculated in cloudy apple juice with ascorbic acid by UV-C in different doses (25, 50, 75, 100, 125 J/mL) in a single pass (black bars) or in recirculation (white bars). Error bars mark the standard deviation, and letters represent significant differences ($p < 0.05$) between sample means of the same treatment. No letters mean that there were no significant differences found between doses.

Table 1. Calculated treatment time in minutes and seconds, and the number of entrances per litre of apple juice through the UV-C reactor at the different doses. The pumping speed for all doses was 0.708 m/s.

UV-C dose (J/mL)	Treatment time per L of juice	Number of entrances
25	14'52"	60
50	29'43"	120
75	44'35"	180
100	59'26"	240
125	74'18"	300
150	89'10"	360
200	118'53"	480

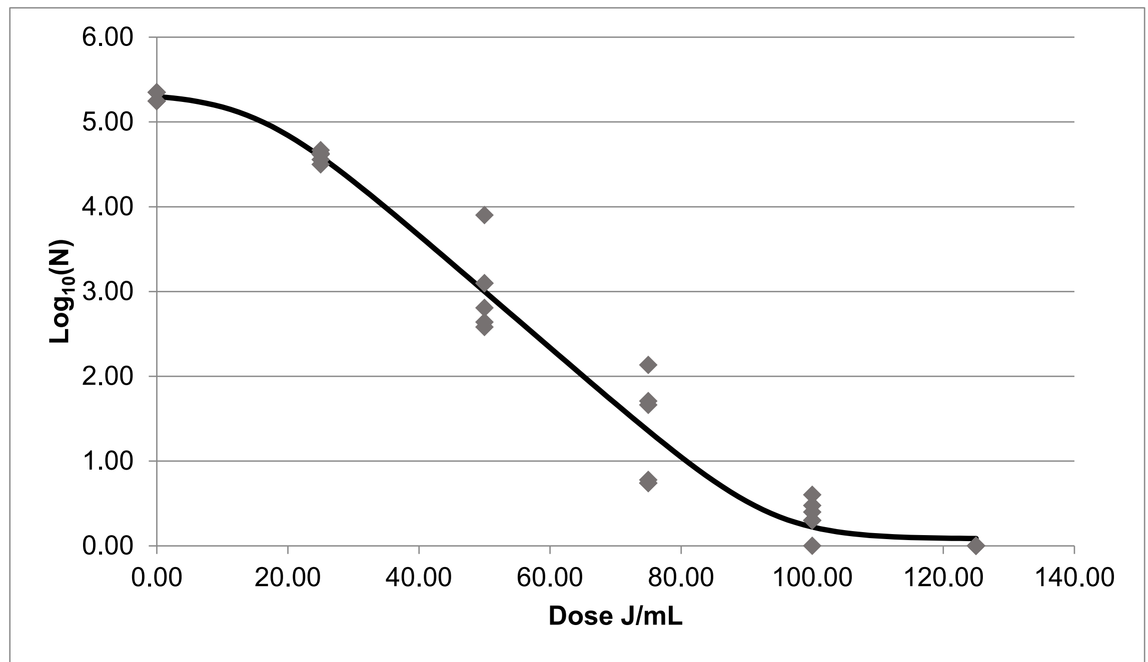

Figure 3. Inactivaction kinectics of *A. acidoterrestris* in cloudy apple juice treated by UV-C with recirculation according to Geeraerd shoulder tail model obtained by using GInaFIT plug-in.

3.3. Physicochemical changes from the application of UV-C treatments with recirculation

Apple juice was irradiated with higher doses than those used to inactivate *A. acidoterrestris* spores in order to better elucidate the changes that the application of UV-C with recirculation can have on some of the physicochemical properties of the apple juice with these characteristics. The first observable change after gathering the samples was an increase of browning in the samples irradiated from 100 J/mL and higher, that was confirmed by the browning assays, as shown in Figure 4, there was an augment of browning in UV-C-treated samples and it became even higher

with higher doses. A possible answer to that could be given by analysing the α_{254} of samples, it was found a reduction of that value as the doses increased. Prolonged exposure to UV-C can degrade ascorbic acid into dehydroascorbic acid and to 2,3-diketogulonic acid (Falguera et al., 2014; Tikekar et al., 2011), and those compounds have different absorbance peaks (Kleinwächter & Selmar, 2004). The addition of ascorbic acid was the main responsible for the increase of α_{254} in apple juice, and also for the inhibition of polyphenol oxidase (PPO) at the moment of the juice extraction. Nevertheless, in lower concentrations, as a consequence of photodegradation, it might not be able to inhibit the activity of that enzyme (Almeida & Nogueira, 1995). Although some investigation show that UV radiation can also inhibit PPO, it has not been able to achieve a total inactivation of the enzyme (Falguera et al., 2014; Guerrero-Beltrán & Barbosa-Cánovas, 2006). A possible degradation of the ascorbic acid in our experiments, which meant lower concentrations in apple juice, as well as a higher contact with oxygen from recirculation might have enabled PPO to oxidise phenolic compounds into pigmented ones (*o*-quinones). Turbidity might have been affected as well by the degradation of ascorbic acid due to the oxidation of polyphenols that would lead to polymerise and react with proteins, and consequently, the formation of particles of large size (Kolniak-Ostek, et al., 2013)

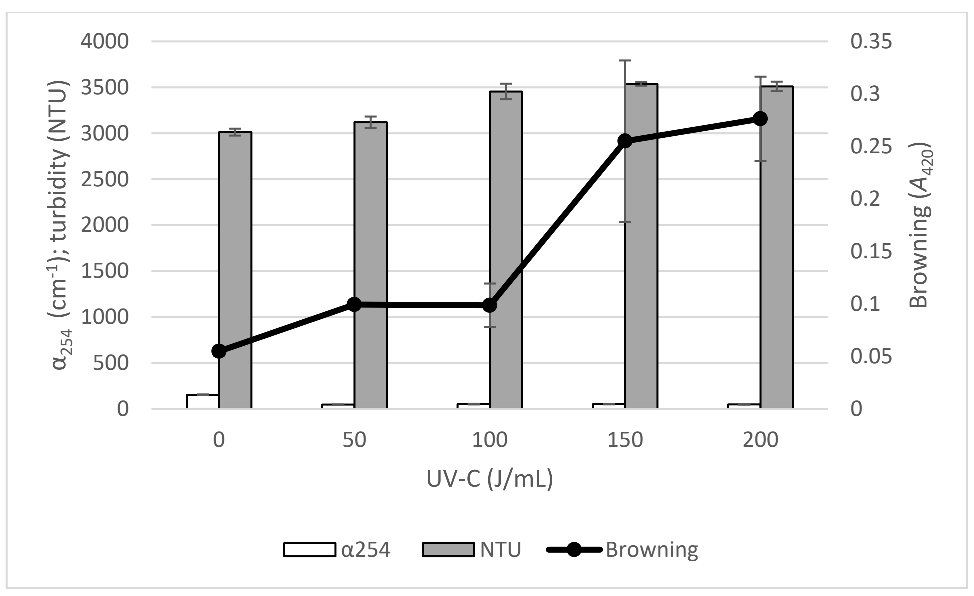

Figure 4. Physicochemical changes in apple juice with added ascorbic acid after UV-C treatments applied with recirculation in different doses (50, 100, 150, 200 J/mL). α_{254} (cm^{-1}) is represented by white bars, turbidity (NTU) is represented in grey bars, and browning (unitless) is represented by the plot line. Error bars mark the standard deviation between samples means.

Conclusions

The knowledge about how turbidity affects the effectiveness of UV-C treatments is important in order to know the intensity of the treatment when trying to inactivate pressure- and heat-resistant spores, like those of *A. acidoterrestris*, by UV-C. But most importantly, fruit matrices as a whole can oppose different types and levels of interferences depending on their singular properties, in this case cloudy apple juice with added ascorbic acid, which showed that its turbidity might not represent the determinant interference when choosing UV-C as the microbicidal treatment. Although there are chances for those types of resistant spores to be present in the product, there are other factors to take into consideration that could affect the quality of the final product like browning reactions. That could open new opportunities to explore the combination with other technologies prior to UV-C treatments that could help to inactivate detrimental enzymes like PPO and also help to inactivate microorganisms at a certain level so as to reduce the intensity in which UV-C treatments are applied to foods.

Acknowledgments

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Paper 6

Evaluation of the addition of chia seed (*Salvia hispanica*) extract in apple juice, and the impact of ultra-high pressure homogenisation, short-wave ultraviolet radiation, high-hydrostatic pressure and thermal pasteurisation on its antioxidant properties.

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Abstract

Chia seed extract was added in two concentrations to clarified apple juice with the concentrations of 24.6 and 60 g of chia seed equivalent per litter of juice (CSE24 and CSE60 respectively) with the purpose of increasing the quantity of polyphenols and the antioxidant activity of apple juice. CSE24 was treated by ultra-high pressure homogenisation (UHPH) at different inlet temperatures (InT), the highest assayed InTs (50 and 75 ºC) could extend better shelf life of the product stored at 5 and 24 ºC, but the juice's antioxidant properties were better preserved at 20 ºC InT. Experiments with CSE60 treated by UHPH, short-wave ultraviolet radiation (UV-C), high-hydrostatic pressure (HHP) and thermal pasteurisation showed that the combination of 200-MPa UHPH and 14.3 J/mL of UV-C could keep the juice from the growth of aerobic mesophiles for 15 days stored at 24 ºC, and there was no observable growth of *Alicyclobacillus acidoterrestris*, which was originally inoculated as spores in a 3 Log/mL concentration, even after a period of 60-days of storage at

42 ºC. In CSE60 treated by 200-MPa UHPH, it was observed a considerable improvement in the quantification of two important dietary polyphenols, caffeic and chlorogenic acids when compared against the control without chia seed extract and CSE60 treated by HHP or thermal pasteurisation.

1. Introduction

Chia (*Salvia hispanica*) seed is a food well recognized for its nutritional properties because of its profile of fatty acids and polyphenols which act as antioxidants among other attributes (Muñoz et al., 2013). It is native of Mexico, but its popularity has increased much so as to be found in many retail markets around the world. However, in 2000 the US Dietary Guidelines suggested no to exceed the consumption of chia seed than 48 g per day. More recently, the European Commission approved the use of chia seeds in 5% or less in baked goods (Valdivia-López & Tecante, 2015). The main uses of chia are not limited to that, but it can also be found in salads as sprouts, or the whole seed in beverages, cereals, salad dressings, snacks, yogurts and others (Mohd Ali et al., 2012).

Their main antioxidants found in chia seeds are the polyphenolic compounds chlorogenic and caffeic acids, and the flavanols myricetin, quercetin, and kaempferol (Valdivia-López & Tecante, 2015). The antioxidant potency might protect against some disorders like atherosclerosis, stroke, diabetes, cancer, Parkinson and Alzheimer's diseases. On the other hand, isoflavones can help as anti-carcinogenic substances and in the prevention of cardiovascular diseases. Among the isoflavones there are phytoestrogens that can be used to treat estrogen-related disorders (Martínez-Cruz & Paredes-López, 2014). For all these reasons, chia seed can be considered as a functional food.

There are numerous researches about the benefits that antioxidants among other components in chia seeds can bring about in terms of human health and nutrition in the form of whole and ground seeds, and in the form of an extract which carries phenolic compounds and flavonoids which are closely related to the antioxidant capacity of foods (Scapin et al., 2016), but up to date there are no products in the market which would use chia seed extract as a way of increasing their antioxidant

content. A good way of incorporating this extract could be in fruit juices like apple juice and with the use of novel technologies such as the ultra-high pressure homogenisation that would help to stabilize the product and grant it a longer shelf-life, as research on the use of this technology to treat fruit juices demonstrate that it can effectively inactivate vegetative spores and detrimental enzymes, while preserving antioxidant content for periods of 4 weeks in refrigeration or longer (Suárez-Jacobo et al., 2012; Velázquez-Estrada et al., 2012; Velázquez-Estrada et al., 2013). Unfortunately, at times these juices are loaded with spores from bacteria and fungi which are resistant to this technology (Reverter-Carrión et al., 2018; Sauceda-Gálvez et al., 2020) and even to pasteurisation processes, for that reason UHPH has also been tested with a combination of inlet temperatures and with other technologies like short-wave ultraviolet radiation (UV-C) in apple juice (Sauceda-Gálvez et al., 2019; Sauceda-Gálvez et al., 2020). This work addresses the use of chia seed extract as an additive in apple juice in order to increase the polyphenols content of that product, and to elucidate how the technologies of UHPH at different inlet temperatures and UV-C would impact in the microbiological load and in the antioxidant capacity of this product. At the same time, they were compared with other technologies that are currently being used for the treatment of juices and other liquid foods, like thermal pasteurisation and high-hydrostatic pressure (HHP).

2. Materials and methods

2.1. Apple juice

Cold-pressed apple juice of the Golden Delicious var. was obtained from a local provider. The extract was clarified in two steps: first was centrifuged in a centrifuge model Eppendorf 5804R (Eppendorf Ibérica, San Sebastián de los Reyes, Spain) in capsules of 250-mL capacity at 3100 *g* per 10 min, and then it was filtered through a Whatman #4 filter.

2.2. Spore suspension of *Alicyclobacillus acidoterrestris*

Alicyclobacillus acidoterrestris CECT 7094 was provided from the Spanish Type Culture Collection (CECT). Spores were revived in K broth (Chang & Kang, 2004) at 43 ºC for 48 h and stored at 4 ºC as stock cultures. Sporulation was induced by transferring 1 mL of the stock culture to a 250-mL Roux bottles with 50 mL of potato dextrose agar (PDA, Oxoid, Basingstoke, UK). Roux bottles were incubated at 43 ºC between 7 to 15 days until about 80% sporulation was observed. Spores were collected by adding 10 mL of sterile distilled water in the Roux bottles and the surface was scratched with a Digralsky spreader. The suspensions were centrifuged at 10,000 *g* for 15 min at 4 ºC in an Eppendorf 5804R centrifuge (Eppendorf Ibérica, San Sebastián de los Reyes, Spain). The pellet was suspended in 30 mL of sterile distilled water. This washing procedure was done four times. Finally, the resulting pellet was suspended in 40 mL of distilled water and heated at 80 ºC for 20 min. Spore preparations were then placed on an ice bath for 10 min and stored at 4 ºC until used.

2.3. Sample preparation

Apple juice was added with chia (*Salvia hispanica*) seed extract provided by the Autonomous University of Nuevo León (San Nicolás de los Garza, México). It was added in two proportions according to the yield of extract from chia seeds; 1) 1.25 g/L which was an equivalent to 24.6 grams of chia seed per litre of juice which was coded as CSE24, and 2) 3.06 g/L which was an equivalent to 60 grams of chia seed per litre of juice which was coded as CSE60. Experiments with *A. acidoterrestris* spores were inoculated into apple juice for a concentration of more than 3 Log right before UHPH and UV-C assays.

2.4. UHPH treatments

These treatments were performed in a ultra-high pressure homogenizer model FPG 12500 (Stansted Fluid Power LTD., Essex, United Kingdom) with a flow rate capacity of 15 L/h and a pressure valve consisting in a combination of a needle and a seat made of zirconium with a cutting angle of 60 ± 0.5° in the needle and 45 ± 0.5° in the seat, which operated at 200 MPa. The temperature of the samples at the

inlet (InT) was adjusted to 20, 50 and 75 ºC before processing CSE24 juice. CSE60 samples were only processed at an InT of 20 ºC.

2.5. UV-C treatments

UV-C treatments were done in a thin-film concentric-type UV-C reactor (UV-Therm, Ypsicon S.L., Barcelona, Spain) of 70-mL capacity, and dimensions of 76.5-cm long and 1-mm width. The reactor is composed by a feeding tank connected to a UV-C lamp (55W) (LAB81055 NNI 150/76 XL, UV- Consulting Peschl España, Geldo, Spain) coated with a quartz tube for an irradiance of 31 mW/cm^2 at a wavelength of 254 nm. This irradiance was measured with an UVM-CP handheld radiometer and a calibrated sensor (90155, UV-Consulting Peschl, Mainz, Germany). CSE60 samples were pumped through the reactor in order to be irradiated with different doses: 7.2, 14.3, and 14.4 (2 passes) J/mL.

2.6. High hydrostatic pressure treatment

HHP processing was carried out in a Hiperbaric 420 (Hiperbaric, Polígono Industrial Villalonquéjar, Spain) with water as the pressure-transmitting medium, and with temperature control. CSE60 samples were processed inside of vacuum-sealed pouches with a volume of 250 mL at a pressure of 600 MPa and a holding time of 15 min.

2.7. Thermal pasteurisation

This treatment for CSE60 was done in a water bath with the samples tightly closed in capped tubes which were heated along with a tube with the same characteristics and a thermometer inside in order to know when the temperature of 85 ºC was achieved. The exposure time was of 5 min and after that, samples were cooled down in iced water.

2.8. Microbiological analysis

All samples were aseptically collected in 15-mL capped tubes and analysed within the 24 h after treatments. Microbial load on samples was screened by colony counting on plate. Serial dilutions of the samples in peptone water (Oxoid,

Basingstoke, U.K.) were employed for this purpose, and these were plated on different media: plate count agar (PCA) (Oxoid) for aerobic mesophilic bacteria, Man, Rogosa and Sharpe agar (MRS) (Oxoid) for lactobacilli, rose-bengal agar supplemented with chloramphenicol (RBA) (Oxoid) for moulds and yeasts, violet red bile glucose agar (VRBG) (Oxoid) for faecal coliforms, and SK agar (Chang & Kang, 2005) for *A. acidoterrestris*. Growth kinetics were performed by aseptically filling sterile capped tubes after the treatments and storing them at different temperatures. CSE24 Samples were screened for aerobic mesophiles at days 0, 5, 15 and 30, or until microbial load reached 5 Log or more at the temperatures of 5 ± 1 ºC or 24 ± 1 ºC. CSE60 Samples were screened for aerobic mesophiles and *A. acidoterrestris* at days 0, 15, 30 and 60, or until microbial load reached 5 Log or more at the temperatures of 24 ± 1 ºC (aerobic mesophiles) or 42 ± 1 ºC (*A. acidoterrestris*).

2.9. Physicochemical analysis

All samples were collected in individual 50-mL Falcon tubes after the treatments and stored at -80 ºC until use.

2.9.1. pH

It was measured by pH-meter pH básico-20 (Crison, Barcelona, Spain).

2.9.2. Total polyphenols

This assay was done based on the method of Singleton et al. (1998). The filtered sample was diluted with distilled water in a 1:1 ratio. The mix consisted on 100 µl of sample or gallic acid standard (Sigma Aldrich), 0.75 mL of 0.2N Folin-Ciocalteau (Sigma Aldrich), and 0.75 mL of 6% sodium carbonate anhydrous (Panreac). Samples were read by spectrophotometer (Implen GmbH, München, Germany) at 760 nm. Results were expressed as gallic acid equivalent in mg/L.

2.9.3. Antioxidant capacity

This assay was done via two methods adapted to a microplates spectrophotometer (Cecil Instruments, Cambridge, UK), and with Trolox (Sigma Aldrich) at different concentrations (50-1000 µM) as the equivalent standard, juice samples were used

in a 1:9 dilution with distilled water: 1) The Ferric-ion Reducing Antioxidant Power (FRAP) method, based on the method described by Benzie & Strain (1996). The mix consisted on 10 µL of sample or standard, 30 µL of distilled water and 300 µL of FRAP reagent pre-warmed at 37 ºC. Samples were incubated at 37 ºC for 30 min and read at 593 nm wavelength. 2) The radical scavenging activity method (DPPH) was based on Bondet et al. (1997). The mix consisted in 80 µL of sample or standard and 2.92 mL of DPPH reagent (Sigma Aldrich). Samples were incubated at 25 ºC for 1 hour and read by spectrophotometer at 515 nm wavelength. Results were expressed in Trolox equivalent (µM).

2.9.4. Partial characterization of phenolic compounds by HPLC

The aglycon content of the phenolic compounds was quantified by doing a hydrolytic excision of polyphenol glycosides following the method of Taga et al. (1984) with some modifications proposed by López-Hernández and Ortega-Villarreal (2014). Briefly, 5 mL of sample were dissolved in 5 mL of methanol with HCL (6N). Samples were tightly closed and heated at 100 ºC for 45 min and cooled down to 25 ºC. The samples were filtered over Whatman 1 and extracted with 2 ml of diethyl-ether three times. Aqueous fraction was discarded and the organic part was evaporated to dryness. The residue was resuspended in 1 mL of methanol 70% HPLC grade.

High-performance liquid chromatography (HPLC) analysis was done in a Thermo Scientific Spectra System (Thermo Fisher Scientific, Waltham, MA USA) provided with a UV/visible detector fixed in 350 nm and a column Purospher Star RP-C18 (250 mm x 4.6 mm x 5 µm) (Merck). It was used the method described by Hempel & Böhm (1996) with some modifications in the elution gradient and in the pH of the mobile phase. The standars were galic acid, caffeic acid, chlorogenic acid, myricetin, apigenin, kaempherol, and quercetin (Sigma-Aldrich). The mobile phase consisted in a gradient of water-acetic acid (98:2) and acetonitrile HPLC grade, with a flux of 1 mL/min and a runtime of 17 min. Ten µl of each one of the samples and standards were filtered by Millipore 0.45 µm and injected for analysis. Both, hydrolysed and non-hydrolysed samples were analysed with the purpose of finding whether the compounds of interest were found in a conjugated state or not.

2.10. Statistical analyisis

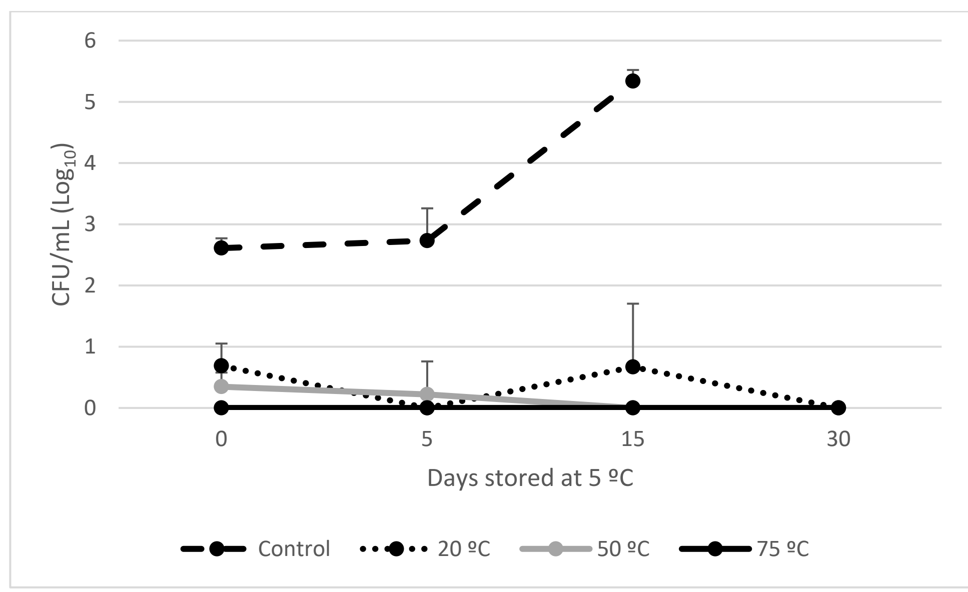
Results came from three individual experiments where samples were analysed by duplicate (n = 6). Averages, standard deviations and analysis of variance (ANOVA) were done by using the linear models (GLM) with the software Statistica ver 7 (StatSoft Inc., Paris, France). Tukey and Games-Howell tests were employed for comparisons between samples means. Differences were considered as significant when $p < 0.05$.

3. Results and discussion

3.1. Microbiological results

UHPH decreased the initial microbial load in CSE24 apple juice at all the assayed InT (Figure 1) and no microbial growth was detected after the UHPH treatment at 75 ºC InT in any of the tested media (Table 1). When samples from this treatment were kept at 5 ºC (Figure 1a) there was no microbial growth detected at any of the screened days in contrast with the samples stored at 24 ºC. This might have happened mostly because to the effect of the different storage temperatures because lower temperatures hinder microbial growth as demonstrated in the different growth curves from the untreated control which surpassed the 5 Log growth at the 5th day in samples stored at 24 ºC, and after the 15th day in samples stored at 5 ºC. Similar to these results, Suárez-Jacobo et al. (2010) reports an important reduction in the indigenous microbiota of apple juice starting from 200-MPa UHPH treatments (4 and 20 ºC InT). In their experiments at 100-MPa UHPH they also report that the most affected microorganisms are faecal coliforms. In our experiments (Table 1, Figure 2) the controls revealed the presence of faecal coliforms but they were not detected in any of the UHPH treatments. The presence of survival microorganisms might have happened due to a possible presence of spores in these samples which are more resistant to stresses including heat and high pressures and could survive UHPH treatments as previous research by our group indicate (Reverter-Carrión et al., 2018; Jezer Noé Sauceda-Gálvez et al., 2020). In both storage temperatures and in treated samples there was a tendency towards a growth peak in day 15, but they decreased again in day 30 (Figure 1), the

highest growth was observed in the samples incubated at 24 ºC with an average of 2.16 Log (Figure 1b). At the end of this screening there was no much difference between the results of the different InT assayed regarding microbial growth.



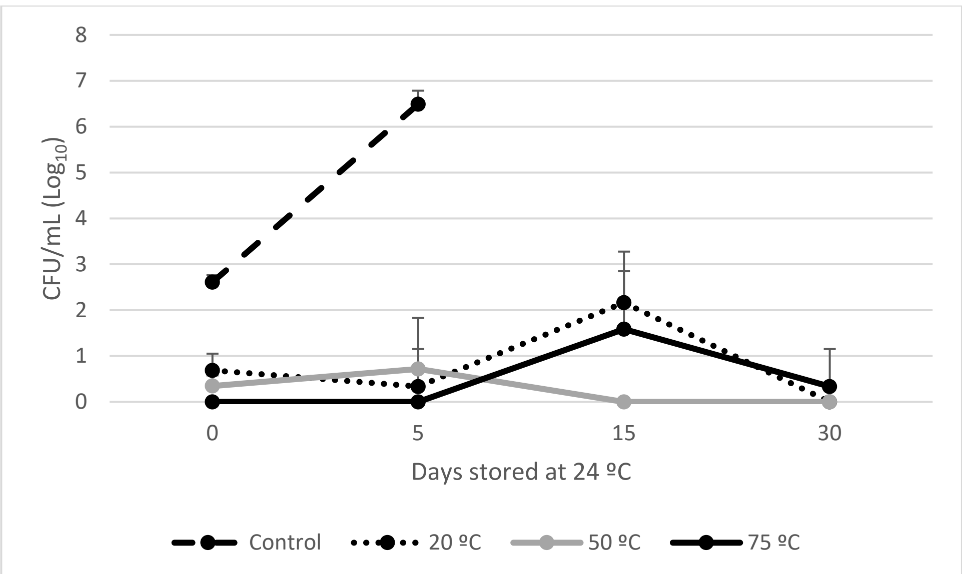


Figure 1. Growth rate of aerobic mesophiles in clarified apple juice with added chia extract (CSE24). The untreated juice (Control) was compared with UHPH-treated juice at 200 MPa and three different inlet temperatures (20, 50 and 75 ºC). The microbial load was screened at days 0, 5, 15 and 30 of samples stored at different temperatures: a) 5 ± 1 ºC, and b) 24 ± 1 ºC. If the microbial load in the sample surpassed the 5 Log count, it was no longer screened. Error bars mark the mean standard deviation.

Table 1. Microbial counts of the many types of indigenous microbiota in clarified apple juice with added chia extract (CSE24) before and after UHPH treatments at 200 MPa and different inlet temperatures (20, 50 and 75 ºC).

	Microbial count in CFU/mL (Log_{10})		
UHPH InT (ºC)	**Aerobic mesophiles**	**Faecal coliforms**	**Moulds and yeast**
Control	2.61 ± 0.16	1.07 ± 1.21	1.9 ± 1.56
20 ºC	0.69 ± 0.36	0	0
50 ºC	0.35 ± 0.23	0	0.39 ± 0.62
75 ºC	0	0	0

Experiments with apple juice with a higher concentration of chia extract (CSE60) seem to have made UHPH more effective into inactivating indigenous microbiota of apple juice, because there were no aerobic mesophiles detected in any of the samples treated by 200 MPa UHPH at 20 ºC. Experiments conducted by Roig-Sagués et al. (2009) explain that higher content of fat in milk could inactivate *Listeria monocytogenes* by UHPH better than in skim milk. A higher chia content might have occasioned a similar effect in microbial lethality, but it can also depend on the types of microorganisms in each of the apple juices batches, and this can also be supported by the fact that the initial microbial counts in them were not the same: this not only included its indigenous microbiota but also a moderate concentration (less than 3.5 Log) of *A. acidoterrestris* spores, a bacterium potentially present in acidic food products that causes spoilage (Chang & Kang, 2004; Walker & Phillips, 2008), to test the sporicidal effect of different treatments (UHPH, UV-C, a combination of UHPH and UV-C treatments, HHP and thermal pasteurisation). *A. acidoterrestris* could be efficiently inactivated by almost any treatment involving UV-C, except for the combined treatment with UHPH and 14.3 J/mL with a total of 0.54 ± 0.18 Log CFU/mL. The least effective treatments (with the lowest lethality) against *A. acidoterrestris* spores was HHP (0.05 ± 0.05) followed by UHPH (0.71 ± 0.11 Log CFU/mL) and thermal pasteurisation (0.74 ± 0.18 Log CFU/mL). These results are in consonance with other investigations with *A. acidoterrestris* spores where it can be seen their high resistance to any kind of pressure-based technologies and thermal pasteurisation (Bevilacqua et al., 2007; Reverter-Carrión et al., 2018; Sauceda-Gálvez et al., 2020; Silva et al., 1999). Previous research conducted by our group (Sauceda-Gálvez et al., 2020) demonstrate that UV-C can inactivate *A. acidoterrestris* spores in clarified apple juice obtaining similar reductions at the same

doses. There was a certain resistance against UHPH and UV-C by some of the indigenous moulds and yeasts in the matrix, but they were not detected in all of the treated samples. That might have happened because of the heterogeneity of microorganisms with different characteristics that could make them resistant to stresses. Due to that variability of microorganisms it is good to submit foods to different types of stresses in order to address a broader range of possible resistances coming from them and from interferences in food matrices. The combined treatment of UHPH and 7.2 J/mL could inactivate moulds and yeasts better than UV-C alone at the same dose, but higher doses of UV-C without UHPH were enough to inactivate them until they were no longer detected. This additive effect between the two technologies has also been reported by Reverter-Carrión et al. (2018) and Sauceda-Gálvez et al. (2020). There were no detectable differences between the UV-C samples of 14.3 J/mL and 14.4 J/mL divided in two passes. Previous investigations show that lethality rates can increase with the number of re-circulations for the same final dose (Martinez-Garcia et al., 2019; Reverter-Carrión et al., 2018; Sauceda-Gálvez et al., 2019)

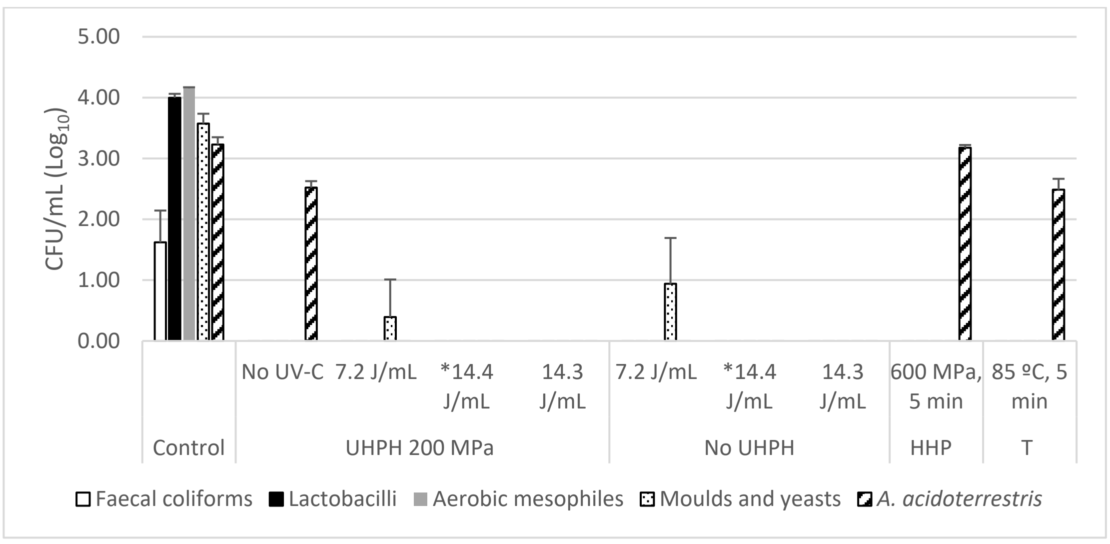


Figure 2. Effect of different treatments (200-MPa UHPH, UV-C, combination of UHPH and UV-C in that order, 600-MPa HHP for 5 min, and thermal pasteurisation at 85 ºC for 5 min) on indigenous microbiota of clarified apple juice with added chia extract (CSE60) and inoculated *A. acidoterrestris* spores. All treatments were performed at 20 ºC, except thermal treatments. Error bars mark the mean standard deviation. (*) means that the UV-C dose was given in 2 passes.

According to the results shown in Fig. 3, there was a growth of aerobic mesophiles in samples stored at 24 ± 1 ºC, although those were not detected in any of the assayed treatments for CSE60, and remaining microbial load was enough to grow

up and keep on multiplying. The treatments that could preserve better CSE60 juice were those ones involving 200-MPa UHPH which had undetectable growth until day 15. Those results are similar to the ones of Suárez-Jacobo et al., 2010 with 200-MPa UHPH, that obtained a reduction in the load of aerobic mesophiles which fluctuated around 1 Log over the days to almost reach a 2-Log growth by day 60 of incubation at 20 ºC. Samples treated with UV-C only surpassed the 5-Log growth by day 15 of incubation, maybe because the dose was not enough to provoke a non-reversible damage on all microorganisms, and the growth of that curve was faster than the ones from HHP and thermal pasteurisation treatments in that order.

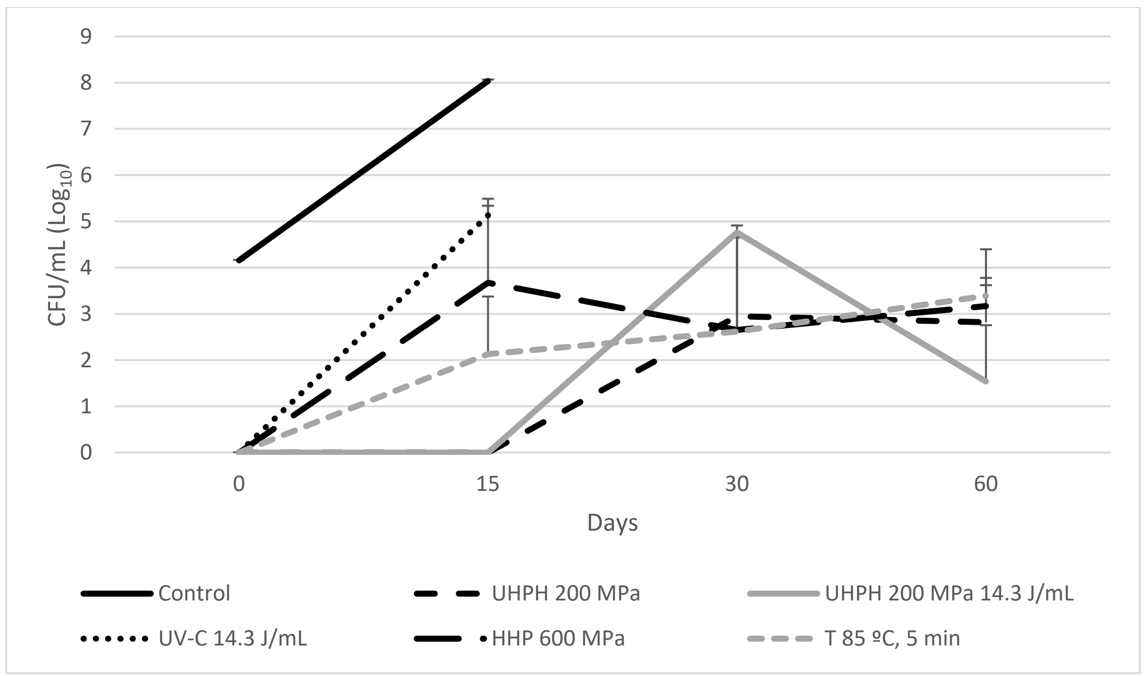


Figure 3. Growth rate of aerobic mesophiles in clarified apple juice with added chia extract (CSE60). The untreated juice (Control) was compared with juice treated by UHPH at 200 MPa, UV-C at 14.3 J/mL and their combination; HHP at 600 MPa and thermal pasteurisation at 85 ºC for 5 min. Non-thermal processes were carried out at 20 ºC InT. The microbial load was screened at days 0, 15, 30 and 60 of samples stored at 24 ± 1 ºC. If the microbial load in the sample surpassed the 5 Log count, it was no longer screened. Error bars mark the mean standard deviation.

Different to the results of aerobic mesophiles, *A. acidoterrestris* growth was not detected in any of the samples treated by UV-C according to Fig. 4. *A. acidoterrestris* spores are known to be resistant to many types of stresses like pasteurisation temperatures and high-pressure technologies, but results here, which were in consonance with our previous results (Sauceda-Gálvez et al. 2020) in apple juice, suggested that those spores inoculated in CSE60 apple juice were vulnerable to UV-C light and also that the damage was not repaired because there was no growth

detected in samples stored even at an incubation temperature within the optimal growth rate (42 - 53 ºC) (Walker & Phillips, 2008). Spore population in HHP-treated samples showed a tendency to diminish during the 60 days of storage, while for the rest of the samples non-treated by UV-C, their average growth kept on fluctuating in between the 3-4 Log.

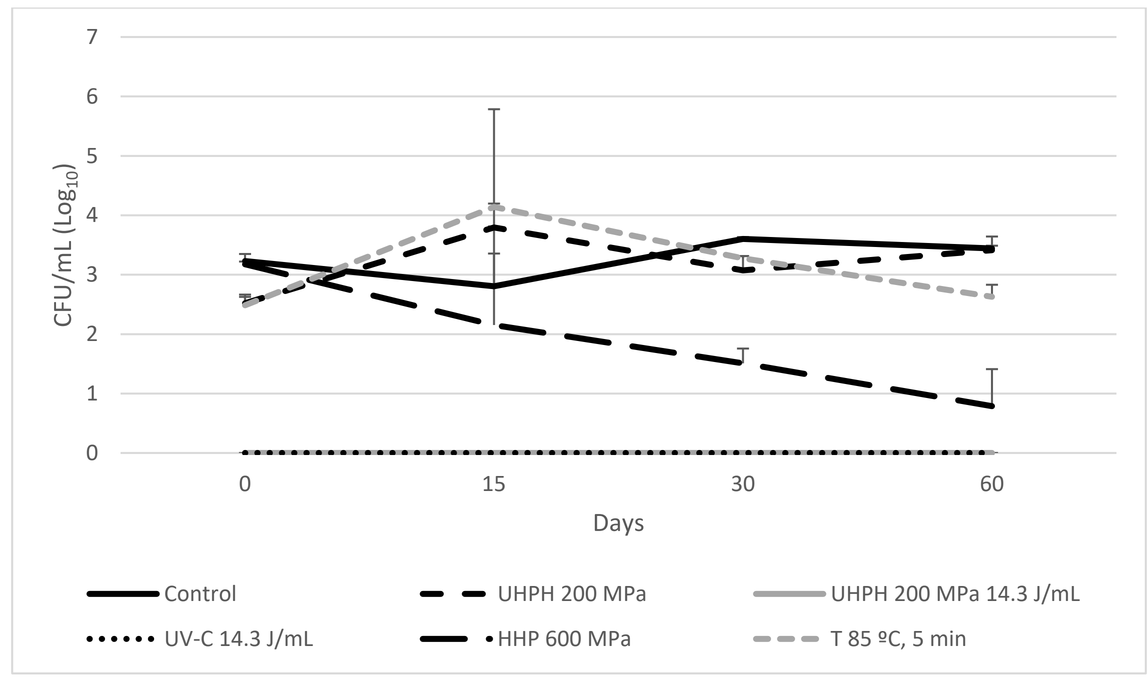


Figure 4. Growth rate of *A. acidoterrestris* in clarified apple juice with added chia extract (CSE60). The untreated juice (Control) was compared with juice treated by UHPH at 200 MPa, UV-C at 14.3 J/mL and their combination; HHP at 600 MPa and thermal pasteurisation at 85 ºC for 5 min. Non-thermal processes were carried out at 20 ºC InT. The microbial load was screened at days 0, 15, 30 and 60 of samples stored at 42 ± 1 ºC. Error bars mark the mean standard deviation.

3.2. Physicochemical results

Both in CSE24 and CSE60 apple juices (Table 3) there was an increase of total polyphenols and antioxidant activity measured by DPPH and FRAP methods when compared with apple juice without chia extract, but there was a remarkable difference between the two assayed concentrations of chia extract as the addition of CSE24 increased total polyphenols in an average of 7.85% while in CSE60 the average increase was of 101%. There was also an increment in the antioxidant activity according to DPPH and FRAP assays with averages of 7.87% and 4.07% respectively in CSE24, and 53.44% and 53.77% in CSE60.

It was also evaluated the effect that the different UHPH treatments (depending on the InT) had on polyphenols and antioxidant activity of the apple juice with CSE24 (Table 2) as well as in their pH value. As InT became higher, there was a tendency for total polyphenols and antioxidant activity measured by FRAP to become lower. In UHPH treatment at an InT of 75 ºC the polyphenol content was the same as in the apple juice before the addition of chia extract, and the antioxidant activity by FRAP showed an average reduction of -9.2%. Antioxidant activity by DPPH showed an increase after 200-MPa UHPH and InT of 20 ºC, but it kept on dropping down with higher InT. pH value augmented with higher InT. Therefore, the UHPH treatment that least affected negatively apple juice with added chia extract was the 200-MPa at an InT of 20 ºC.

Table 2. Physicochemical changes in apple juice after the addition of chia extract equivalent to 24.6 g chia seeds per litre of apple juice, and after UHPH treatments at 200 MPa and different inlet temperatures (20, 50 and 75 ºC). The different measured parameters were pH, total polyphenols, and antioxidant activity done by DPPH and FRAP methods. All values included their standard deviations.

UHPH - InT (MPa - ºC)	**Increment in percent**			**pH**
	Total polyphenols	**Antioxidant activity DPPH**	**Antioxidant activity FRAP**	
0 - 20ºC	7.85 ± 3.44	7.87 ± 3.41	4.07 ± 18.06	3.69 ± 0.006
200 - 20 ºC	7.97 ± 0.27	14.76 ± 0.49	-1.03 ± 10.21	3.7 ± 0.006
200 - 50 ºC	6.17 ± 3.2	12.79 ± 4.51	-4.09 ± 12.42	3.73 ± 0.006
200 - 75 ºC	0.2 ± 3.55	11.8 ±4.51	-9.2 ± 9.07	3.81 ± 0.011

Table 3. Physicochemical changes in apple juice after the addition of chia extract equivalent to 60 g chia seeds per litre of apple juice. Juices were treated by 200-MPa UHPH and different doses of UV-C (7.2, 14.3, and 14.4# J/mL). Those were also compared with other treatments like HHP at 600 MPa and thermal pasteurisation (85 ºC, 5 min). The different measured values were total polyphenols, and antioxidant activity done by DPPH and FRAP methods. All values included their deviation standards. Significant differences ($p < 0.05$) were marked by lowercase letters and they were obtained by Tukey test (*), and Games-Howell test (**). # UV-C dose was done in two passes of half the final dose

Treatment		**Increment in percent**		
	UV-C (J/mL)	**Total polyphenols***	**Antioxidant activity DPPH***	**Antioxidant activity FRAP****
Non-treated		101.01 ± 12.17^{abc}	56.44 ± 18.45^{abc}	53.77 ± 56.03^{abc}
UHPH 200 MPa	**0**	325.11 ± 82.38^{d}	11.92 ± 12.4^{ac}	63.99 ± 42.22^{abc}
	7.2	217.91 ± 40.34^{bcd}	6.16 ± 46.94^{abc}	126.85 ± 63.87^{c}
	14.3	277.06 ± 30.69^{cd}	11.37 ± 87.04^{abc}	124.34 ± 23.76^{c}
	14.4#	289.52 ± 95.13^{cd}	12.13 ± 95.61^{abc}	120.25 ± 32.96^{bc}
UV-C only	**7.2**	176.11 ± 13.97^{bc}	96.35 ± 31.24^{abc}	-4.77 ± 38.75^{a}
	14.3	208.78 ± 22.12^{bcd}	44.04 ± 25.05^{abc}	15.74 ± 30.47^{abc}
	14.4#	205.29 ± 23.97^{bcd}	-15.93 ± 18.4^{c}	39.52 ± 2.2^{abc}
HHP 600 MPa		97.51 ± 43.92^{ab}	112.49 ± 27^{ab}	10.41 ± 45.62^{abc}
Pasteurisation 85 ºC 5 min		314.47 ± 50.32^{d}	136.89 ± 18.78^{b}	35.13 ± 42.68^{abc}

Experiments with UHPH, UV-C and their combination showed that the treatment that better preserved the content of total polyphenols was UHPH at 200-MPa. UV-C treatments can affect the polyphenol content in apple juice with CSE60, but according to Tukey test, these changes were not significant when compared with the sample treated by 200-MPa of UHPH. A lower content in total polyphenols from UV-C treatments alone might have happened due to the activity of the polyphenol oxidase (PPO). According to previous research, although UV-C treatments are capable of inactivating PPO at a certain degree, it seems that they will not be able to inactivate that enzyme completely, even after prolonged treatments (Falguera et al, 2014; Guerrero-Beltrán & Barbosa-Cánovas, 2006). PPO is an enzyme that is capable to oxide phenolic compounds that contain two *o*-dihydroxy groups to the corresponding *o*-quinones (Kolniak-Ostek et al., 2013). Some other research point out that UHPH is able to inactivate PPO at different degrees in fruit juices depending on the utilized pressure and in the number of passes through the system, up to a 90% in grape must using 300-MPa UHPH (Bot et al., 2018; Morata et al., 2019). Probably the combination of those two technologies helped to prevent some phenolic compounds to degrade by the activity of PPO. Another advantage from using UHPH in treating apple juice with chia extract came from the homogenisation

of the product itself. Although the extract can be dispersed in the juice, it would need constant agitation or stirring so as to prevent phase separation, that was not the case for UHPH-treated samples. That could also be the reason why antioxidants from chia extract were better quantified in UHPH treated samples (Table 4) as they were more homogenous than in the other treatments. In chia seed extract, two of the predominant antioxidants are chlorogenic acid and caffeic acid (Taga et al., 1984), those compounds were better quantified in UHPH-treated samples. Chlorogenic acid was found at a concentration of 0.656 $\pm$ 0.08 mg/100 mL of juice, that was 4.6 times higher comparing it with CSE60 before UHPH-processing; and caffeic acid, which was below quantification limits, its value rose up to 2.263 $\pm$ 0.33 mg/100 mL of juice after it passed through 200-MPa UHPH. In comparison with UHPH alone, the concentration of those phenolic compounds, and many of the quantified antioxidants, decreased after the combination with UV-C treatments, being non-significant those from the combined treatments at 7.2 J/mL that was the lowest assayed dose, but they still remained significantly higher than in untreated samples or in juices treated by 600-MPa HHP and thermal pasteurisation. The preservation of those two compounds is important because they have exhibited antimutagenic, carcinogenic and antioxidant activities *in vitro*, and a protective effect against intestinal ischemia-reperfusion injury *in vivo* (Sato et al., 2011).

Table 4. Phenolic compounds in apple juice with CSE60 treated by 200-MPa UHPH, different UV-C doses, HHP (600 MPa), and thermal pasteurisation (85 ºC, 5 min). Results were obtained by HPLC. The averages and standard deviations of the compounds were expressed in mg/100 mL of juice. Lowercase letters mark significant differences ($p < 0.05$) between sample means obtained by Tukey test. (*) UV-C dose was given in two passes of half the final dose. (#) From non-hydrolysed samples. (BQL) Below quantification limits.

Phenolic compound	Untreated		UHPH and UV-C				UV-C			HHP	T ºC
	Apple juice	Apple juice with chia extract	0 J/mL	7.2 J/mL	14.3 J/mL	14.4 J/mL*	7.2 J/mL	14.3 J/mL	14.4 J/mL*		
Galic acid	0.408 ± 0.10 ^{a}	0.413 ± 0.15 ^{a}	0.300 ± 0.00 ^{ab}	0.133 ± 0.04 ^{b}	0.425 ± 0.03 ^{a}	0.390 ± 0.00 ^{a}	0.158 ± 0.07 ^{b}	0.215 ± 0.11 ^{b}	0.150 ± 0.00 ^{b}	0.263 ± 0.05 ^{ab}	0.370 ± 0.00 ^{a}
Chlorogenic acid#	0.062 ± 0.03 ^{a}	0.142 ± 0.07 ^{ab}	0.656 ± 0.08 ^{g}	0.553 ± 0.02 ^{fg}	0.465 ± 0.05 ^{ef}	0.485 ± 0.09 ^{f}	0.285 ± 0.03 ^{bcd}	0.290 ± 0.06 ^{cd}	0.340 ± 0.04 ^{de}	0.161 ± 0.03 ^{ab}	0.185 ± 0.01 ^{abc}
Caffeic acid#	BQL	BQL	2.263 ± 0.33 ^{f}	1.970 ± 0.21 ^{ef}	1.042 ± 0.30 ^{cd}	1.606 ± 0.34 ^{d}	0.421 ± 0.19 ^{bc}	0.640 ± 0.35 ^{c}	1.819 ± 0.52 ^{ef}	0.124 ± 0.03 ^{a}	0.188 ± 0.03 ^{ab}
Myricetin	0.063 ± 0.06 ^{ab}	0.025 ± 0.03 ^{a}	0.160 ± 0.05 ^{b}	0.038 ± 0.03 ^{ab}	BQL	0.063 ± 0.03 ^{ab}	0.113 ± 0.05 ^{ab}	0.075 ± 0.03 ^{ab}	0.145 ± 0.05 ^{ab}	0.138 ± 0.13 ^{ab}	0.060 ± 0.00 ^{ab}
Luteolin	0.100 ± 0.07 ^{a}	0.110 ± 0.02 ^{a}	0.200 ± 0.12 ^{ab}	0.133 ± 0.08 ^{ab}	0.075 ± 0.03 ^{a}	0.110 ± 0.02 ^{a}	0.263 ± 0.06 ^{ab}	0.193 ± 0.07 ^{ab}	0.338 ± 0.16 ^{b}	0.338 ± 0.13 ^{b}	0.155 ± 0.11 ^{ab}
Quercetin	0.050 ± 0.00 ^{a}	0.050 ± 0.00 ^{a}	0.188 ± 0.05 ^{bc}	0.140 ± 0.00 ^{abc}	0.063 ± 0.03 ^{ab}	0.075 ± 0.03 ^{ab}	0.250 ± 0.00 ^{c}	0.158 ± 0.08 ^{abc}	0.230 ± 0.09 ^{c}	0.125 ± 0.09 ^{abc}	0.153 ± 0.08 ^{abc}
Apigenin	0.088 ± 0.10 ^{a}	0.098 ± 0.04 ^{a}	0.200 ± 0.06 ^{ab}	0.260 ± 0.06 ^{ab}	0.125 ± 0.13 ^{ab}	0.095 ± 0.05 ^{a}	0.200 ± 0.04 ^{ab}	0.228 ± 0.08 ^{ab}	0.335 ± 0.07 ^{b}	0.275 ± 0.12 ^{ab}	0.138 ± 0.14 ^{ab}
Kaempferol	0.565 ± 0.05 ^{ab}	0.565 ± 0.11 ^{ab}	0.500 ± 0.04 ^{bc}	0.457 ± 0.03 ^{bd}	0.713 ± 0.06 ^{a}	0.130 ± 0.05 ^{e}	0.495 ± 0.08 ^{bcd}	0.395 ± 0.09 ^{cd}	0.568 ± 0.05 ^{ab}	0.340 ± 0.02 ^{d}	0.595 ± 0.08 ^{ab}

Conclusions

UHPH and UV-C combination could be a good treatment for fruit juices with added chia extract, in this case with clear apple juice. They showed complementarity in the microbiological and physicochemical aspects. UHPH could homogenise the product and diminish some of the bacterial population, while a combination with UV-C at the lowest assayed dose could reduce the population of *A. acidoterrestris* spores down to undetectable limits and those cells were unable to repair the damage to its genome. That combination apparently did not represent any significant decrease in the physicochemical properties of the product. Those were things that some of the most utilized technologies currently used for juices production were unable to do. The incorporation of the combination of these technologies in a serial process could represent an opportunity for the introduction of novel food products in the market with an improved content of important antioxidants to health.

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