Universidade de Aveiro Departamento de Química
2012

FABIANA NEVES VIEIRA

EFEITO DA PASTEURIZAÇÃO E DA ALTA PRESSÃO NO SUMO DE LARANJA

EFFECT OF PASTEURISATION AND HIGH PRESSURE ON ORANGE JUICE PROPERTIES

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EFFECT OF PASTEURISATION AND HIGH PRESSURE ON ORANGE JUICE PROPERTIES

Dissertation submitted to the University of Aveiro to fulfil the requirements for the degree of Master of Biochemistry in the field of Food Biochemistry, done under the scientific supervision of Dr Jorge Manuel Alexandre Saraiva, Assistant Researcher of the Department of Chemistry of the University of Aveiro and Dr. Eliana Jerónimo, Researcher at Centro de Biotecnologia Agrícola e Agro Alimentar do Baixo-Alentejo e Litoral – CEBAL.

Dedico este trabalho aos meus pais e irmão por me terem feito crescer a nível pessoal e profissional ao longo destes 23 anos

O júri

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"A vida é uma constante aprendizagem, de convívio, de partilha e de emoções que tanto nos fazem crescer."

Palavras-chave atividade antioxidante, compostos bioativos, processamento por alta pressão, sumo de laranja, métodos não térmicos

Resumo

As frutas e os produtos derivados de fruta recebem cada vez mais atenção, não só pela sua elevada estabilidade após o processamento através de técnicas tradicionais, mas também devido ao seu elevado conteúdo em compostos bioativos. As características únicas de sabor, aroma e textura são as principais razões da grande aceitação do sumo de laranja, por parte dos consumidores. A utilização de novas técnicas de processamento não térmico para os alimentos tem vindo a aumentar ao longo dos últimos anos. Além disso, a maior procura por produtos microbiologicamente seguros que mantenham as suas características e qualidades originais é a razão mais evidente para o desenvolvimento do processamento por alta pressão como alternativa não térmica.

Assim, o principal objetivo deste trabalho foi estudar possíveis benefícios da utilização desta técnica aplicada aos sumos de laranja, comparando esta tecnologia inovadora com o método comumente utilizado no processamento de sumos de laranja, a pasteurização térmica. Foi assim avaliado o efeito da aplicação de tratamento térmico (70°C, 30 seg) e de alta pressão (550 MPa, 70 seg, 18°C) no processamento de sumo de laranja ao longo de 36 dias de armazenamento sobre alguns compostos bioativos, como antocianinas, flavonoides, carotenoides e compostos fenólicos, na atividade antioxidante, cor e sólidos solúveis totais. Observou-se que, comparativamente ao tratamento térmico, a alta pressão promove a retenção dos compostos fenólicos como antocianinas e flavonoides, aumentando atividade antioxidante do sumo de laranja. Relativamente à cor verificaram-se alterações bastante importantes podendo estar associadas às grandes perdas no conteúdo total de carotenoides. No que respeita a identificação e quantificação dos compostos fenólicos, foram verificados um ácido orgânico (ácido quínico) e 4 compostos fenólicos (ácido elágico, narirutina, vicenina II, e hesperidina). Observou-se que, à excepção do ácido quínico não ocorrem diferenças significativas ao longo do tempo de armazenamento e entre ambas as técnicas de processamento. No entanto, a sua concentração é alterada verificando-se em alguns casos, diminuição significativas no sumo processado por alta pressão, quando comparado com o sumo tratado termicamente.

Dessa forma, este trabalho permitiu verificar que a alta pressão promove efeitos benéficos no sumo de laranja podendo, assim, ser utilizada como alternativa às técnicas de processamento térmicas.

Keywords antioxidant activity, bioactive compounds, orange juices, high pressure processing, non-thermal processing

Abstract

Fruits and fruit products receive more and more attention, not only because its low stability when processed by traditional technologies, as pasteurisation; but also due to its high content of bioactive compounds. The favourable ratio of sugar to acid along with the unique orange flavour, gives orange juice its universal high consumer acceptance. The use of novel non-thermal processing food technologies has emerged during the past few years. Moreover, the increasing demand of safer products that maintain their original qualities is the major driver of high pressure processing (HPP) technique development as an alternative to thermal treatment.

So, the principal objective of this work was to study the possible benefits on the utilisation of this technique applied on orange juices, when compared to the most commonly used method, thermal pasteurisation. Thus, it has been verified thermal (70°C, 30 sec) and high pressure (550 MPa, 70 sec, 18°C) processing effects during 36 days of storage regarding some bioactive compounds, such as anthocyanins, flavonoids, carotenoids and phenolics, on antioxidant activity, total soluble solids and colour.

Here was observed that, comparing with thermal treatment, HP promotes the retention of phenolic compounds such as anthocyanins and flavonoids, increasing the orange juice antioxidant activity. Regarding the colour of the samples were verified some important changes which might be associated mainly to total content of carotenoids losses. Also the characterization and quantification of phenolics present on orange juice samples allowed the identification of one organic acid (quinic acid) and four phenolic acids (ellagic acid, narirutin, vicenin II and hesperidin). Was observed that with the exception of quinic acid, there are no significant changes within the storage time and type of process. However their concentration it's changed and in some cases it was observed significant decreases on HPP orange juice when compared with TP samples.

Thus, with this work was verified that HPP promotes some beneficial effects on orange juice being a great alternative to thermal processing methods used before for this type of products.

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LIST OF ABBREVIATIONS

AA	Ascorbic Acid
ANOVA	One-way analysis of variance
ARP	antiradicalar power
CHOOH	formic acid
CIE*Lab*	CIE L*, a*, b* uniform colour space
cy3glu	cyaniding-3-glucoside
DNA	Deoxyribonucleic acid
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
$ESI\text{-}MS_n$	electrospray ionization mass spectrometry-tandem mass spectrometry
HHP	High Hydrostatic Pressure
HPLC	High-performance liquid chromatography
HPLC-ESI-MS	High-performance liquid chromatography - electrospray ionization mass spectrometry
HPP	High Pressure Processing
LDL	Low Density Lipoprotein
LOX	Lipoxygenases
PA/PE-90	Polyamide/ polyethylene
PEF	Pulsed Electric Fields
PME	Pectin methylesterase
PPO	Polyphenoloxidase
TP	Thermal Processing
t_R	Retention Time
UHP	Ultra High Pressure
UV-Vis	Ultraviolet-Visible
ΔE	Total variation of colour

I. Introduction

In the past few years, human diet has been the focus of attention of some nutrition researchers [1]. Some epidemiological studies have shown a relationship between foods and health, finding the specific function of some food components on human body [2]. One of the most illustrative effects of fruit consumption on health are the phytonutrients, such as polyphenols and vitamins that prevent degenerative diseases inhibiting the oxidative mechanism [3-7]. However, it is known that these capabilities highly depend on its processing and so, the demand for processed foods containing ingredients with a specific body function – functional foods [8] – has led to the development of new minimally processing techniques [9, 10].

Physical and chemical preservation methods continue to be extensively used. Heat treatments, such as pasteurisation and sterilisation, are the most used methods to process and preserve food, mainly due to its ability to inactivate a wide range of microorganisms and spoilage enzymes [11, 12]. However, heat processing (normally using 90 °C) may induce several chemical and physical changes, reducing the content or even the bioavailability of some bioactive compounds [13, 14]. Therefore, considerable research efforts have been done to develop non-thermal processes used as preservation techniques, and thus respond to consumers' desires for nutritious, fresh and natural products [15]. In the search for new processing methods, a few non-thermal techniques to process fruit and fruit juices have emerged. Pulsed electric fields (PEF), high-intensity light pulses, magnetic fields and high-pressure processing (HPP) are the most used ones [16], and all of them are used as alternative technologies to heat treatments regarding the quality and safety of minimally-processed food products [17-19]. These methods have significant differences, particularly regarding to advantages, disadvantages and used conditions, that may differently affect fruit components as review by Gomez *et al*. (2011) [20]. Among the non-thermal processes, the HPP has shown to be effective preserving the microbial and sensory qualities, as well as the bioactivity of fruit juices during storage [21]. This technique uses pressure ranges from 100 to 800 MPa to inactivate some harmful and pathogenic microorganisms and enzymes responsible for the loss of quality of some products, including several fruit juices [22].

Taking into account all the research that has been already done on HPP of fruit juice, the aim of this work is to evaluate the main effects of this novel technology on some bioactive compounds and antioxidant activity of orange juice The effects of HPP on orange juice were compared with those observed on thermal pasteurization, because it keeps to be the most used method to process orange juice.

II. Review

II.1. Orange juice composition

During last two decades the dietary influence on health has been highly studied and new healthier foods have been created to reduce the risk of several diseases [23]. At the same time, the vital role of fruits and vegetables have been widely considered to be critical for human health and a wide range of studies demonstrate their effects on the reduction of the risk of cardiovascular diseases and some types of cancers [1, 10, 24]. These results have created a new tendency on fruit juices consumption in many countries, mainly because they are an important dietary source of bioactive compounds, such as polyphenols, vitamins and carotenoids [15, 25]. Additionally, and due to their nutritional benefits, fruit juices are highly consumed worldwide, and because their well known profits on prevent cardiovascular disease and some cancers, orange juices are receiving higher attention [26, 27]. Globally, orange juices are the most consumed fruit juices among apple, grape, grapefruit and other fruit juices (Figure 1) [28].

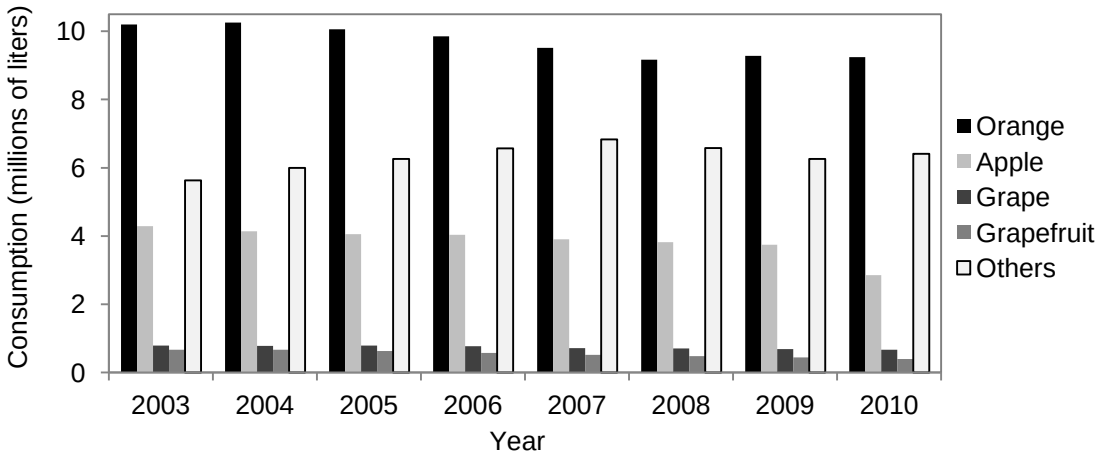

Figure 1: Global consumption (in millions of litres) of orange, apple, grape, grape fruit and other fruit juices (adapted from [28]).

The composition of orange is not homogeneous, varying over the maturation of the fruit and with the orange specie. Orange juice provides a wide range of bioactive compounds and nutrients for human diet [29]. Beyond vitamin C (Table 1), orange juice is also one of the main sources of carotenoids (xanthophylls, cryptoxanthins, carotenes), antioxidant flavanones (hesperetin and naringenin predominantly as glycosides) and other beneficial phytochemical compounds such as folate [25, 30]. In addition, orange juice has some important organic acids responsible for its low pH, such as citric acid, malic acid, tartaric acid, benzoic acid and succinic acid [31].

Table 1: Basic nutrient composition of orange juice (adapted from [32])

Nutrients (units)	Amount in 100 g juice
Water (g)	88.40
Soluble solids (°Brix)	11.0
Protein $N \times 6.25$ (g)	0.80
Lipids (g)	0.27
Carbohydrates (g)	10.06
Energy (kcal)	44
Sugars (g)	9.7
Ash (g)	0.48

The well known primary composition of orange juice together with the higher consumption of orange and orange products demonstrate the importance of full studies regarding its main bioactive compounds, bioavailability and beneficial effects on human's health.

II.1.1. Carbohydrates

Carbohydrates are highly energetic (nutritional value – 17 kcal/g) organic compounds, being the main source of energy in the human diet [33] and acting as regulatory compounds and bulk materials to human organism [34]. About 10% of orange juice composition (Table 2) are carbohydrates such as fructose, glucose, sucrose, pectin and cellulose [32, 35].

Table 2: Main sugar composition on orange juice (g/100mL) (adapted from [32]).

Sugars (per 100 mL)	Glucose (g)	2.1
	Fructose (g)	2.5
	Sucrose (g)	5.1
	Total (g)	9.7

The sucrose, an oligosaccharide formed by glucose and fructose (Figure 2a), is one of the most important carbohydrates presents in orange and orange juice, since the degradation of this carbohydrate might provoke browning colours on food products, namely on orange juice due to thermal degradation and Maillard reactions [36]. Regarding human

health sucrose is degraded into glucose and fructose providing a great source of energy; however it can contribute to obesity and dental caries if consumed in excess [37].

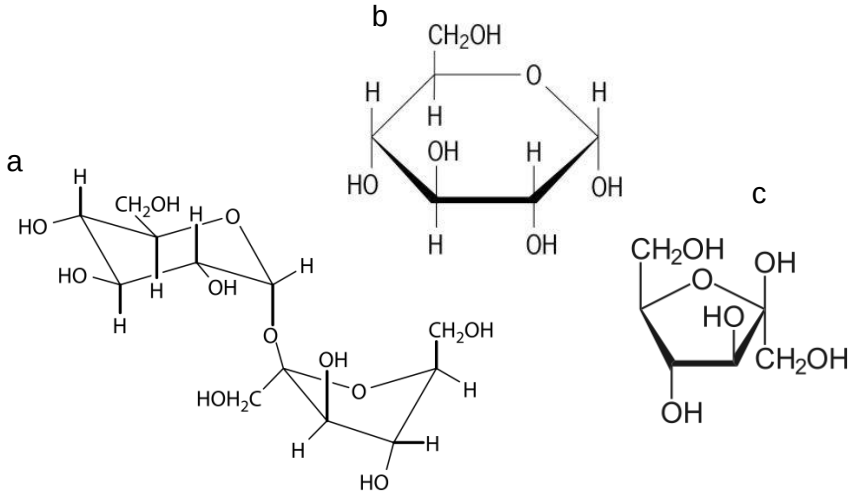

Figure 2: Sucrose (a), glucose (b) and fructose (c) [36]

Glucose (Figure 2b) is also an important carbohydrate present in orange juice, since its degradation to pyruvate (glycolysis) and further use of this derivative product in the citric acid cycle is one of the most important sources of adenosine triphosfate (ATP). Fructose (Figure 2c) is also used in glycolysis to form fructose-6-phosphate and fructose-1-phosphate, important intermediates to pyruvate production [38]. Although there are other important plant carbohydrates such as starch and cellulose, its content on orange and orange juice is residual and so no studies were found in this area.

II.1.2. Organic acids

As observed before (Table 1), organic acids are not the major component of orange juice, nevertheless, they have an important role as preservative compounds in a whole number of food products [39]. The major organic acids found on orange juice are citric and malic, whose concentrations are indicated on Table 3.

Table 3: Main organic acids on orange juice (10^{-4}g/100mL) (adapted from [32]).

Organic acids	Citric acid	0.50-0.66
	Malic acid	0.09-0.14

Organic acids (Figure 3) are constituted by a carbon chain and a carboxyl group and, in general, are the main responsible by the acidity of fruits [40]. In one hand, one of the main group of organic acids are the phenolic acids such as caffeic and chlorogenic [41].

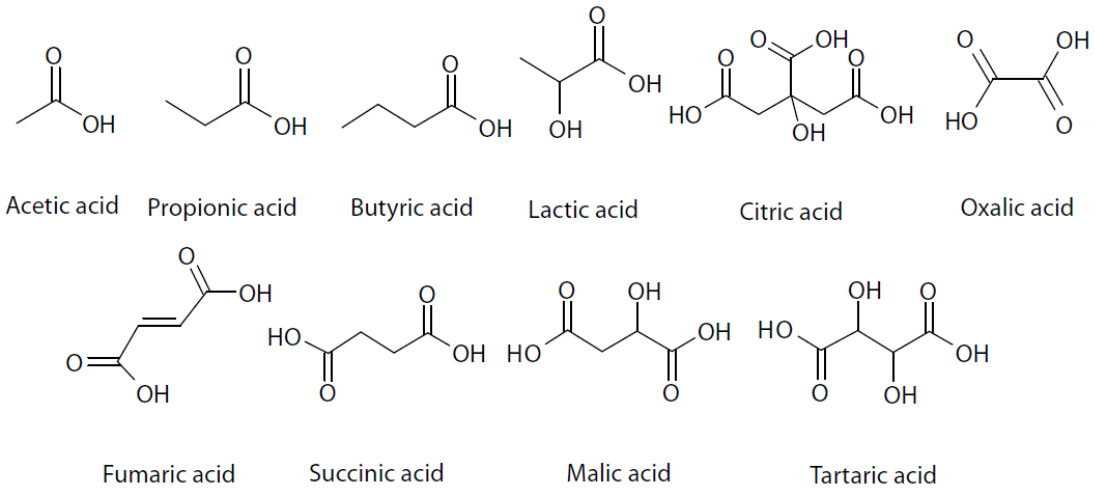

Figure 3: Main organic acids present in fruits [40]

By the other hand, citric and malic acids are the major organic acids of fruits, namely orange [41-43], and the main contributors of the flavour and acidity of orange juice [29].

II.1.2.1. Phenolic compounds

Phenolics are a wide range of compounds mainly found in plants. They are generally constituted by at least one aromatic ring and one or more hydroxyl groups [44, 45]. Due to those carboxyl groups, associated to the antioxidant and free radical scavenging abilities, and their effects on human health, food phenolics have been the focus of attention of some fruit studies [46-49]. The content of phenolic compounds in plants is regulated by many factors, such as germination and degree of ripeness. Temperature, processing and storage also influence phenolics concentration [50-52]. One of the major parts of phenolic compounds are phenolic acids which possess carboxylic groups and a remarkable effect on sensory and nutritional quality of plant products [40]. They also play an important role on enzymatic browning as polyphenoloxidase (PPO) substrates and over a few years the antioxidant capacity of phenolic acids in food received a lot of attention [49]. The production of free radicals in human body might provoke damages on proteins, lipids and deoxyribonucleic acid (DNA). Despite endogenous mechanisms presents in the human organisms, in the most cases, those mechanisms are not enough and cells need exogenous antioxidants, such as phenolic acids, to prevent free radical damages [53, 54]. In fact, this compounds act as chain breakers or terminators of lipid radical chain oxidation preventing degenerative diseases [55].

The other major part of phenolics compounds are flavonoids. Flavonoids are natural substances with phenolic structures found in fruits, vegetables, grains and many other sources [56]. Those pigments are usually classified as anthocyanins, catechins (flavonols)

and anthoxanthins (flavones, flavanones and flavonols) and are important antioxidants compounds because of hydrogen-donating capacity [57]. In addition, flavonoids have been associated with prevention of some cardiovascular disorders, chronic diseases and cancer, as well as with antiviral, antimicrobial and anti-inflammatory activities [58]. Orange is one of the main sources of anthocyanins and flavanones, two of the flavonoids pigments responsible for red and blue coloration and for the bitter taste of oranges, respectively [59].

II.1.3. Vitamins

Vitamins are important compounds in human diet, however their daily intake should be controlled since human needs for these type of compounds are reduced [60]. Over the years have been identified 13 vitamins and they are generally found on food or supplements, with the exception of vitamin D, which can be synthesized in the skin (as inactive form), and vitamin K, which is synthesized by intestinal microflora [61]. Regarding orange juice, it has been observed a major concentration of vitamin C and vitamin A when compared to other vitamins (Table 4).

Table 4:Main vitamins content on 100 mL of orange juice (adapted from [32])

Vitamins	Vitamin C (mg)	3.95
	Vitamin A (IU)	78.0
	Thiamine (mg)	0.096
	Riboflavin (mg)	0.021
	Niacin (mg)	0.280
	Pantothenic acid (mg)	0.191
	Vitamin B_6 (mg)	0.054
	Folic acid (mg)	0.029

Vitamin C is a water-soluble vitamin usually used to refer the ascorbic acid (Figure 4). However it is known that this term also refer the dehydroascorbic acid. This vitamin have some important roles on biosynthesis pathways such as collagen (structural protein found in skin, bones, cartilage and tendons), carnitine (transport of long chain fatty acids from the cell cytoplasm into the mitochondrial matrix) and neurotransmitter synthesis [62].

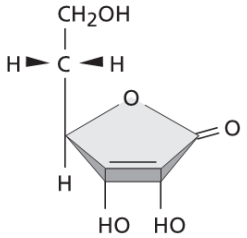

Figure 4: Chemical structure of ascorbic acid [63].

Moreover, vitamin C is involved in hydroxylation reactions such as biosynthesis of catecholamine, hydroxyproline and corticosteroids, and it is absorbed and mainly distributed by adrenal and pituary glands [64]. In addition, vitamin C shows a significant capacity as reducing agent and consequently an important antioxidant activity because of its acidic hydroxyl group [65]. Vitamin C also plays an important role as inhibitor of enzymatic browning [64]. Those facts make clear the importance of orange on human diet linking its role on human biosynthesis pathways with its antioxidant activity [65, 66], to the prevention of scurvy and some cardiovascular diseases [62]. Despite all the advantages of its presence on food products, ascorbic acid (AA) might suffer degradation and non-enzymatic browning leading to important losses on commercial value of those products, namely on colour and flavour parameters [62, 67-69].

Vitamin A (or retinol) is the normally used name for the group of structurally similar compounds with *all*-trans retinol activity [70]. The importance of vitamin A in protein metabolism of cells is well known and the lack of retinol may affect epithelial tissue and cause night blindness [71]. Additionally, vitamin A is recognized as being essential for vision, being responsible for the formation of rhodopsin – an important pigment protein that is cleaved in the presence of light, releasing opsin and consequently inducing conformational changes on cis-retinal [70]. Vitamin A also plays an important role in gene expression, bone growth and development, reproduction and cell division [61].

II.1.4. Minerals

Minerals are used by human body in different ways, being essential to several life processes and acting as activators of enzymes, hormones or other molecules [65]. There are two groups of minerals, the macrominerals, which the daily intake needs to be at least 100 mg/day, and the microminerals needed in very small quantities [72]. Potassium is one of the main minerals present in orange juice (Table 5) and it is essential in human nutrition due to its important role on electrolyte balance, muscle contraction and neural impulses [73].

Table 5: Minerals composition on orange juice (g/100mL) (adapted from [32]).

Minerals	Sodium	0.4
	Potassium	151
	Calcium	10.2
	Iron	0.11
	Magnesium	12.9
	Phosphorous	16.7
	Zinc	0.043
	Copper	0.031
	Manganese	0.023

Calcium, magnesium and phosphorus are also important minerals which might be found on orange juice. Calcium is the most important mineral in the human body, being mainly present in bones and teeth (99%), and playing an important role in normal physiological functions [74]. Also phosphorus and magnesium are accumulated in the skeleton and have important roles in human body regulatory system [73]. Despite the importance of those minerals in human body only a few studies where found regarding its content on fruits, especially on orange.

II.1.5. Carotenoids

Carotenoids, a group of vitamin A precursors responsible for retinol synthesis (Figure 5), are lipid soluble pigments, normally produced by plants, that can be converted to retinol [1]. They are the main responsible for the colours of fruits and vegetables [75]. Structurally, carotenoids have an expanded carbon chain with conjugated double bonds and an unsubstituted β-ionone ring (Figure 6) [70]. These pigments are highly sensitive to oxygen and light but are stable even at high temperatures [71]. Therefore, a wide range of aroma compounds (*eg*. lycopene, β-carotene, neoxanthin) are formed during oxidative degradation of carotenoids and that could be a problem in food processing [76].

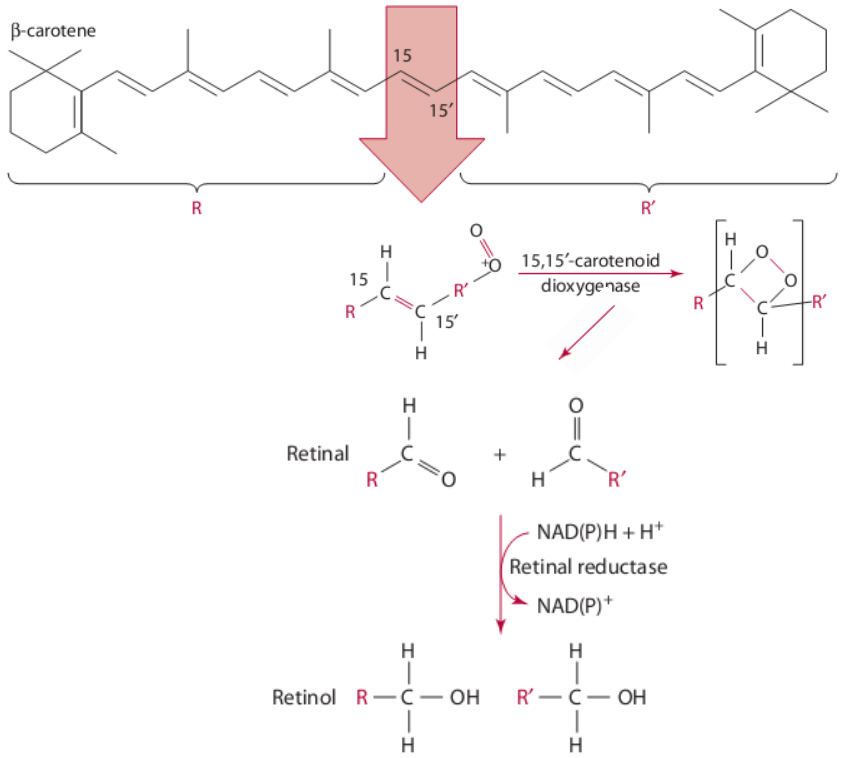

Figure 5: Retinol formation by β-carotene cleavage and retinal reduction (adapted from [70]).

Both vitamin A and carotenoids are found naturally in foods from animal origin (*eg.* dairy products, fish and meat), but the main sources of this important bioactive compounds are fruits and vegetables [70]. The most frequent carotenoids are β-carotene, α-carotene, and β-cryptoxanthin [77]. β-carotene is the most abundant of all, but on orange juice β-cryptoxanthin (Figure 6a), lutein (Figure 6b) and zeaxantin (Figure 6c) [27, 71] are the main carotenoids.

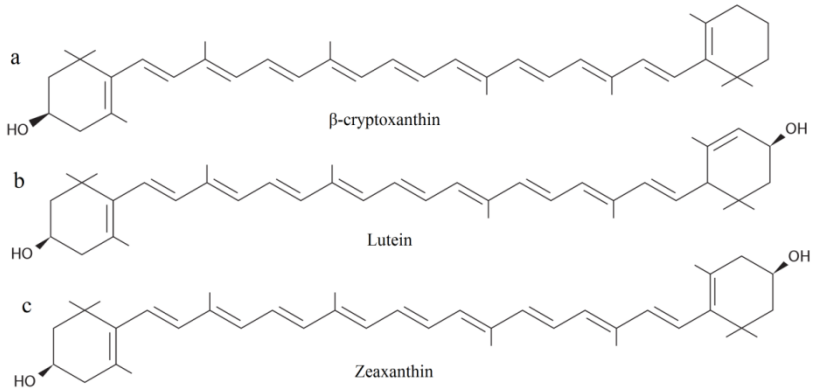

Figure 6: β-cryptoxanthin (a), Lutein (b) and Zeaxanthin (c) structure (adapted from [70, 71]).

Carotenoids content on orange, and consequently, on orange juice are quite variable and highly influenced by the variety, environment and maturation stage of the orange. For instance, some researchers reported that the carotenoids content can vary within the variety

as function of climatic and geographical area features [78]. Although structurally similar, carotenoids have different functions. For example, β-carotene shows an important antioxidant activity, reacting with some radical species and protecting against degenerative diseases [33]. In fact, the main role of those pigments relies on prevention of low density lipoprotein's (LDL) oxidation, an so, preventing atherosclerosis [70]. Moreover, because of its provitamin A activity, β-carotene has extremely important functions in immunitary system, bone development, and cellular differentiation [79]. On the other hand, lutein and zeaxanthin, found in the macula, prevent oxidation of cell membranes protecting eye against UV induced damage [70]. A wide range of carotenoids also acts on cells, inhibiting for example cellular growth in tumour cell lines [75].

II.2. The ongoing high-pressure processing technique

Nowadays, consumers require safe, high quality and fresh-like products which implies treatment with fewer or even no additives. Although there are several food preservation methods, such heat, freeze, dehydration and acidification, the most commonly used is thermal treatment such as pasteurisation. Although the use of high temperatures affects positively the microbial safety of TP fruit juices, the opposite effect is observed on bioactive compounds. In fact bioactive compounds and microorganisms are affected negatively by thermal processing, with inactivation of enzymes and microorganism proliferation, but also with important changes on bioactive compounds [11, 80]. So, use of non-thermal technologies that ensures the food safety and at the same time preserves its bioactivity, appears to be a good compromise for food production with high nutritional value.

High pressure processing (HPP), high hydrostatic pressure (HHP) or ultra high pressure (UHP) processing is a non-thermal technique used to inactivate some harmful pathogens, microorganisms and enzymes [11, 22]. The first known research about HPP was Hite's report (1899), that investigated the application of high pressure as a mean of preserving milk, and later extended the study to preserve fruits, fruit juices and vegetables [80, 81]. In 1899 Hite and his co-workers designed and constructed the first known HPP unit to pasteurise milk and other products [82], and years later showed that yeast and lactic acid bacteria associated with fruit were more susceptible to pressure than other organisms, especially spore forming bacteria associated with vegetables [80].

After Hite's studies a wide range of researchers report some of the advantages and disadvantages of HPP as a potential method for producing products with natural qualities, leading to an extensive exploration of this technique. During the past two decades several HHP-processed commercial fruit products are available in some countries [83] being seafood, juices, smoothies, pickled vegetables, ham, cheese and sauces (Figure 7a) the main products treated by HPP in United Kingdom, Japan, United State of America and Europe [84].

The great development of the HPP method is the direct precursor to global growth of this technology in food industry (Figure 7b). Over the past decade, the number of installations units increased from 19 to 158 and it is estimated that in the coming years this number tends to grow even more, due to its effectiveness for microorganisms inactivation [85].

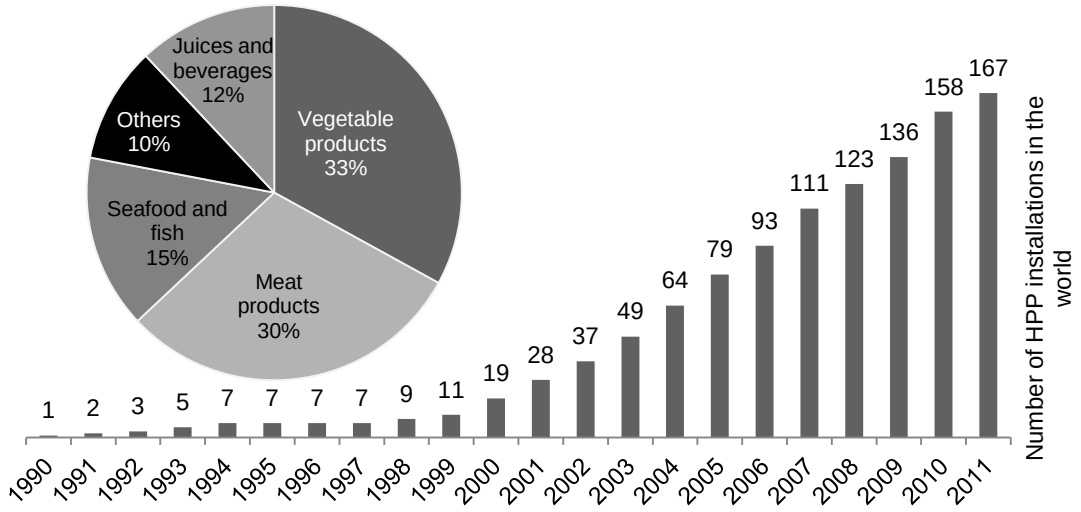

Figure 7: Application of high pressure processing on food (a) and food industry growth of high pressure processing technology (b) [85].

Nowadays, food industry has been given more attention to one of HPP advantages: establish new products with new functionalities and characteristics, enhancing the effect of bioactive compounds in human organisms and improving its taste, texture and aroma [86, 87]. Despite all the advantages, there are some dilemmas with regard to the inactivation of microorganisms by HPP [88]. Food enzymes and bacterial spores are highly resistant to pressure and require very high pressure for their inactivation. Furthermore, the residual enzyme activity and dissolved oxygen results in enzymatic and oxidative degradation of food components [89]. Another disadvantage of HPP is that pressure processed foods needs low temperatures during storage and distribution to retain their sensory qualities [90].

Other food processing techniques have also been studied, namely pulsed electric fields (PEF) and irradiation but some researchers found that when compared to HPP products, the consumer acceptance is lower, and was observed a negative response to irradiation products. Consumer's acceptance for PEF foods is similar to HPP products; however they verified that consumers noticed the naturalness, improved taste and high nutritional value of HPP [91, 92].

II.2.1. High pressure process – system and principles

A typical HPP unit consists basically of a high hydrostatic vessel and the pressure generating system [85]. The food product is placed in the pressure vessel, closed and then placed in the pressure chamber. Hydrostatic pressure is generated mainly by volume reduction [93] pumping the medium liquid, such as silicon oil, ethanol, glycol, water or even a mixture of fluids into the pressure chamber [9]. When the desire pressure is reached the pump is stopped and the pressure is maintained during the require hold time and the system

is then depressurised [94]. One of the most important parameters of HP systems is the working pressure value, not only because the equipment price that increase significantly with the increase of the maximum pressure needed, but also because, the equipment life time grows with the low pressure use, in opposite with the high pressure [12]. Usually the pressures range used to process food are between 50 and 1000 MPa and during the treatment compression increase the temperature of the foods through adiabatic heating [12].

There are two relevant principles to the use of HPP in food processing. The first one is the “Le Chatelier’s Principle” which is related to the response of the system to a disturbance on the equilibrium. This means that HPP enhance those phenomenon’s that are accompanied by a decrease in volume, such as changes in molecular configuration and chemical reactions [87]. The second principle, the Isostatic Rule, states that pressure is transmitted instantaneously and uniformly throughout the food sample, independently of the size, shape and constitution of the product [85]. These principles are the key drivers for using HPP as a non-thermal alternative to the commonly used heat pasteurisation. The main differences between thermal and non-thermal processing technologies are related with the effects induced by temperature on nutritional and quality parameters of the products [88, 94, 95]. Although fruit derived products are traditionally produced by thermal technologies, after Hite’ studies, the researchers proved that HPP could be more effective than any other thermal treatment. On grape and apple juices for example, the fermentation is stopped if processed at high pressure and room temperature (680 MPa for 10 min and 410-820 MPa for 30 min, respectively) [80]. Another study shows that pressures above 300 MPa cause irreversible protein denaturation at room temperature [96] and HPP affects cell membranes in vegetables. On the other hand, Ferrari *et al*. (2010) [97] investigated the effects of high pressures (400–600 MPa) at different temperatures and treatment times on total content of anthocyanins and polyphenols in pomegranate juice. This work showed that the anthocyanin content is influenced mainly by pressure and temperature. In fact, at room temperature an increase in the pressure level and processing times leads to lower concentrations of total anthocyanins in orange juice [97]. Moreover, if treated at higher temperatures, the application of 400 MPa causes a small decrease in anthocyanins content but if pressure levels are higher than 400 MPa, concentration of anthocyanins increases compared to the unprocessed juice values [97]. In this processing range both temperature and pressure decreases anthocyanin degradation rates by affecting enzymes which are involved in kinetics of the reaction [90, 98]. Although thermal processes affect the major properties of food, some studies show combinations of PEF, high-intensity light pulses, magnetic fields and HPP with thermal treatments as powerful preservation processes, due to the effectiveness of microbial thermal inactivation [95].

II.2.2. High pressure processing to improve food safety and stability

Every food preservation technique requires a significant effectiveness on microbial inactivation. The ability to eradicate pathogenic microorganisms enhancing the product's safety and to inactivate spoilage microorganism improving the shelf-life of the food are the primarily objectives of HP process [99]. Here the inactivation mechanisms are based on the changes in the morphology, cell membrane or biochemical reactions of microorganisms causing some lethal injuries to microbes [100]. Additionally, each species of microorganism has a particular temperature and pressure range in which it can grow best and in the case of HP process, generally vegetative cells such as yeast and moulds are inactivated at pressure around 400-600 MPa, while bacterial spores can survive pressures higher than 1000 MPa [94]. The main problem regarding the inactivation of microorganism is the ability of some cells to recover from a sublethal injury caused by HP process, observed for instance in prolonged storage of some products [87]. Furthermore, the use of high pressure (about 400-600MPa) at soft temperatures, allow some foods to be preserved with minimal effect on taste, texture, appearance and nutritional value [101], maintaining unaffected covalent bonded compounds, smaller molecules such as volatile compounds, pigments, vitamins and other compounds associated with the sensory and nutritional properties [14, 102, 103]. One of the most known cases of success of HPP method is the increase of avocado products shelf life and the reduction of oysters poisoning outbreaks. In both cases producers look for a process that efficiently reduces spoilage and harmful microorganism, and HPP shows the best results [104].

II.2.2.1. Bacteria and Spores inactivation

Nowadays, elimination of pathogens and extension of products shelf life are the main applications of HPP method [93] have been extensively studied over the past years. Inactivation mechanisms for bacterial spores or vegetative bacteria are different and result from a combination of factors [12, 87], and strongly depends of food composition and environmental conditions (such as pH, water activity, salt and sugar content) [20]. As said before, HP process and other chemical treatments provoke sublethal injuries, such as modifications in cytoplasmatic membrane, however the microorganisms might recover from those injuries and become a serious risk for safety and preservation of food during storage of processed products [105]. Generally, bacterial inactivation by HPP involves membrane disruption, damage on nuclear material and consequently the interruption of some cellular functions [85]. Moreover, it have been reported that HP process also provoke cell compression, separation of the cell wall from cytoplasmatic membrane, and damage to mitochondria, cytoskeleton and lysosomes which are all involved on reproduction and

survival of the cell [106]. In fact, some researchers reported slight reductions (Table 6) of *S. cerevisiae, Leuconostoc mesenteroide* and *L. innocua* on orange juice samples treated with different pressure, temperature and time conditions [89, 107-109].

Table 6: High pressure effect on some microorganisms of orange juice (adapted from [89, 107-109]).

Ref.	Microorganism	Conditions	Main conclusions
[107]	*L. mesenteroides*	100 to 350 MPa, 120 minutes	Reduction from 7 to 1.5 log CFU/mL after 15 min at 350 MPa
[107]	*S. cerevisiae*	100 to 350 MPa, 120 minutes	Reduction from 5 to 2 log CFU/mL after 20 min at 250 MPa
[108]	*C. botulinum*	600 MPa, 80°C, 2 seconds	Slight variations (5.5 to 0 log)
[89]	*L. plantarum*	200 to 350 MPa, 35°C	Higher pressure conditions provoke better reduction
[109]	*L. innocua* e *E. coli*	103 to 241 MPa	More efficient reduction using higher pressure conditions

Regarding bacterial spores, they are not *per se* an hazard to food products, however its eventual germination and proliferation may result in toxification or spoilage of food during the post-processing storage [93]. To deal with spores researchers have been using three strategies. The first one is based on the full inactivation by using severe temperatures or combination of high pressure and temperature [85, 93, 102]. In fact, using pressures of 500 MPa during orange juice processing shows no significant differences on microbial quality when compared to thermal treated samples [110], but on the other hand a large variation on the reduction of *Clostridium botulinum* spores was observed after treatment with 600 MPa during 1 second at 80°C (from 5.5 log units to no reduction) [108]. Germinate spores by temperature or pressure and then inactivate by a subsequent treatment of pressure/temperature is another strategy used to minimise the risk of spore contamination. Finally, the last strategy involves the inducing of injuries on spores, preventing its germination or outgrowth in the food [87, 93, 111].

To prevent food spoilage, a wide range of researchers have been doing great efforts, studying the effect of both thermal and non-thermal processing methods in different types of microorganisms [89, 112, 113]. Those studies show that generally microorganisms are efficiently inactivated by pressure treatments, but in some cases a combination of thermal and high pressure allow a better spoilage reduction and so, a better shelf life of orange juice [85].

II.2.2.2. Enzymes activity

Enzymes are a special class of proteins formed by a three-dimensional conformation and an active site stabilized by non-covalent interactions [94]. Enzymes such as PPO, pectin methylesterase (PME) and Lipoxygenases (LOX) are the main target on fruit and vegetable studies because the enzymatic activity of those enzymes appear to be one of the most important parameters regarding food organoleptic characteristics such as colour and viscosity [114]. While PPO catalyzes the enzymatic browning on fruits and vegetables, PME and LOX are responsible for texture and flavour changes respectively, acting on the desesterification of pectin and lipid oxidation of some fruits and meats [115]. Pressure effect on enzymes activity seems to be variable. Although in some cases HP process inactivates food enzymes, normally lower pressures, between 100 and 200 MPa, may activate them [85]. Generally pressure induces structural rearrangements by affecting the weakest bonds that maintain tertiary and quaternary structures of proteins [85, 114, 116]. Actually, some studies regarding enzymes activity on orange juice showed cloud losses and gelation of orange juice concentrates [115, 117, 118]. Pressure inactivation of enzymes is a complex phenomenon that depends on both enzyme structure and environmental conditions [67, 68, 119]. Since there were not been yet observed changes in covalent bonding after pressure processing, primary structure of enzymes is only minimally affected by this technology and thus constitute one of the most important characteristics in HPP method [115]. On the other hand, changes in secondary structure only take place at pressures above 700 MPa and tertiary structures might be lost during pressure denaturation beyond 200 MPa [14, 29, 89, 110]. In addition, HP process may provoke changes in catalytic rate by affecting the enzyme-substrate interactions or the reaction mechanism, and in some cases, the cell membrane or the membrane of intracellular organelles is altered and those reactions are facilitated [94].

II.2.3. High pressure and orange juice quality

II.2.3.1. Carbohydrates

Carbohydrate composition may be affected by a wide range of factors, such as variety, processing conditions and seasonal variations. During heat treatment and storage of orange juice, carbohydrates can suffer changes such as inversion of sucrose and formation of oligosaccharides. Other food processing methods also provoke important changes in carbohydrates thus being mainly involved in two kinds of reaction: Maillard reaction and caramelisation [33]. Maillard reaction promotes some important nutritional changes, reducing proteins digestibility or amino acids availability [29] and results from the reaction between carbonyl compounds and amines [120]. This reaction can be divided into three

major phases that lead to the formation of some volatile and non-volatile compounds (Figure 8). The early stage begins with a simple reaction between a reducing sugar and a primary amine, forming a Schiff's base and Amadori products that are later clived at the intermediate stage, forming degradation products, reactive intermediates and volatile compounds [29]. At the end, Maillard reaction produces brown nitrogen containing polymers and copolymers named melanoidins (low molecular weight substances capable of cross-link proteins) [29].

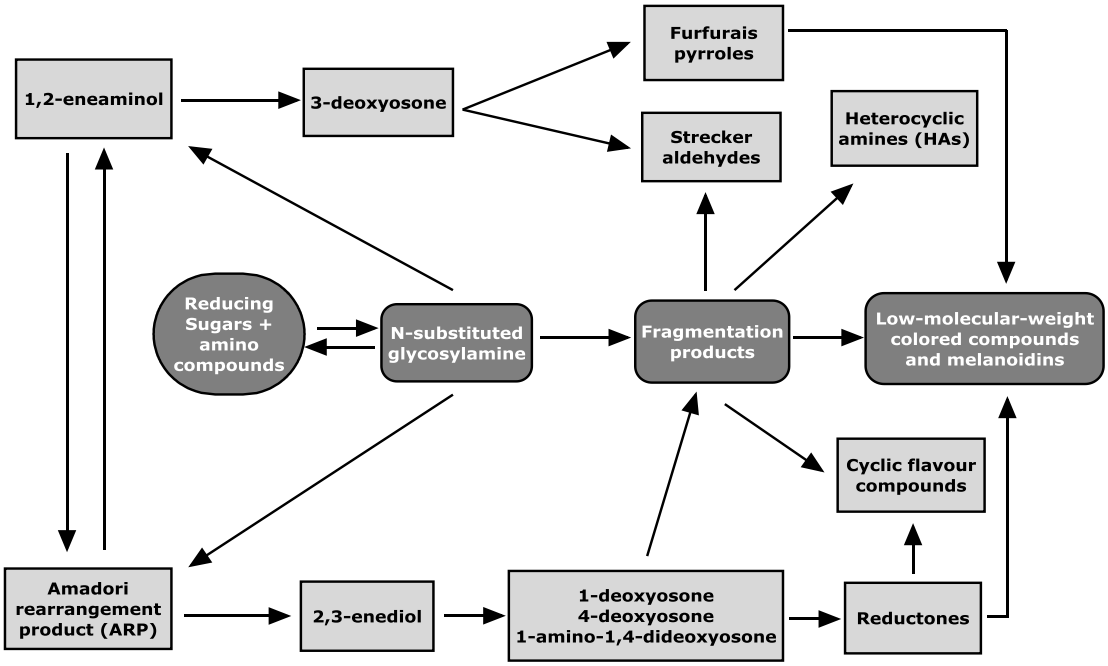

Figure 8: Chemical pathways of Maillard reaction (adapted from [95])

In processes such as pasteurisation, storage, sterilisation and drying, Maillard reactions may produce different effects in food, leading to important organoleptic and nutritional changes [34]. In addition to aroma, flavour and colour changes, in some products this effect may lead to beneficial changes in the nutritional field [121], resulting in products with more antimutagenic, antimicrobial and antioxidant capacity [119, 122].

Moreover, caramelisation of carbohydrates is the result of high temperature heating of foods with high sugar content [123]. In general, dehydration of carbohydrates during caramelisation results in the formation of double bond compounds which absorb different light wavelengths causing food browning [121]. Due to the mutagenic compounds and physical changes in colour and flavour, which may result from those reactions, caramelisation is not always a desirable reaction [124]. Furthermore, in the case of some fruit products, such as juices and smoothies, researches are seeking ways to prevent them, by using for example lower temperatures to pasteurise those products, or even the addition of some chemical compounds to avoid or minimize the browning reactions, such as AA [67].

Although the well known importance of carbohydrates in fruit juices, only few studies shows the degradation mechanism during thermal treatment of those products and until the date no studies were found regarding the effect of HP treatment on orange juice carbohydrates.

II.2.3.2. Vitamin C

Orange juice heat processing can have a negative effect on vitamin C retention and so, HPP method has been introduced as a non-thermal alternative to minimally affect vitamin C and inactivate spoilage microorganisms [125]. Polydera *et al*. (2005) [126] report a decrease of AA loss rates on HPP samples when compare with pasteurised orange juice, however the reduction in total vitamin C content is not significant. The same authors proved that PEF provoke more significant changes in total vitamin C content than HPP during refrigerated storage. A wide range of researchers found significant differences in AA retention after thermal and pressure treatments [126, 127]. Those studies showed higher retentions of AA on pressurised samples of orange juice after treatments of 200, 400, 500 and 600 MPa [126, 127]. Polydera *et al.* (2005) pressurised the orange juice at 600 MPa during 4 minutes and 40 °C and found an AA retention of about 84% while thermally processed samples (80°C for 6 seconds) showed only 72% of retention. Additionally, Polydera *et al*. (2005) [126] observed that during storage time the AA content decreases slowly on HPP processed samples, leading to a significant extension of orange juice shelf life comparatively with thermally processed juice [126].

Application of high temperature in combination with pressure seems determinate strongly the AA and total vitamin C contents in orange juice. Some researcher observed that using higher temperatures provoke higher decreases in total vitamin C content, explained by the thermal degradation of AA [25, 26]. When compared with unprocessed samples, some researchers found slightly decreases of vitamin C content in HPP orange juice [26, 109]. Moreover, it was observed that both thermal and pressure processes provoke a decrease on AA levels of orange juice [26] showing that even HPP method causes AA degradation.

II.2.3.3. Carotenoids

Carotenoids, as a wide range of other compounds, are modified during storage and processing of food products. In general, thermal processing methods may decrease carotenoids concentration; however, the disruption of food matrices facilitates the liberation and solubilisation of carotenoids [79, 128]. Some researchers have demonstrated that people with higher carotenoids intake levels, especially lutein and zeaxanthin, have significantly lower risk of macular degeneration [86, 129]. So, studies regarding the effects of processing

techniques in those compounds are very important. Despite generally HPP don't affect significantly carotenoids content, some researchers showed higher extraction rates of carotenoids in orange juice subjected to HPP treatment comparatively with control samples[100, 130]. More recently, Plaza *et al.* (2011) [27] studied the effect of HPP, TP and PEF on orange juice. The effects of pressure processing at 400 MPa, 40°C for 1 minute were compared to those of thermal pasteurisation (70°C for 30 seconds) and PEF (35kV cm^{-1}) during 40 days of refrigerated storage (4°C). They observed a significant increase (45.19%) in total carotenoids extractability of HP juice, just after treatment. Moreover, HP juice showed the highest carotenoids content among all juices [27]. Once the carotenoids are small pigments localized in plastids (chromoplast and chloroplast), the HPP treatment probably improves its extractability due to plastids disintegration [100], and so, it extractability increase. Although processing and storage provoke instability of polyene chain of carotenoids, AA protection activity may prevent carotenoids oxidation [131]. Another study showed that when compared with pasteurised samples (90°C during 30 seconds), the carotenoid content was less affected using non-thermal methods – HPP and PEF [132]. Independently the storage temperature, in pressurised orange juice it is observed better retention of carotenoid content during storage than in thermally processed juice [132, 133]. Results obtained for carotenoids, in addition with those described in previous sections, demonstrate that HPP in processing industries may produce high nutritional value orange juices with similar characteristics to fresh ones.

II.2.3.4. Flavonoids

Generally, canning, bottling and thermal processing affect both anthocyanins and flavanones content in fruits [58]. In addition, temperature, pH, oxygen and the presence of enzymes such as oxidase, anthocyanase and peroxidase affect the stability of those two flavonoids [68]. There are a few studies of the effect of HPP on anthocyanins content in orange juices and its bioavailability. Nevertheless, many of those studies report that there are no significant changes on anthocyanins content in fruit juices after processing [134]. Additionally, some researchers reported that samples treated with high pressures, have higher anthocyanin retention than unprocessed or TP samples [26, 98, 135] which could be due to thermal degradation [90].

Until the date, there are no studies regarding the effect of HPP on bioavailability of flavonoids, but some researchers found interesting effects on the extraction of those compounds. Recently, Sanchéz-Moreno *et al*. (2005) [25] compared unprocessed and HPP (400 MPa/40 °C/1 min) orange juice. They have shown an increase of 26.2% and 39.9% on naringenin and hesperetin (two of the main flavonoids presented in orange juice) content

respectively on HPP treated juices when compared to unprocessed samples [25]. Also Plaza *et al*. (2011) [27] reported similar results with regard those two flavonoids. These results appeared to be related to the residual activity of PPO and peroxidise on orange juices [136]. In addition, as a result of experimental studies, authors assume that in the case of denaturation of protein-carotenoid complex by HPP, cell walls of juice vesicles undergo structural changes releasing phenols from proteins and increasing flavanones extraction [25, 27, 56, 137].

II.2.3.5. Antioxidant activity

One of the most known food deteriorative mechanisms is the oxidation, which results in a decrease of the nutritional value and sensory quality of products. Application of lower temperature, inactivation of catalytic enzymes, reduction of oxygen pressure and use of food additives are the most applied methods to inhibit the oxidation [138], and consequently the development of adverse changes in taste, colour, texture and flavour of foods [139]. There are many chemical compounds that efficiently reduce the oxidation and those compounds are called antioxidants [140]. The great number of antioxidants in orange juice is associated with its high protective effect regarding some degenerative diseases [141]. Antioxidants can be used in food as preservative additives and antimicrobial substances contributing to significantly increase food preservation [139].

Although orange juice is one of the most studied juices, the effects of new technologies, including HPP, on its antioxidant capacity are not well known yet. Some researchers showed that although the HPP increase the content of several compounds with recognized antioxidant activity in orange juice , application of this non-thermal technology did not affect the antioxidant activity of juice, comparatively with unprocessed samples [25, 127] in short storage times [137, 141]. Plaza *et al*. (2006) [127] studied the antioxidant activity of untreated and processed juice. In this study, the group compared freshly squeezed orange juice with HP, PEF and thermal treated samples, during 40 days of storage, After 10 days of storage, only HPP juice showed no significant differences, regarding EC_{50} parameter when compared with untreated samples. However, at the end of refrigerated (4°C) storage HPP, PEF and TP juices showed significant increases of EC_{50} when compared to unprocessed juice. Those results are, in fact, very important to understand that even thermal treatments may negatively affect antioxidant activity of orange juice in long storage times.

Additionally, the interest in natural antioxidants increased in the last years due its importance for the prevention of several diseases mediated by free radical reaction *in vivo*. There are many degenerative processes linked to the excess of free-radicals in human organism. One of the most important mechanisms is associated with the increased

superoxide formation by intracellular triglycerides. Superoxide stimulates the production of inflammatory cytokines and consequently the formation of oxidative radicals which might be involved in degenerative processes such as cancer and aging [30]. So, health problems such as cancer, atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, immune system decline, brain dysfunction, cataracts, and malaria may be delayed by natural antioxidants which prevent those mechanism mainly by scavenging of free radicals [142]. So, the importance of knowing the effects of different processing techniques on antioxidants and antioxidant activity, becomes evident the need to extend all research done so far.

II.2.3.6. Colour

Consumer's acceptance of HPP products is greatly affected by the colour differences observed between fresh product and processed one [143]. Due to the protein denaturation during HP treatment, some colour changes might be observed. For instance, on meat products, this changes may be related to changes in myoglobin, namely globin denaturation, oxidation of ferrous atom and heme displacement or release [144]. Regarding fish, fruit and vegetable products, HP treatment is unlikely to cause significant changes on their colour, however some fruit purees gradually suffer a colour decrease due to browning reactions resulted from the residual activity of PPO [116]. Colour studies based on CIE *L* a* b** system (where *L** is lightness, *a** redness and *b** yellowness) shows different results. While some authors reported that colour slightly change with HPP treatment [126, 145, 146], other showed significant changes on colour parameters [26, 109, 125, 126]. Additionally, Hartyáni *et al*. (2011) [109] showed big differences (total colour difference of 9.3) for juices processed with a combination of high pressure and low temperature [109]. Thus, when compared to thermal treatments, HPP demonstrates better effects on orange juice improving it colour even when treated at different pressure levels. Once again, HPP enhance orange juice characteristic, demonstrating the great importance of this method in food industry.

II.2.3.7. Flavour and aroma

Organic acids are the principal contributors to taste and flavour of many fruits and vegetables. So, the flavour changes that occur between production and consumption represent a enormous interest to the food industry [147]. Few studies were made in order to establish the influence of HPP on fruit juice flavours. Those studies generally show no significant differences between unprocessed, thermal and HPP juices [103]. Baxter *et al*. (2005) [148] showed different behaviours during storage at 4 °C and 10 °C after 12 weeks of thermal (85 °C for 25 seconds) and pressurised (600 MPa for 60 seconds at 18 to 20 °C)

treated samples concerning flavour attributes, with increment of the sour taste and bitterness among storage days, while sweet and processed flavours decreased on HPP juices [148].

Regarding aroma, there are several volatile components responsible for the wide range of aromas of orange juice. Monoterpenes, esters, organic acids, aldehydes, ketones and alkanes are the most well known [149]. Hartayáni *et al*. (2011) [109] observed that pressurisation did not affect significantly the main aroma compounds of orange juice comparatively with control samples.

II.2.3.8. Organic acids

Regarding phenolic compounds there only a few studies where its content on fruits is determinated. Patras *et al*. (2009) [90] studied the effect of TP (70°C, 2 min) and HPP (400, 500 and 600 MPa, 20°C) on phenolics composition of strawberry and blackberry purées. Here, the research group found that phenols are relatively resistant to processing effects, by comparing processed samples with raw purées, and showed that phenolic levels of HP treated purées at 600 MPa significantly increased when compared to unprocessed samples. Concerning TP samples, they reported no significant changes when compared to unprocessed samples. The HP increase on phenolics may be related to the increase extractability of some of the organic acids components after HPP [90].

II.2.3.9. pH and total soluble solids

Organic acids such as citric, malic and succinic acid present in orange are the main responsible for the acidity of orange juices [150]. Orange juice pH, of about 3.4, inhibit the growth of pathogenic microorganisms, however yeast moulds and lactic acid bacteria remain active and causing juice spoilage [125]. There is also some degradation with carotenoids and vitamin C reactions depend on pH. Nevertheless, Timmermans *et al*. (2011) [145] and Hartyáni *et al*. (2011) [109] shows no significant differences on pH of pressurised samples, when compared with untreated ones or even when processed with other technologies such as PEF and pasteurisation [145].

Regarding total soluble solids (°Brix) parameter, the results are similar to those observed for pH and there are only a few studies comparing the effect of processing technologies on this parameter. Total soluble solids is a fundamental measure of quality of citrus juices that depends on sugar content and its values usually ranged between 12 and 14 [150]. Also for this parameter no significant differences between HPP and untreated samples were found [145]. Although pH and °Brix do not show differences, verification of the behaviour of both parameters with different processing conditions is very important, regarding the effect of pH and soluble solids on a wide range of biochemical parameters.

III. Objectives

Because is the most consumed juice in the world, the production of a high quality and natural orange juice is a very important issue and nowadays more researchers try to understand which mechanisms are affected by the different processing technologies. The application of HPP for orange juice industrial production has been studied over the past years however some studies are incomplete and does not provide the information needed to understand the differences between thermal and non-thermal processing methods.

Although the great number of studies on the effects of HPP and TP methods on orange juice, only a few compare those effects over the storage time and none of them uses lower temperatures on HPP. Thus, the main objective of this work was to evaluate the main bioactive compounds (phenolic compounds, anthocyanins, flavonoids and carotenoids) and other important parameters (soluble solids, colour and antioxidant activity) on HPP and TP orange juice during a storage period of 36 days.

In order to understand some of the mechanism affected by HPP and TP, the phenolic fraction was quantified in addition to the kinetic study of the bioactive compounds. Those to topics were defined as secondary objectives to this work.

IV. Material and Methods

IV.1. Orange juice, treatments and sample collection

Orange juice was provided by Frubaça – Cooperativa, a fruit and food products producer of Alcobaça, Portugal. Oranges were squeezed on an industrial juice wringer and then carried trough refrigerated tubes to a tank where were kept at low temperatures (4°C) until bottling and processing stages. Orange juice samples have a life-time of 21 days which was determinate by Frubaça – Cooperativa. All treatments were performed on samples collected from the same orange juice lot. Then, samples were taken to the laboratory on a refrigerated reservoir.

High-pressure treatment was conducted in Frubaça-Cooperativa using a hydrostatic press from Avure, Model QFP 100L-600, with a pressure vessel of 100 L surrounded by a horizontal frame and connected to the water system. After bottling 250 mL, orange juice was treated at 550 MPa for 70 seconds at 18°C. Those pressure conditions were set up by Frubaça-Cooperativa after optimisation studies.

The thermal treatment was performed in Chemistry Department of Aveiro University, using raw orange juice. Polyamide/polyethylene (PA/PE-90) plastic bags with 2 cm of width containing 5 mL of orange juice each were placed into the water bath at 70°C. The time needed to reach 70°C was previously estimated with water, taking about 70 seconds. Once that temperature was reached, the samples were held in the water bath for 30 seconds After pasteurization, the thermal processed (TP) plastic bags where quickly cooled in ice.

Samples of both processed methods were storage at 4°C and collected in the processing day (day 0) and after 4, 15, 22, 29 and 36 days of storage, frozen at -80°C and stored at -20°C until further analysis. All analysis was performed at most after one month of frozen storage.

IV.2. Physicochemical analysis

IV.2.1. Soluble solids

Soluble solids (°Brix) were determinate at room temperature (~25°C) using a handheld refractometer (ATAGO) [137]. There was not enough orange juice to perform duplicates in this study.

IV.2.2. Colour

Colour of the samples was evaluated by the spectrophotometer method described by Cao *et al*. (2012) [151] using the transmittance mode in a PerkinElmer Lambda 35 UV Vis spectrophotometer (PerkinElmer Instruments) at 25°C. The results were expressed according to the CIE L*, a*, b* uniform colour space (CIE*Lab*) where L* indicates lightness, a* indicates hue on a green (-) to red (+) axis and b* indicates hue on a blue (-) to yellow (+)

axis. It was measured 5 mL of orange juice to a centrifuge tube. The samples were centrifuge at 2500 rpm, during 10 minutes in a Compact Tabletop Centrifuge 2010 (Kobota Corporation). The supernatant was collected and the sample was inserted in the spectrophotometer. The results were collected and was calculated the colour difference (ΔE) by the equation (1). The L^*_0, a^*_0 and b^*_0 represents the standard values for the non-processed juice. There was not enough orange juice to perform duplicates in this study.

$$\Delta E = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \quad (1)$$

IV.2.3. Bioactive compounds

IV.2.3.1. Total content of phenolic compounds

Total content of phenolics was arrayed using the Folin–Ciocalteu reagent, following the method of Singleton (1985) [152] modified by Falleh *et al.* (2008) [153]. Six standards were prepared with concentrations between 0 and 0.24 µg/mL, by dilution of a gallic acid solution (210 µg/L) (CAS 5995-86-8, Panreac). Aliquots of each sample and diluted orange juice were added to 2.25 mL of distilled water and 1.5 mL of the 10% Folin–Ciocalteu solution (F9292, Merck). The mixture was shaken and allowed to stand for 5 minutes in the dark before adding 1.5 mL of a Na_2CO_3 solution (60 g/L) (CAS 497-19-8, Fluka). The mixtures were then shaken and incubated in the dark for 30 minutes. After incubation, the absorbance at 415 nm was read versus the prepared blank, where the orange juice was substituted by distilled water. Total phenolic content of juice was expressed as milligrams of gallic acid equivalents per millilitre of juice through the calibration curve. All tests were performed in duplicated.

IV.2.3.2. Total content of anthocyanins

The methodology was performed as described by Rapidsara *et al.* (2000) [154]. One mL of orange juice was diluted to 10 mL using a 10.15:39.85 (v/v) mixture of 95% ethanol (CAS 64-17-5, Sigma) and 37% HCl (CAS 7647-01-6, Riedel-de Haen). Absorbance was measured at 535 nm by a 6405 UV/Vis spectrophotometer (Jenway) and cyaniding-3-glucoside (cy-3-glu) as standard. Concentration of anthocyanins in mg equivalents of cy-3-glu per mL (C) was calculated by the Beer-Lambert law, using the molar absorptivity coefficient of 60.45 mL/mg.cm.

IV.2.3.3. Total content of flavonoids

To measure the total content of flavonoids it was used the modified method described by Chang *et al* (2002) [155]. This colorimetric method is based on the formation of a complex with aluminium chloride and some flavonoids. For the calibration curve 1.05 mg of rutin were dissolved in ethanol (CAS 64-17-5, Sigma) and then diluted to 10, 20, 40, 60, 80 and 100 µg/mL standards in 0.24 mL of a 10% aluminium chloride solution (M&B), 0.4 mL of a 1M

potassium acetate solution (CAS 6131-90-4, Sigma) and distilled water until final volume of 2 mL. An aliquot (0.2 mL) of the diluted standard solutions and the diluted orange juice samples were separately mixed with 0.6 mL of ethanol, 0.04 mL of 10% aluminium chloride solution, 0.04 mL of 1M potassium acetate solution and of 1.12mL distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a 6405 UV/Vis spectrophotometer (Jenway). The amount of 10% aluminium chloride solution and the sample amount were substituted by the same amount of distilled water in blank.

IV.2.3.4. Total content of carotenoids

The concentration of total carotenoids was determined following adapted methods from George *et al.* (2011) and Rajchl *et al.* (2010) [156, 157]. First it was mixed orange juice with distilled acetone (1:8 v/v) in a SpeedVac tube, previously wrapped in aluminium paper. The samples were then left at 4°C for 30 minutes and stirred every 10 minutes. After incubation, the tubes were introduced into the SpeedVac (Univapo 100H) and waited between 30 and 40 minutes, until the acetone was almost completely evaporated. At each SpeedVac tube was added 3.50 mL of petroleum ether (CAS 8032-32-4, Lab-SCAN Analytical Sciences). Stirred and incubated during 1 hour at 4°C. Then, it was added 1 mL of distilled water and centrifuge the samples at 2500 rpm for 5 minutes in a Compact Tabletop Centrifuge 2010 (Kobota 2010). Finally, the absorbance was measured in an UV-VIS spectrophotometer at 450nm. This protocol used petroleum ether as blank and the concentration of total carotenoids in β-carotene equivalents was made using the molar absorptivity coefficient of 259.2 mL/mg.cm.

IV.2.3.5. Individual phenolic compounds identification and quantification

The several phenolic compounds presents in orange juice where identified and further quantified by the high-performance liquid chromatography (HPLC-MS) method described by Santos *et al.* (2012) [158].Samples preparation for phenolics characterization was performed according to Ross *et al.* (2009) [159] with slight modifications. An aliquot (0.4 mL) of orange juice was diluted with 4.0 mL of 80% HPLC grade methanol solution (CAS 67-56-1, Sigma) in water. The extraction samples were left during 24 hours in the dark at 4°C until injection. The HPLC system consisted of a variable loop Accela autosampler (200 vial capacity set at 15°C), an Accela 600 LC pump and an Accela 80 Hz PDA detector (Thermo Fisher Scientific, San Jose, CA, USA). The separation of orange juice compounds was carried out at room temperature with a gradient elution program at a flow rate of 0.2 mL/min. The mobile phase consisted of water/acetonitrile (90:10, v/v) (A) and acetonitrile (B) both with 0.1% of formic acid (CHOOH), applying the following linear gradient: 0-3 minutes, 0% B; 3-10 minutes, 0-10% B; 10-30 minutes, 10-20% B; 30-35 minutes, 20-25% B; 35-50 minutes, 25-50% B; 50-

55 minutes, 50-100% B; followed by a ten minutes re-equilibration before the next run. Before the injection, orange juice extraction samples were filtered through a 0.2 µm PTFE syringe filter (VWR International). Double online detection was carried out in the diode array detector, at 280, 325 and 365 nm, and UV spectra in a range of 210–600 nm were also recorded. To the electrospray ionization mass spectrometry-tandem mass spectrometry (ESI-MSn) analysis the HPLC was coupled to a LCQ Fleet ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an electrospray ionization source and operating in negative mode. The spray voltage was 5kV and the capillary temperature, 300°C. The capillary and tune lens voltage were set at -28 V and -225V, respectively. Collision-induced dissociation-mass spectrometry (CID-MS^n) experiments were performed on mass-selected precursor ions in the range of *m/z* 100–1000. The isolation width of precursor ions was 1.0 mass units. The scan time was equal to 100 ms and the collision energy was optimized between 15 and 45 (arbitrary units), using helium as collision gas. The data acquisition was carried out by using Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA). Using HPLC chromatograms and MS/MS spectra, molecular ions ($[M-H]^-$) and consequent fragmentation patterns where listed in order to found on literature the respective compound name and structure. To phenolic compounds quantification, calibration curves were obtained by HPLC injection of ellagic acid (476-66-4, Fluka), quercetin dihydrate (CAS 6151-25-3, Sigma) and naringin in 80% methanol solution. The concentrations of identified phenolics were calculated in triplicate using the calibration curve equation.

IV.3. Antioxidant Activity

The antioxidant activity was determined according to the modified method of Kelebek, *et al.* (2008) [160] using 2,2-diphenyl-1-picril-hidrazil ($DPPH^{\bullet}$) as a free radical. First were prepared five different dilution samples of the orange juice (5%, 10%, 15%, 20% and 30%) in distilled water (v/v). A set of standard DPPH (CAS 1898-66-4, Sigma) solutions in methanol, with concentration ranged between 0 and 95 µM were also prepared. These standard solutions were used to make the calibration curve. An aliquot of 75 µL of diluted orange juice solution was added to 2.93 mL of a 60 µM DPPH solution in methanol and left to react during 45 minutes at a dark place and room temperature. Finally the absorbance of each sample was measured at 515 nm by a 6405 UV/Vis spectrophotometer (Jenway), using methanol as blank solution. The DPPH concentration in mg/mL (C_{DPPH}) in the reaction medium was calculated from the calibration curve with the equation (2). All tests were performed in duplicated.

$$Abs(515nm) = 25{,}595 \times (C_{DPPH}) - 0{,}0945\ ;\ (R^2 = 0{,}9955) \qquad (2)$$

Effective concentration (EC_{50}) was then calculated using the remaining DPPH percentage (% $DPPH_{remain}$) as shown in equation (3). Antioxidant values are represented as antiradicalar power (ARP) which is the inverse of EC_{50} [161] and represents the antioxidant efficiency.

$$\% DPPH_{remain} = \frac{CDPPH_{t=0}}{C_{DPPH}} \times 100 \quad (3)$$

IV.4. Statistical analysis

The effects of treatment method and storage time were tested in a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tuckey's HSD) to find which samples were significantly different from one another. Differences between treatments were tested at a 0.05 level of significance. All data are expressed as the mean ± standard deviation. For the colour and soluble solids it was not possible to carry out statistical analysis due to the lack of orange juice samples to perform duplicates or triplicates.

IV.5. Kinetic data analysis

Total content of phenolics, flavonoids, carotenoids, anthocyanins and individual phenolics were subjected to reaction kinetic analysis. These results could be described by a zero-order model (4). According to Eq. (4) the parameter's content loss rate (*dA/dt*) is proportional to the rate constant (*k*).

$$\frac{dA}{dt} = -k \quad (4)$$

The reaction rate constant was determinate from a zero-order kinetic (5), were A_t represents the response value after HPP and TP treatments and A_0 the initial value.

$$A_t = -kt + A_0 \quad (5)$$

V. Results and Discussion

V.1. Effect of HPP and TP treatments on physicochemical properties of orange juice

V.1.1. Total soluble solids content

Soluble solids determination was used to indicate the percentage of soluble sugars, and so, it is an important quality value for orange juice. Some researchers reported values ranging 10.60 and 11.59 % for total soluble solids on orange juice [24, 109, 145], however the studies regarding the effect of HPP and TP on this parameter and during storage are limited. As observed in Table 7, at the processing day the total soluble solid content was similar among raw juice and treated samples, ranging from 12.9 to 13.0; values that were maintained during storage time for both processing methods. The variations between the values found on literature and those obtained in our study are normal since this parameter depend on the orange specie, time of maturation and environmental issues such as the temperature conditions during the maturation process [145, 162].

Table 7: Total soluble solids content (°Brix) of raw, thermal (TP) and pressure (HPP) processed orange juice during 36 days of storage.

Time of storage (days)	Raw	TP	HPP
0	13.0	12.9	13.0
4		12.9	12.9
15		12.9	12.9
22		12.9	12.9
29		13.0	13.0
36		13.0	13.0

Being directly linked to sugar content in orange juice, soluble solids content is very important to quality and flavour of orange juice. So, this stable behaviour observed both in HP and thermal processed orange juice represents a great advantage regarding HPP technology as there are no changes between thermal and pressurised samples.

V.1.2. Colour

The CIE*Lab* scale is based on the perception of colours as opposite pairs, in this case, as light-dark, red-green and yellow-blue [163]. So, the colour scale is composed of three parameters, *L**, *a** and *b** which defines the product colour according to represented in Figure 9.

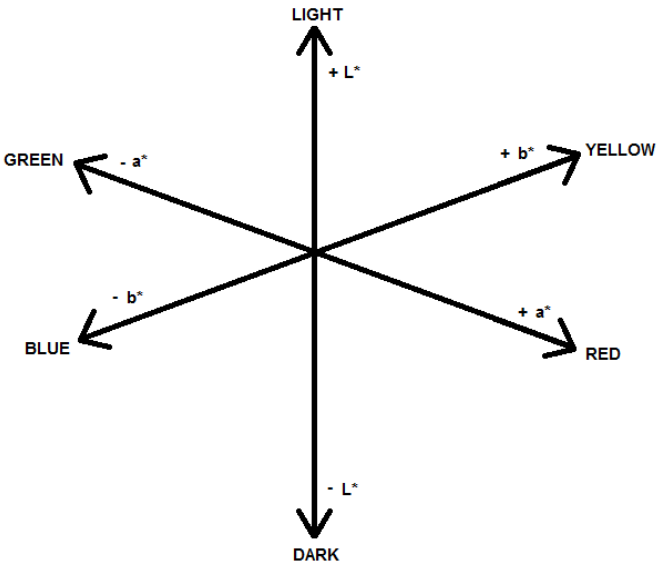

Figure 9: CIELab scale axis (adapted from [163])

As observed in a wide range of orange juice colour reviews [26, 109, 145, 163], in this study there are some changes between the different parameters of CIE*Lab* system after TP and HPP methods (Table 8) in the processing day, when compared to unprocessed juice. Although *L**, *a** and *b** values in raw juice are different from those found on literature [26, 145, 164] it is observed a slight decrease of *L** and *b** after HPP of orange juice which, as reported by Melendez-Martinez *et al.* (2011), might be correlated with the AA loss, formation of a jelly-like translucent structure or even with the vacuole disruption during HP treatment [165]. Regarding the observed decrease on TP it may be possibly related to non-enzymatic browning and caramelisation due to thermal treatment.

Table 8: *L**, *a** and *b** on the processing day of orange juice on raw, thermal (TP) and pressure (HPP) processed samples.

	*L**	*a**	*b**
Raw	78.02	0.43	38.20
TP	81.93	0.45	33.43
HPP	71.89	0.42	31.78

The overall trend during storage time (Figure 10) showed a slight increase on lightness and a decrease in both *a** and *b** parameters on TP and HPP samples. When compared with TP method, HPP caused higher reductions in both *a** and *b** parameters, and after 15 days of storage. Negative values of *a** and lower values of *b** may result in a brownish hue of the HPP orange juice. During storage PPO activity increases both in thermal and pressurised samples, however as demonstrated in a wide range of enzymatic studies [166-168] the PPO

activity after TP treatments is lower than in HPP samples, leading to higher rates of enzymatic browning and consequently higher variations in *a** and *b** parameters of HPP orange juice.

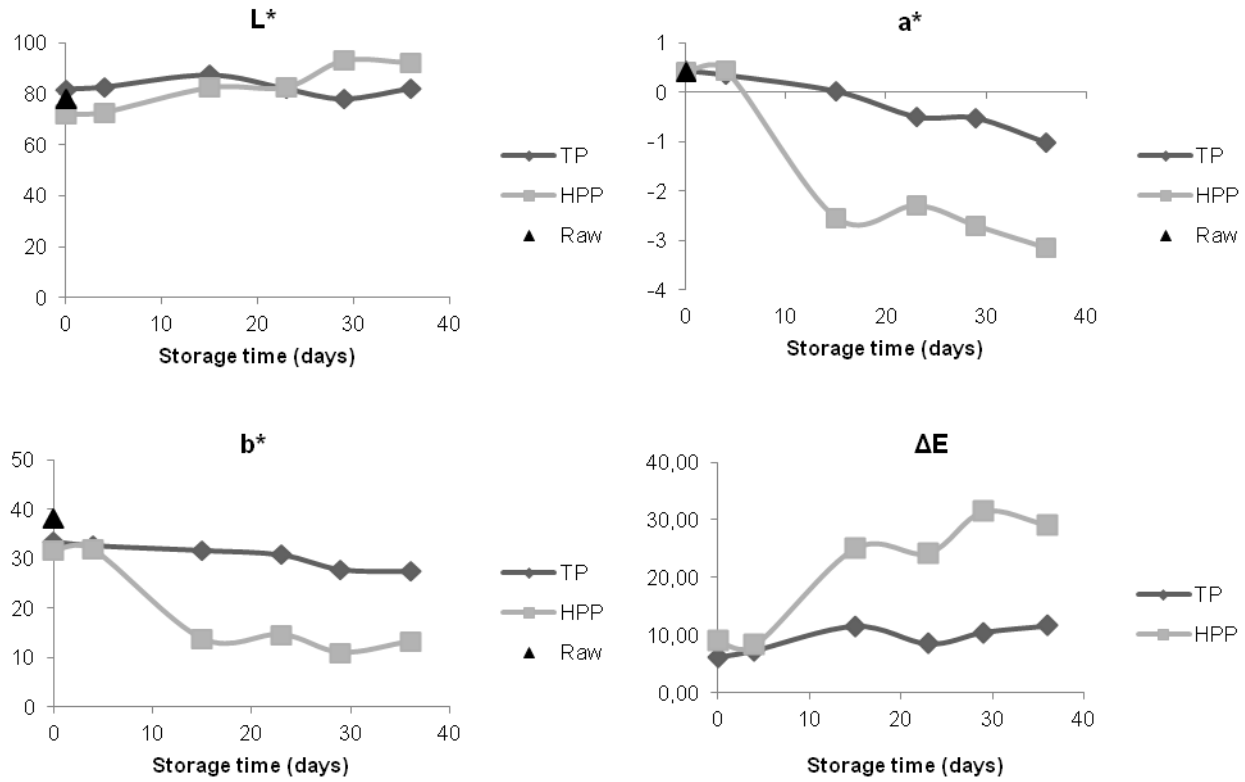

Figure 10: *L**, *a**, *b** and colour variation (ΔE) after thermal (TP) and pressure (HPP) processed samples during 36 days of storage at 4°C.

Carotenoids and flavonoids (mainly responsible by yellowness and redness hue in orange juice) degradation is also related to food colour changes. For instance, it was observed by some researchers, an increase in carotenoids extraction yields [169, 170] after food processing. On the other hand, the possible degradation mechanisms of anthocyanins cause some changes mainly in *a** and *b** parameters, as observed in this study [145].

To easily compare colour changes both in HPP and TP samples, the total variation of the colour perception (ΔE) was calculated, using the raw parameters as standard. Right after treatment (at day 0) HPP and TP orange juice showed visible (6.15 and 8.88 respectively) colour variations. In fact, the ΔE found for HPP samples are in agreement with that reported by Hartyáni *et al*. (2011) which treated orange juice at 600 MPa during 10 minutes. After 36 days of storage, it was observed a ΔE increase from 9 to 29 units, which might be due to the ineffectiveness of HPP in some enzymes activity inactivation, as said before. It is generally accepted that during storage this increase in colour degradation is more associated with TP due to the formation of degradation products. However, the colour losses observed for HPP

treatment could be due to the releasing bound cell constituents such as PPO from the cell vacuole [163]. Although TP also cause cellular disruption, the temperature applied in this study may inactivate enzymes potentially released during processing in contrast to what happens with HPP [171], and so, ΔE only varies between 6 and 12 units. Additionally, colour changes might be associated to oxidation or endogenous microorganisms' growth [172].

V.1.3. Effect of HPP and TP treatments on bioactive compounds of orange juice

V.1.3.1. Total content of phenolic compounds

Phenolic acids are an important class of organic acids and, as said before, have important roles in human health and development. The total content of phenolic compounds was investigated in order to understand their behaviour after orange juice processing. In Figure 11 it is possible to compare the total phenolic content in equivalents of gallic acid on raw (at day 0), HPP and TP (during 36 days of storage) of orange juice samples. As reported by Patras *et al.* (2009) [90] for blackberry purée, our results showed no significant changes ($P<0.05$) between raw, thermal treated and HPP orange juice at the day of the treatments (day 0). However processing technologies may affect phenolics extractability increasing or even decreasing their level on orange juice. During storage it was noticed a significant decrease (about 25%, $P<0.05$) in total phenolics after 36 days of storage in thermally processed juice.

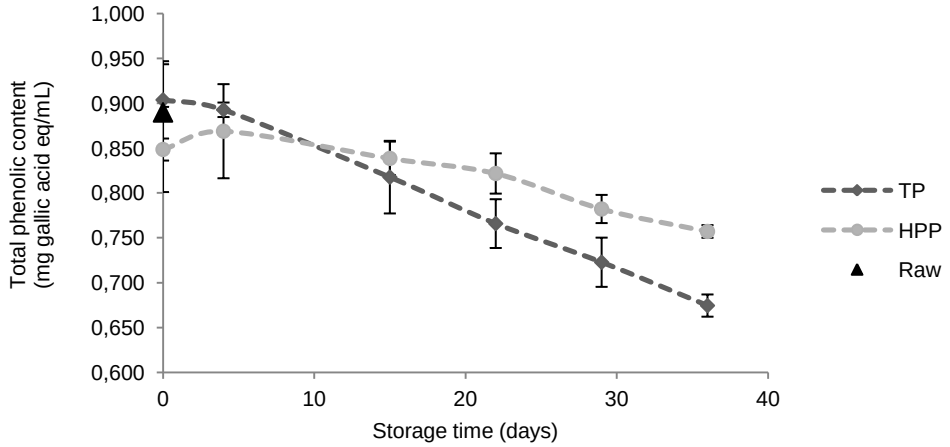

Figure 11: Total phenolics content of raw, thermal (TP) and pressure (HPP) processed orange juice.

Comparing both methods it was possible to conclude that there are no significant ($P<0.05$) differences between them until the 22nd day of storage and so, the effects of HPP on phenolic compounds of orange juice induce the same behaviour than TP treatment.

Although in some cases TP might cause the previously referred releasing of phenolics, the inadequate inactivation of PPO after HP treatments may contribute to their oxidative and enzymatic degradation. This opposite response might be the explanation why both methods have no significant differences ($P<0.05$) between them until 22 days of storage. Another interesting behaviour emerges when total phenolics content of raw juice is compared with treated samples among the storage time. As observed in Table B1 (Appendix B) after 22 days of storage only HPP treated samples do not show significant differences ($P<0.05$) comparing with raw juice at day 0, but regarding TP juice the total phenolic content is significantly ($P<0.05$) lower after the same 22 days of storage, decreasing from 0.890±0.054 mg/mL to 0.674±0.012 mg/mL. Thus, it is possible to conclude that HPP method maintains original phenolics content during more time and with fewer variations through all storage period.

V.1.3.2. Total content of anthocyanins

The main anthocyanin present in orange juice is cyanidin-3-glucoside (cy3glu) [173] and so, the total anthocyanins content was measured in equivalents of cy3glu. As has been demonstrated in several studies, degradation of anthocyanins may be catalyzed by the presence of oxidase enzymes [98, 171, 174].

Although anthocyanin degradation has been reported to arise in processed orange juice as a result of indirect oxidation by phenolic quinines generated by PPO [171], in this study (Figure 12) was observed a decrease in about 11% on anthocyanin content of thermal treated samples when compared to raw samples, maybe due to simple thermal degradation of those compounds. In the case of HPP samples, it was observed a significant increase ($P<0.05$) from 4.87E-02 ± 5.03E-04 mg/mL on raw orange juice to 5.03E-02 ± 1.26E-04 mg/mL. As mentioned before, processing technologies might lead to the disruption of some membranes (such as the plant vacuole membranes), releasing some of their components, namely anthocyanins. Although the lower changes verified on anthocyanin content in both TP and HPP samples, their content decreases during storage, being significantly lower ($P<0.05$) in TP samples. This behaviour has been associated with two main hypotheses. The first one is the PPO and peroxidase action as said before, and the second one is based on the effect of AA which accelerates the anthocyanin degradation [90].

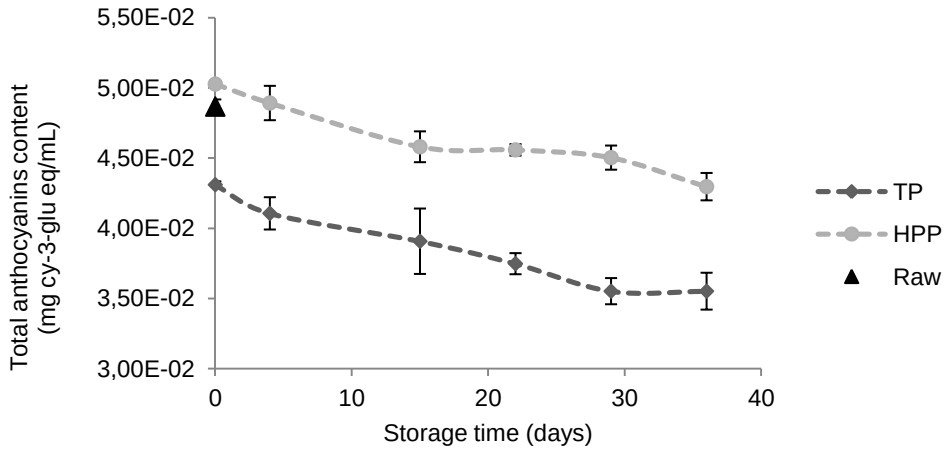

Figure 12: Total anthocyanins content of raw, thermal (TP) and pressure (HPP) processed orange juice.

These effects on anthocyanin were observed both in TP and HPP orange juice samples being higher on TP treated ones. In fact, it was observed loses of about 18% on the anthocyanin content of TP samples, when compared to a 15% loss on HPP samples during storage period. Also during storage it is possible to verify a quasi stable and linear behaviour on anthocyanins. As observed both on Table B2 (Appendix B) and Figure 12 HPP induces better anthocyanins retention on orange juice since during all storage time HPP total anthocyanins values are closest from raw values than in the case of TP orange juice. Additionally it was verified at day 4 and 15 of storage that the differences between HPP and raw orange juice were no significant ($P<0.05$). As in the case of total phenolics content, also here has been concluded that HPP prevent higher losses on those bioactive compounds.

V.1.3.3. Total content of flavonoids

Flavonoids are also important pigments and antioxidants present in orange juice. As in the case of anthocyanins, it was used one of the flavonoids present in orange juice, in this case rutin, to express total flavonoids content. It was found that HPP treatment does cause a significant increase ($P<0.05$) in this compound (Figure 13) right after treatment (day 0). Comparing with unprocessed samples, HP orange juice flavonoids increase from 0.573±0.010 to 0.708±0.003 which means an increase in about 24% of total flavonoids content. In fact, it is possible that HPP induce some structural changes that release phenols from proteins and consequently increase the flavonoids extraction [58]. On the other hand thermal treatments might induce several damages on the structure of flavonoids, leading to a decrease of concentration, in this case, to a decrease of about 22%.

The evolution of flavonoids concentration on both TP and HPP samples during 36 days of storage shows that those two methods have distinct effects on total content of flavonoids

during storage time. Regarding thermal treatment, it was observed a non trending behaviour with a significant increase (P<0.05) from 0.448±0.010 to 0.608±0.020 at the first 4 days of storage.

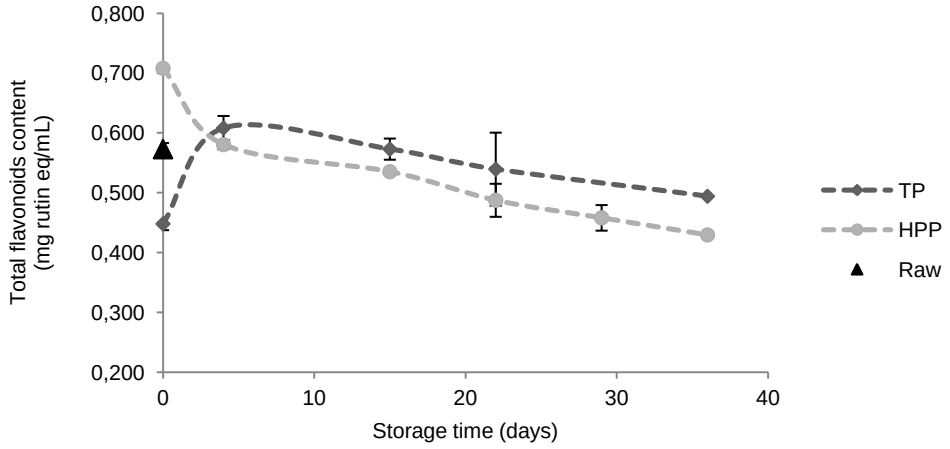

Figure 13: Total flavonoids content of raw, thermal (TP) and pressure (HPP) processed orange juice.

From day 4 to day 22 total flavonoids content on TP orange juice was not significantly affected (P<0.05). From the 22th day of storage until the end of storage period TP orange juice suffers a decrease of 19% in total flavonoids content. Unlike the observed on total anthocyanins, HPP shows more significant differences (P<0.05) comparing with raw juice during all time of storage (Table B3 – Appendix B) which could be linked to the highest reduction on flavonoids content than in TP samples. Also in the case of flavonoids content, PPO activity might be related with flavonoids degradation on processed orange juice [90], however as in the case of anthocyanins, there are no studies that compare PPO activity with flavonoids stability on fruit and fruit juices.

V.1.3.4. Total content of carotenoids

Carotenoids are one of the most important indicators of orange juice quality as they contribute both for the colour and nutritional value of the juice. One of the main carotenoids present in this fruit is β-carotene and so it was used to express the total carotenoids content on orange juice samples. As observed in Figure 14, the HPP and TP processing methods significantly (P<0.05) reduces the total carotenoids content in 20 and 12% respectively. These results were not in agreement with other reported, which has shown highest content of carotenoids in HPP and TP samples comparing with unprocessed juice [25, 27, 113]. This behaviour is observed even during all time of storage since both TP and HPP samples showed significant (P<0.05) differences when compared to raw juice (Table B4 – Appendix B).

During storage was observed a stable behaviour both in thermal as in pressurised samples. Both in the case of TP and HPP orange juice, was verified a significant decrease ($P<0.05$) of total carotenoids content during storage which might be associated to the disruption of the matrix and the instability of the polyene chain of carotenoids provoking processes of isomerisation and oxidation [78], one of the main carotenoids degradation mechanisms.

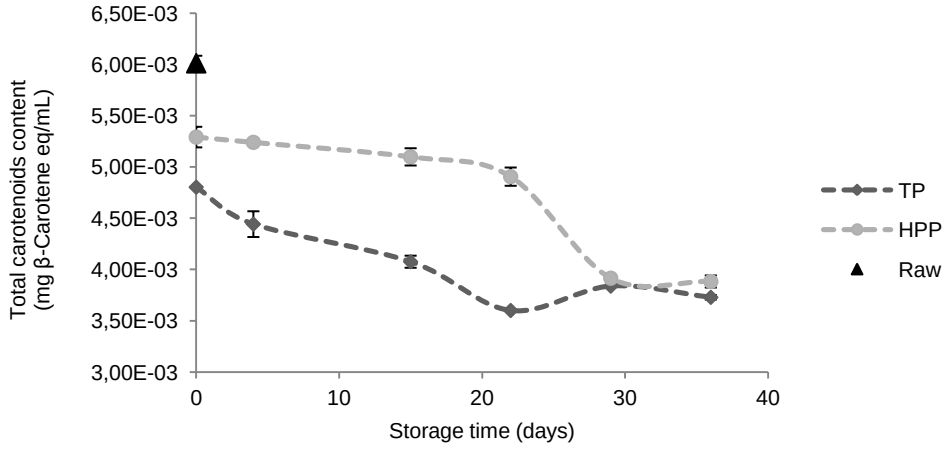

Figure 14: Total carotenoids content of raw, thermal (TP) and pressure (HPP) processed orange juice.

The most stable behaviour observed in the case of HPP samples could be probably related with the AA protective effect from oxidation [27]. Comparing the TP and HPP methods were observed more interesting results. The total carotenoids content was higher on HPP samples than on TP samples during the 36 days of storage. These results can be related with the effect of HPP on macromolecules since carotenoids are bonded to macromolecules (proteins and membranes) and consequently, HP increases the release of carotenoids and its accessibility by affecting the carotenoids-binding protein [78].

V.1.3.5. Individual phenolic compounds identification and quantification

The HPLC-DAD-MS analysis of orange juice samples allowed the identification of five compounds (Figure 15): quinic acid, ellagic acid, naringenin 7-O-rutinoside (narirutin), apigenin 6,8-di-*C*-glucoside (vicenin II) and hesperetin 7-O-rutinoside (hesperidin). All these compounds were previously reported to occur in citrus or orange juice [58, 175, 176].

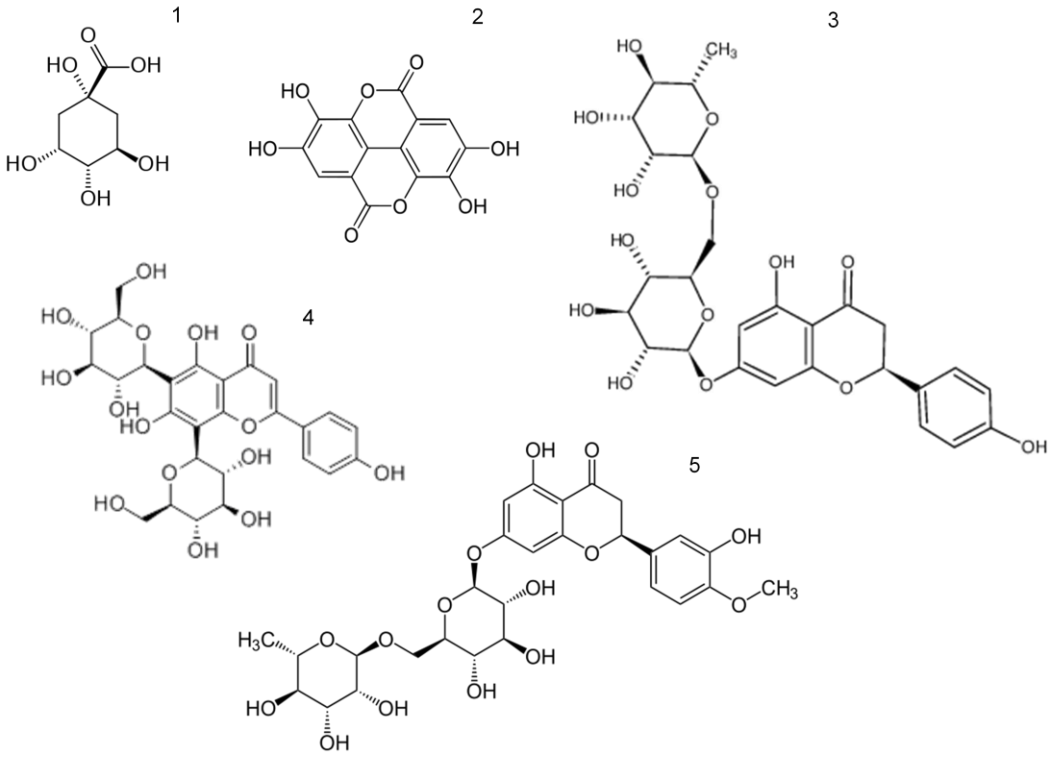

Figure 15: Chemical structures of identified compounds. (1) quinic acid, (2) ellagic acid, (3) narirutin, (4) vicenin II and (5) hesperidin.

Table 9 shows the compounds found in samples of day 0 on raw, TP and HPP samples. Our results show that the main phenolic group identified in orange juice samples were flavonoids glycosyl derivatives (compounds 3-5) as reported by Gattuso *et al.* (2007) [58]. Additionally, to correctly identify all the five compounds were used ESI-MS spectra, which allow the verification of the fragmentation pattern.

Table 9: Compounds identified in orange juice samples in raw, thermal (TP) and pressure (HPP) processed orange juice.

Nº	**$[M-H]^-$ (*m/z*)**	**Compound name**	**Fragments (*m/z*)**		**Retention Time (min)**		
			MS^2	MS^3	Raw	TP	HPP
1	191	Quinic acid	173, 111	-	2.90	2.88	2.85
2	301	Ellagic acid	257, 229, 285	301, 173	23.23	23.12	23.16
3	579	Naringenin 7-*O*-rutinoside	271	-	20.84	20.85	20.83
4	593	Apigenin 6,8-di-*C*-glucoside	575, 503, 473, 383, 353, 323	353, 293, 189	13.45	13.36	13.34
5	609	Hesperetin 7-*O*-rutinoside	301	283, 256	23.20	23.15	23.11

Figure 16 shows quinic acid MS^2 (a) and vicenin II MS^3 spectra (b) as examples of those obtained for the other compounds. Regarding the fragmentation pattern of compound 1, *m/z* 173 ion on MS^2 spectrum, suggest a loss of a water molecule ([M-H-18]$^-$). Also *m/z* 111 ion is characteristic of 1, being related with a loss of another water molecule and a CO_2. Thus, this fragmentation pattern allowed the unequivocally identification of the respective organic acid. Those results were additionally verified by the UV-Vis spectrum which has an absorption band on 237 nm, also characteristic of 1.

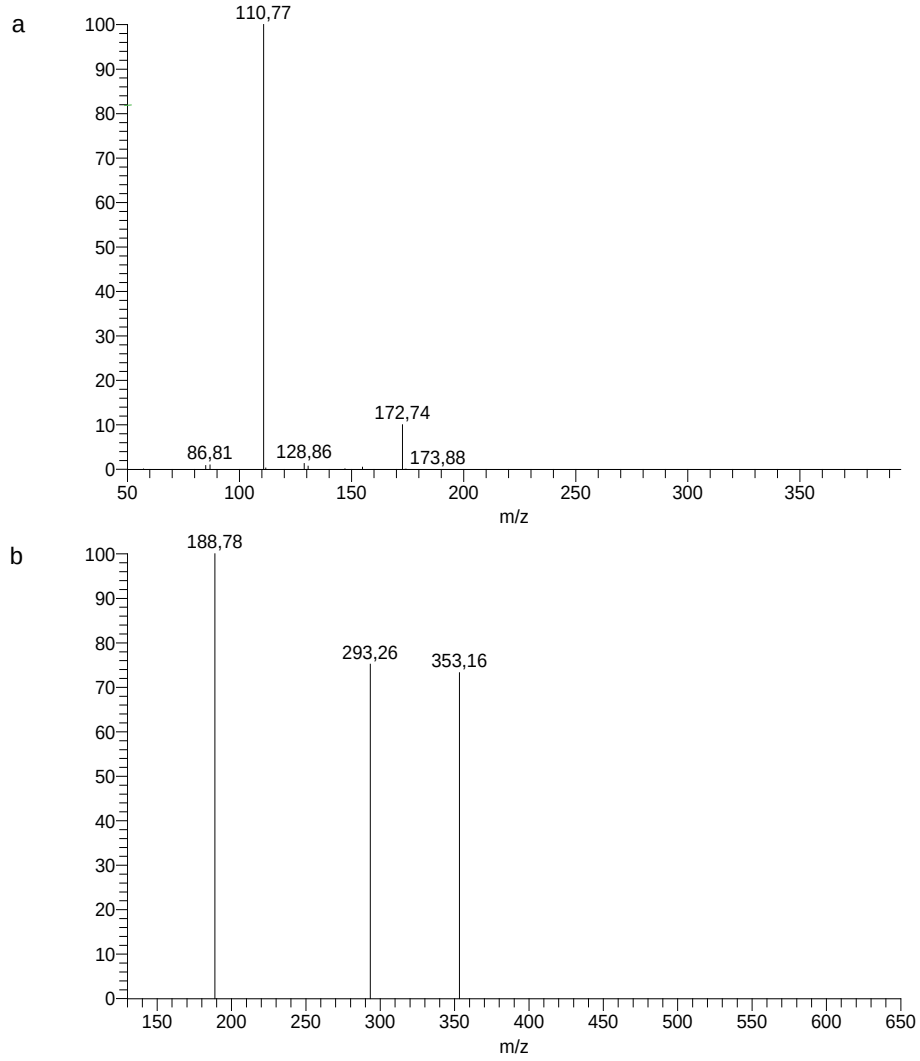

Figure 16: Example of ESI-MS data. (a) MS^2 spectrum of quinic acid and (b) MS^3 spectrum of vicenin II.

Compound 2 had [M-H]$^-$ ion at *m/z* 301 and MS^2 fragmentation ions at *m/z* 257, 229 and 185 which are typical fragments of ellagic acid [177]. Compounds 3 and 5 produced [M-H-308]$^-$ ion in the MS^2 spectrum which suggests the loss of a rutinoside [175]. Also the [M-H]$^-$ ions with *m/z* 579 and 609, and the UV-Vis spectrum o both compounds (Figure 17 and Figure 18) proves the existence of naringenin and hesperetin rutinoside derivates, respectively as observed by Shi *et al.* (2007) [175].

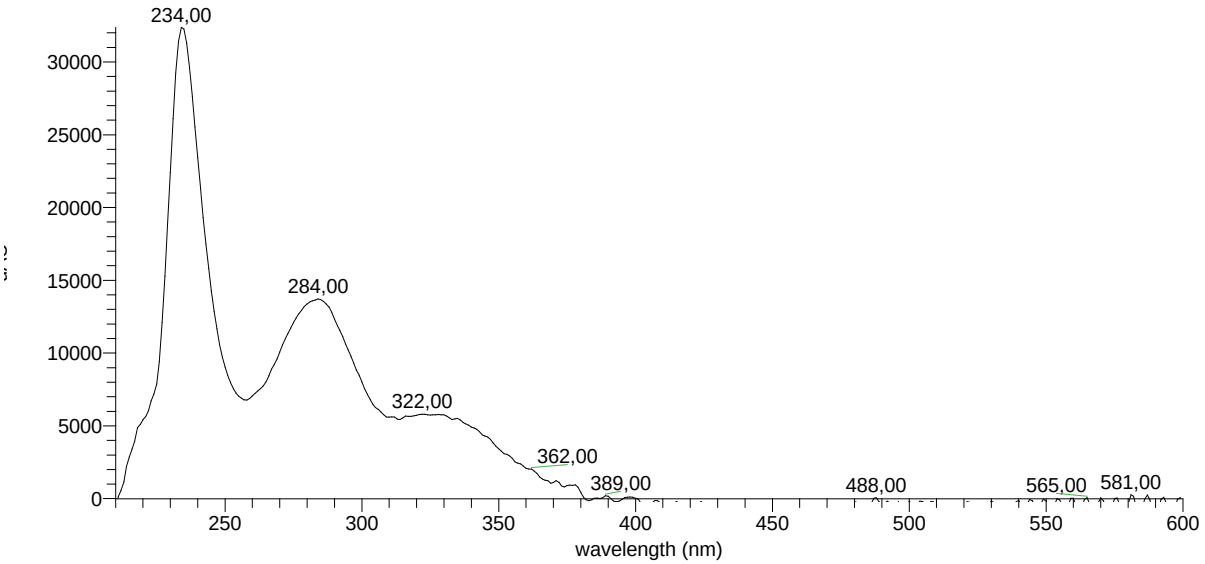

Figure 17: UV-Vis spectra on retention time 20,84 min of the raw orange juice sample, associated to compound 3

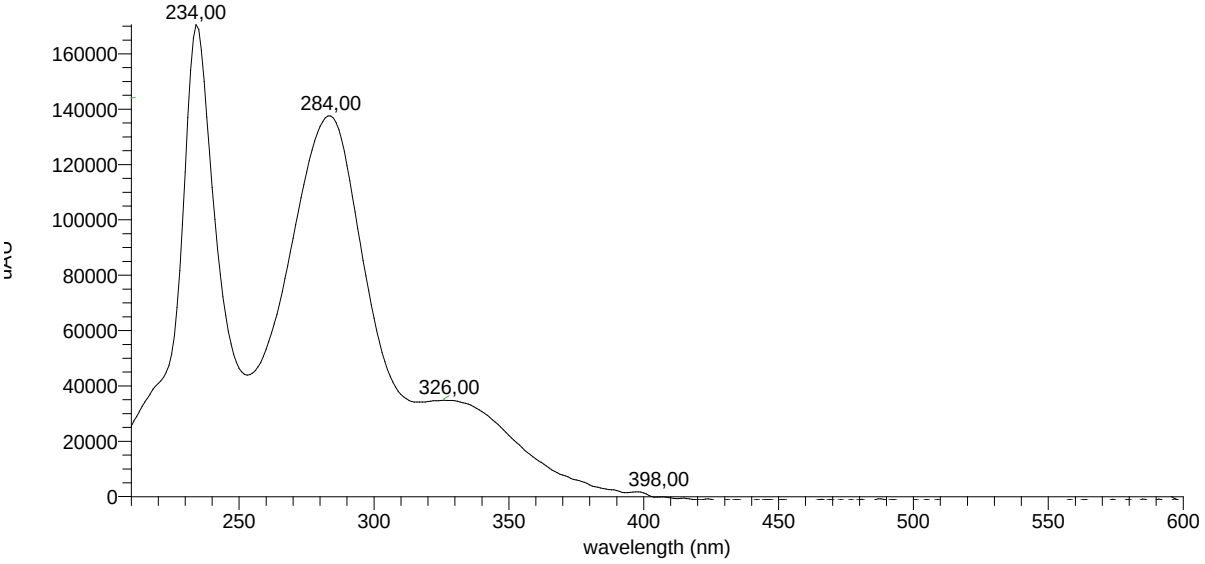

Figure 18: UV-Vis spectra on retention time 23,20 min of the raw orange juice sample, associated to compound 2 and 5.

The MS/MS spectra in negative mode of compound 4 exhibit a $[M-H]^-$ ion at *m/z* 593 and the fragmentation patterns starts with the loss of a water molecule, proceeding with the appearance of a well-defined series of characteristic ions which correspond to the sugar ring dissociation: $[M-H-18]^-$, $[M-H-90]^-$, $[M-H-120]^-$, $[M-H-120-90]^-$, $[M-H-120-120]^-$, [178]. Comparing the data obtained with those reported on literature it was possible to indentify compound 4 as being apigenin 6,8-di-*C*-glucoside also known as vicenin II [176].

To compare the effect of both thermal and pressure processing methods on identified compounds, a quantitative analysis was made. It is important to mention that compounds 2 and 5 are observed at the same retention time and comparing the UV-Vis at the specific

retention time (Figure 18) of all chromatograms allowed to conclude that the most abundant phenolic between the two overlapping was compound 5 due to its 284 nm band. As observed on Figure 19, Figure 20, Figure 21 and Figure 22 all compounds identified have a different response on the HPP and TP effect. At the processing day, only compound 5 (Figure 22) shows significant differences ($P<0.05$) between raw and pressurised orange juice demonstrating that generally both processing methods do not affect the concentration of these phenolic compounds.

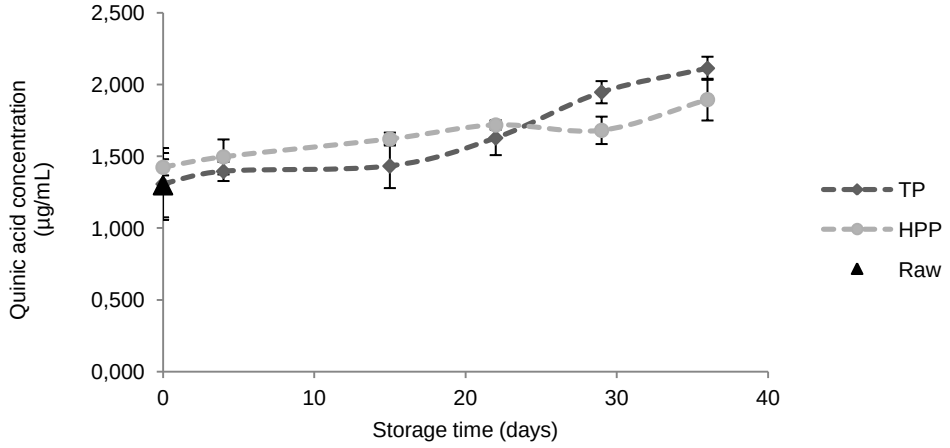

Figure 19: Quinic acid concentration of raw, thermal (TP) and pressure (HPP) processed orange juice.

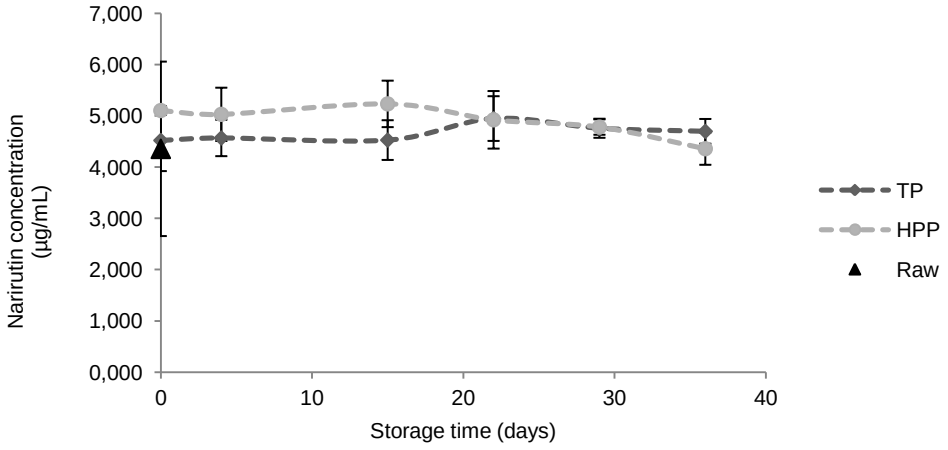

Figure 20:Narirutin concentration of raw, thermal (TP) and pressure (HPP) processed orange juice.

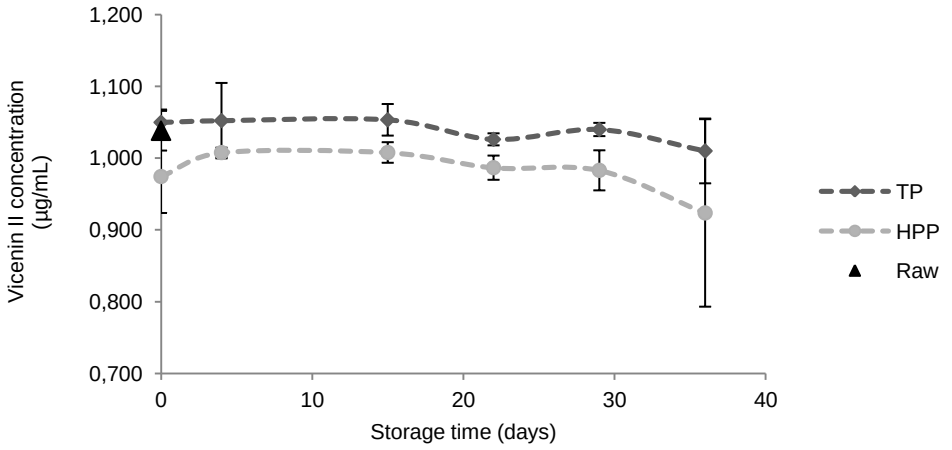

Figure 21: Vicenin II concentration of raw, thermal (TP) and pressure (HPP) processed orange juice.

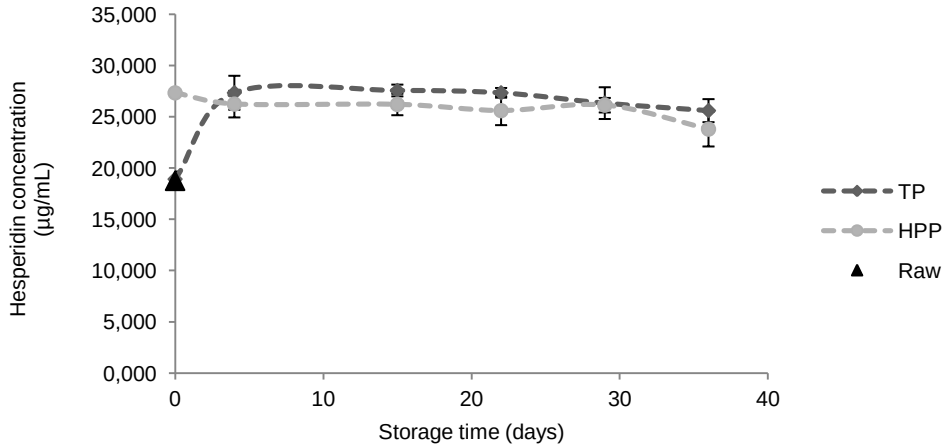

Figure 22: Hesperidin concentration of raw, thermal (TP) and pressure (HPP) processed orange juice.

Regarding quinic acid (Figure 19) it was possible to observe a slight increase on its content after 22 days of storage in HPP and TP orange juice. Compounds 4 and 5 (Figure 21 and Figure 22) showed the opposite behaviour on TP samples, where was observed a slight but not significant decrease ($P<0.05$) of their concentration before 22 days of storage. Additionally HPP and TP showed no significant effects ($P<0.05$) in compound 3 (Figure 20) being observed a quasi stable behaviour. In general, was not observed significant changes ($P<0.05$) on the concentration of the identified compounds after HPP which demonstrate once again the feasibility of this method on the processing food industry. Comparing with raw juice, generally, both HPP and TP methods during the 36 days of storage induce significant changes ($P<0.05$) on individual phenolics (Table B5 – Appendix B). Although the impossibility to identify and quantify more orange juice phenolics, the results allowed to show that for the identified compounds, in general, HPP technology does not significantly affect

(P<0.05) their concentration in orange juice when compared with TP juice. These results are interesting toward the use of high pressure as an alternative to thermal processing.

V.2. Antioxidant activity

The importance of antioxidants, and so antioxidant activity, is associated with the availability of neutralise free-radicals, preventing damages caused by free-radicals. DPPH radical scavenging method is one of the main methods used to evaluate antioxidant activity in fruit juices, and antiradicalar power (ARP) one of the parameters quantify it, since the higher the ARP, the more efficient the antioxidant [179]. Comparing to unprocessed samples (Figure 23), thermal and pressure treatments induce a significant decrease (P<0.05) on ARP parameter at day 0, being this reduction of 26 and 13% respectively. Also during storage time were noticed significant changes (P<0.05) (Table B6 – Appendix B) between raw, TP and HPP orange juice, being observed lower reductions on ARP of HPP orange juice (34%). This behaviour might be associated with the observed decrease of total phenolics, carotenoids and other antioxidants presented in orange juice.

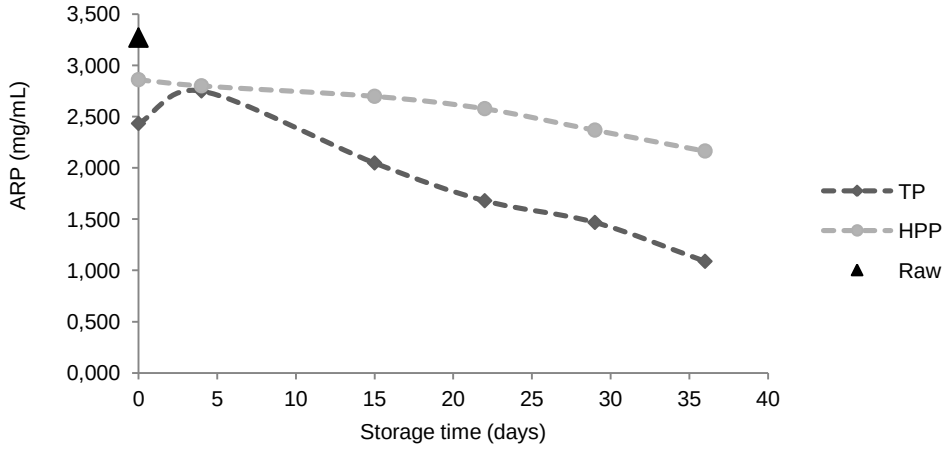

Figure 23: Antiradicalar power of raw, thermal (TP) and pressure (HPP) processed orange juice.

Moreover, during storage were observed interesting results, with a decrease on ARP during all 36 days in both treatments. However, during 36 days of storage were verified a major loss in TP juice (about 55%) than in HPP juice (about 24%). The higher values of ARP on HPP treated samples, even through storage time, might be associated to higher content of some bioactive compounds such as carotenoids, flavonoids and vitamin C.

V.3. Kinetic analysis of bioactive compounds and antioxidant activity changes of HPP and TP orange juice

The observed changes detected after TP and HPP orange juice processing and during storage time were subjected to reaction kinetic analysis (Appendix D). The kinetic parameter *k* (reaction rate constant) was calculated for ARP and bioactive compounds, and is present in Table 10. To our knowledge there are no reports data of kinetic analysis of HP and TP effects on phenolics, anthocyanins, flavonoids, carotenoids and ARP on orange juice. The differences on *k*-values for HPP and TP orange juice are no higher than 3-fold. When comparing the reaction rates for HPP and TP orange juice samples it was verified that only in the case of total flavonoids content the *k*-value of HPP is higher than the observed for TP orange juice (about 1.3-fold).

Table 10: Kinetic parameters orange juice ARP and bioactive compounds, assuming a zero-order reaction.

Parameter	Condition	*k*-value (days^{-1})	r^2
Total phenolics	TP	6.82E-03	1.000
	HPP	3.55E-03	0.976
Total anthocyanins	TP	2.13E-04	0.959
	HPP	1.85E-04	0.942
Total flavonoids	TP	3.62E-03	0.995
	HPP	4.85E-03	0.992
Total carotenoids	TP	5.03E-05	0.972
	HPP	1.68E-05	0.965
Quinic acid*	TP	3.37E-02	0.986
	HPP	1.14E-02	0.921
ARP	TP	5.06E-02	0.986
	HPP	1.85E-02	0.945

* for quinic acid the reaction rates refers to an increase in concentration

Additionally, total phenolics, anthocyanins and carotenoids show *k*-values 1.9, 1.5 and 3-fold higher on TP samples. In the case of quinic acid it was observed an increase of its concentration and kinetic data shows that when compared to TP samples, HPP orange juice reaction rate is about 2.6-fold lower. Also in the case of ARP it was observed a higher *k*-value for TP orange juice - about 2.7-fold higher than on HPP juice. These results give valuable information about the evolution of the parameters studied with storage time, after thermal and HP pasteurisation.

VI. Conclusion

During the past few years, the use of non-thermal processing methods has emerged with the increasing demand of fresh and natural products. High pressure processing technique represents a rapid, efficient and reliable method to improve the quality of food products, namely fruit juices. The effect of HPP on orange juice, for example, relies on the improvement of carotenoids, flavanones and vitamin C retention, keeping also the organoleptic characteristics such as aroma and texture, almost unaffected.

Although there has been a large development of HPP in the past ten years, there are numerous parameters that have not been studied. The application of HPP to produce orange juice has been extensively explored, but the effects of HPP at even lower processing temperatures are unknown and appear to be important in fruit processing industries. Thus, this experimental work evaluated TP and HPP effects on phenolic acids, anthocyanins, flavonoids, carotenoids and antioxidant activity and some organoleptic properties, such as colour, and soluble solids. At day 0 of storage time, some interesting results were observed. When comparing with raw orange juice, total flavonoids content is 22% lower in TP orange juice samples and total anthocyanins content increase from 4.87E-02±5.03E-04 to 5.03E-02±1.26E-04. Total carotenoids content and ARP decrease in both TP and HPP orange juice right after treatment, when comparing with raw orange juice. During storage HPP orange juice showed higher retentions of phenolics, anthocyanins and carotenoids. In fact there are higher reductions on total phenolic content of TP orange juice (about 25%) from day 0 to day 36. Regarding total carotenoids content, it was verified a decrease of about 62% on its content after 36 days of storage for TP orange juice, comparing with HPP. However, the verified losses on HPP orange juice carotenoids were higher than those of TP orange juice and so it is possible that those pigments are more affected by HPP during storage time. This behaviour might be associated with colour changes observed after HPP treatments. In fact, a* and b* parameters suffer more changes on HPP samples when compared to TP samples. Comparing raw juice with HPP and TP orange juice after 36 days of storage was observed that only in the case of total flavonoids content HPP shows higher losses (25% when compared with the 14% of losses in TP orange juice). The antioxidant activity is the most affected parameter and it was observed a decrease of about 67% on ARP of TP orange juice after 36 days of storage when compared with raw juice. Regarding the four individual phenolics identified, it was possible to concluded that both thermal and pressure does not significantly affect ($P<0.05$) its content, although some slight changes were observed.

With these results it is possible to conclude that HPP appear to be a generally better alternative to thermal treatments. In what concerns orange juice, further investigation it is necessary, not only to understand what mechanisms are actually affected by HPP and TP processing technologies.

VII. References

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VIII. Appendices

Appendix A. Standard curves

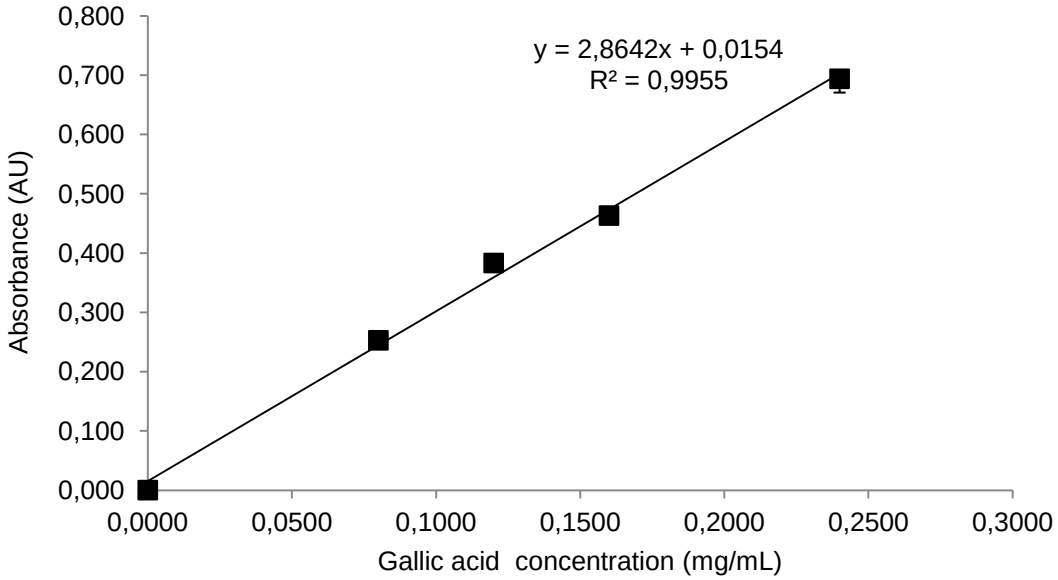

Figure A1: Standard curve used for determining total phenolic content on orange juice.

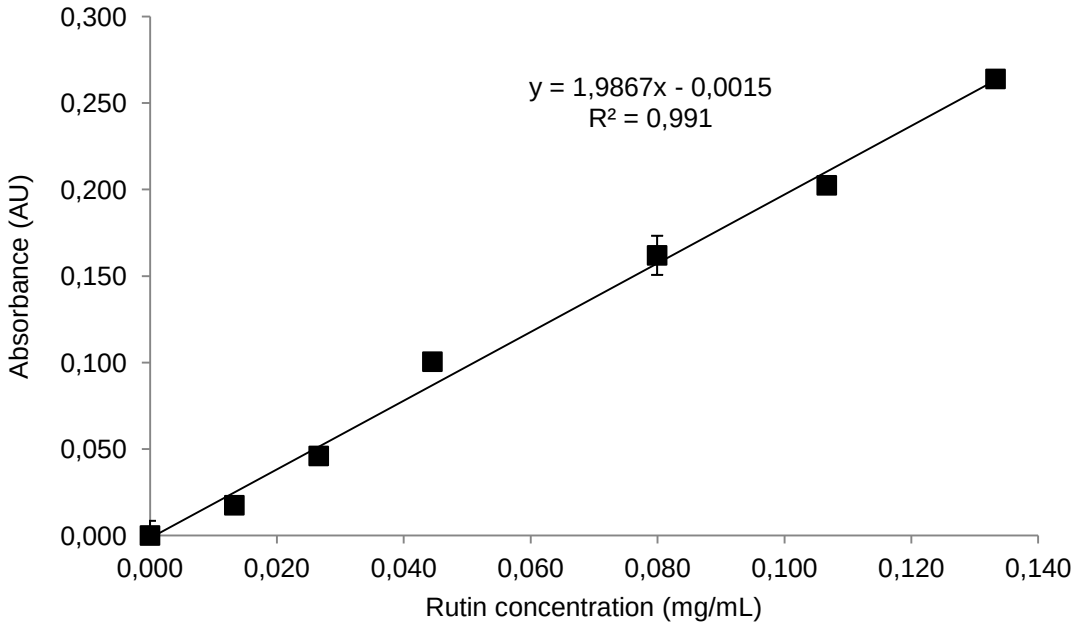

Figure A2: Standard curve used for determining total flavonoids content on orange juice.

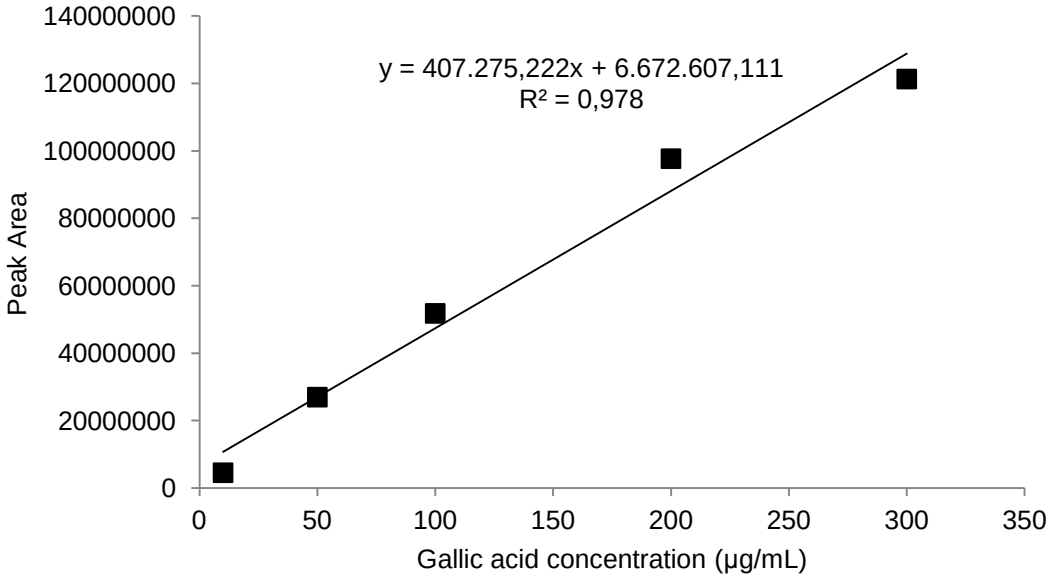

Figure A3: Standard curve used for determining quinic acid concentration on orange juice.

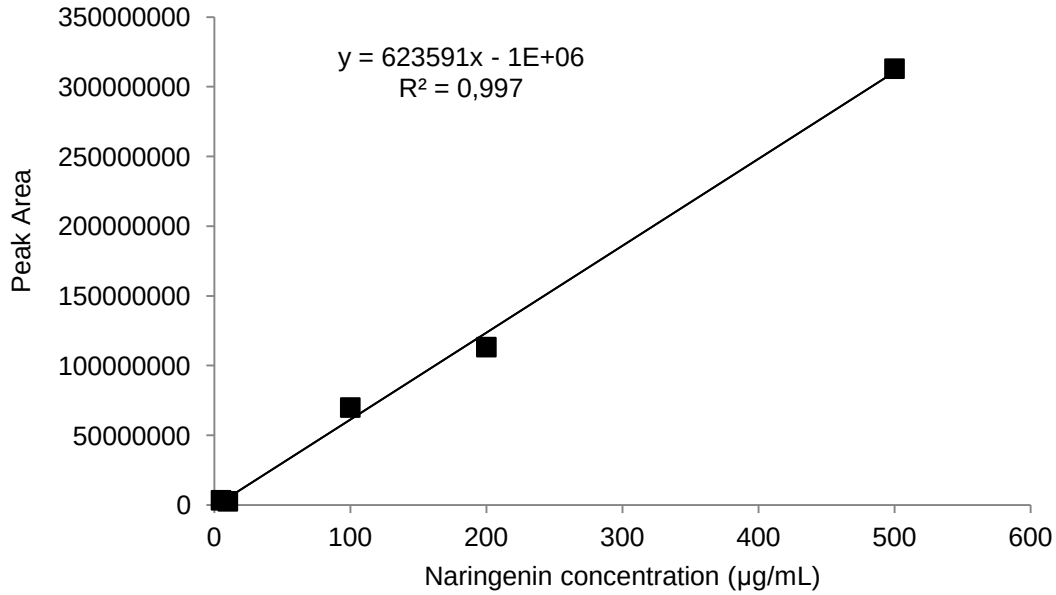

Figure A4: Standard curve used for determining narirutin and hesperidin concentration on orange juice.

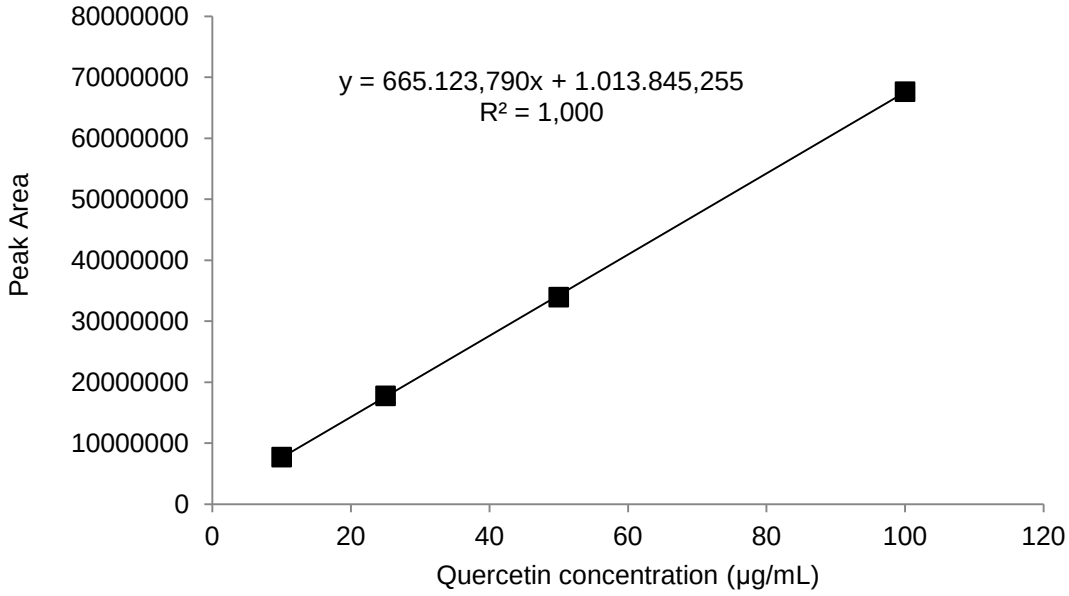

Figure A5: Standard curve used for determining vicenin II concentration on orange juice.

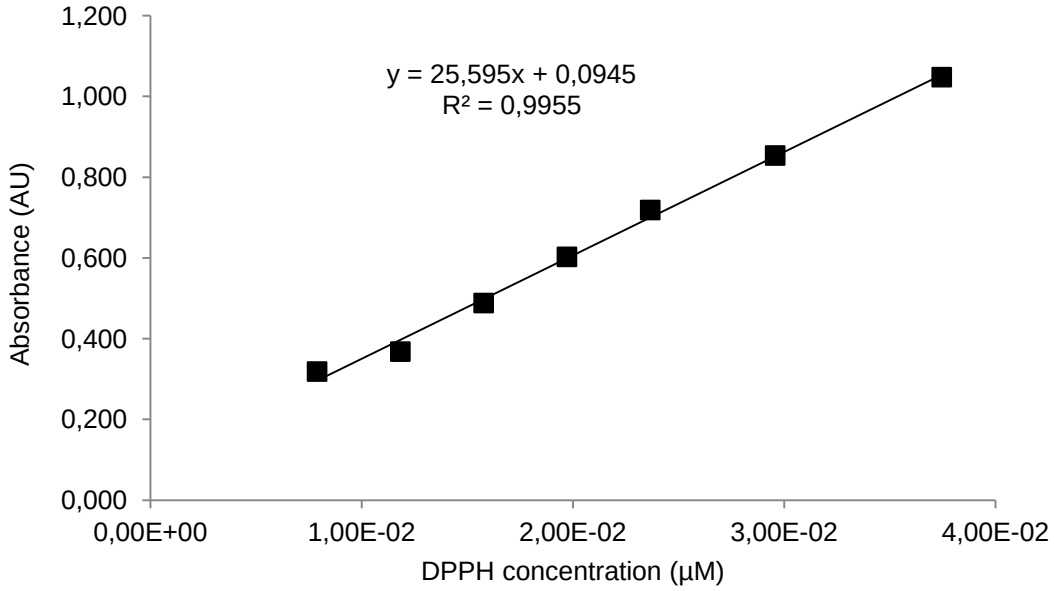

Figure A6: Standard curve used for determining DPPH concentration on orange juice.

Appendix B. Statistical data

Table B1: Total phenolic compounds content (mg equivalent gallic acid/mL) of raw, thermal (TP) and pressure (HPP) processed orange juice samples[a]. Values are means±standard deviation.

Time of storage (days)[b]	Raw[c]	TP	HPP
0	0.890±0.054 A	0.904±0.043 eA	0.848±0.047 aA
4	A	0.893±0.008 deA	0.869±0.052 aA
15	A	0.818±0.040 cdA	0.839±0.019 abA
22	B	0.766±0.027 bcA	0.822±0.022 abAB
29	B	0.723±0.027 abA	0.782±0.016 abA
36	C	0.674±0.012 aA	0.757±0.007 bB

a Different non-capital letters in the same treatment during storage indicate significant differences (P<0.05).

b Different capital letters in the same day of storage indicate significant differences between raw, TP and HPP (P<0.05).

c Capital letters in raw column at days 4 to 36 indicates statistical data used to compare with TP and HPP.

Table B2: Total anthocyanins (mg equivalent of cy3glu/mL) content of raw, thermal (TP) and pressure (HPP) processed orange juice samples[a]. Values are means±standard deviation.

Time of storage (days)[b]	Raw[c]	TP	HPP
0	4.87E-02 ± 5.03E-04 B	4.31E-02 ± 2.39E-04 dA	5.03E-02 ± 1.26E-04 cC
4	B	4.11E-02 ± 1.15E-03 cdA	4.89E-02 ± 1.22E-03 cB
15	B	3.91E-02 ± 2.33E-03 bcA	4.58E-02 ± 1.10E-03 aB
22	C	3.75E-02 ± 7.51E-04 abA	4.56E-02 ± 4.08E-04 aB
29	C	3.55E-02 ± 9.32E-04 aA	4.50E-02 ± 8.60E-04 bcB
36	C	3.55E-02 ± 1.31E-03 aA	4.30E-02 ± 9.75E-04 bcB

a Different non-capital letters in the same treatment during storage indicate significant differences (P<0.05).

b Different capital letters in the same day of storage indicate significant differences between raw, TP and HPP (P<0.05).

c Capital letters in raw column at days 4 to 36 indicates statistical data used to compare with TP and HPP.

Table B3: Total flavonoids content (mg equivalent of rutin/mL) of raw, thermal (TP) and pressure (HPP) processed orange juice samples[a]. Values are means±standard deviation.

Time of storage (days)[b]	Raw[c]	TP	HPP
0	0.573±0.010 B	0.448±0.010 cA	0.708±0.003 eC
4	A	0.608±0.020 aB	0.581±0.008 dAB
15	A	0.573±0.018 abA	0.535±0.004 cB
22	A	0.539±0.061 abA	0.487±0.028 bA
29	A	-	0.458±0.021 abB
36	C	0.494±0.028 bcB	0.430±0.001 aA

a Different non-capital letters in the same treatment during storage indicate significant differences (P<0.05).

b Different capital letters in the same day of storage indicate significant differences between raw, TP and HPP (P<0.05).

c Capital letters in raw column at days 4 to 36 indicates statistical data used to compare with TP and HPP.

Table B4: Total carotenoids content (mg equivalent of β-Carotene/mL) of raw, thermal (TP) and pressure (HPP) processed orange juice samples[a]. Values are means±standard deviation.

Time of storage (days)[b]	Raw[c]	TP	HPP
0	6.01E-03 ± 1.39E-05 C	4.80E-03 ± 1.11E-05 eA	5.29E-03 ± 1.00E-04 eB
4	C	4.44E-03 ± 1.26E-04 dA	5.24E-03 ± 1.11E-05 bcB
15	C	4.08E-03 ± 5.89E-05 cA	5.10E-03 ± 8.41E-05 bB
22	C	3.60E-03 ± 5.31E-19 bA	4.91E-03 ± 8.91E-05 dB
29	B	3.84E-03 ± 1.11E-05 abA	3.92E-03 ± 1.93E-05 aB
36	C	3.73E-03 ± 2.23E-05 aA	3.88E-03 ± 5.89E-05 aB

a Different non-capital letters in the same treatment during storage indicate significant differences (P<0.05).

b Different capital letters in the same day of storage indicate significant differences between raw, TP and HPP (P<0.05).

c Capital letters in raw column at days 4 to 36 indicates statistical data used to compare with TP and HPP.

Table B5: Individual compounds content (µg/mL) of raw, thermal (TP) and pressure (HPP) processed orange juice samples[a]. Values are means±standard deviation.

Nº	[M-H]⁻ (*m/z*)	Time of storage (days)[b]	Content (µg/mL)			λ (nm)	Standard
			Raw[c]	TP	HPP		
1	191	0	1.300±0.224 A	1.308±0.251 aA	1.424±0.057 bA	280	Gallic acid
		4	A	1.395±0.067 aA	1.496±0.057 abA		
		15	A	1.433±0.153 aA	1.621±0.045 abA		
		22	A	1.628±0.120 abAB	1.719±0.032 acB		
		29	B	1.947±0.077 bcA	1.681±0.095 acA		
		36	B	2.114±0.081 cA	1.895±0.145 cA		
2	301	0	trace			280	Gallic acid
		4					
		15					
		22					
		29					
		36					
3	579	0	4.356±1.701 A	4.520±0.598 aA	5.103±0.086 aA	280	Naringenin
		4	A	4.568±0.354 aA	5.028±0.520 aA		
		15	A	4.527±0.387 aA	5.232±0.453 aA		
		22	A	4.946±0.436 aA	4.922±0.561 aA		
		29	A	4.757±0.186 aA	4.780±0.150 aA		
		36	A	4.697±0.241 aA	4.358±0.313 aA		
4	593	0	1.038±0.028 A	1.050±0.018 aA	0.974±0.051 aA	365	Quercetin
		4	A	1.052±0.053 aA	1.008±0.007 aA		
		15	A	1.053±0.022 aA	1.008±0.014 aA		
		22	B	1.026±0.008 aAB	0.987±0.017 aA		
		29	A	1.040±0.009 aA	0.983±0.028 aA		
		36	A	1.010±0.045 aA	0.924±0.131 aA		
5	609	0	18.761±0.793 A	18.922±0.744 bA	27.335±0.211 bB	280	Naringenin
		4	B	27.347±1.656 aA	26.241±1.298 abA		
		15	B	27.596±0.574 aA	26.204±1.046 abA		
		22	B	27.342±0.473 aA	25.601±1.411 abA		
		29	B	26.336±1.548 aA	26.134±0.709 abA		
		36	B	26.606±1.122 aA	23.801±1.691 aA		

a Different non-capital letters in the same treatment during storage indicates significant differences (P<0.05).

b Different capital letters in the same day of storage indicate significant differences between raw, TP and HPP (P<0.05).

c Capital letters in raw column at days 4 to 36 indicates statistical data used to compare with TP and HPP.

Table B6: Antiradicalar power (mg/mL) of raw, thermal (TP) and pressure (HPP) processed orange juice samples[a]. Values are means±standard deviation.

Time of storage (days)[b]	Raw[c]	TP	HPP
0	3.270±0.007 C	2.433±0.007 fA	2.860±0.009 eB
4	B	2.749±0.007 eA	2.800±0.001 fA
15	C	2.048±0.008 dA	2.698±0.020 dB
22	C	1.679±0.020 cA	2.577±0.022 cB
29	C	1.467±0.009 bA	2.368±0.020 bB
36	C	1.088±0.001 aA	2.164±0.002 aB

a Different small letters in the same treatment during storage indicates significant differences (P<0.05).

b Different capital letters in the same day of storage indicate significant differences between raw, TP and HPP (P<0.05).

c Capital letters in raw column at days 4 to 36 indicates statistical data used to compare with TP and HPP.

Appendix C. Calibration data used for determining individual compounds concentration.

Table C1: Calibration data used for the HPLC-UV quantification of individual compounds of orange juice.

Compound	λ (nm)	Concentration range (μg mL^{-1})	Calibration curve[a]	r^2	LOD[b] (μg mL^{-1})	LOQ[c] (μg mL^{-1})
Gallic acid	280	10-300	y = 407.275,222 x + 6.672.607,111	0.978	61.07	203.58
Naringenin	280	5-200	y = 623.591,000 x – 1.103.161,000	0.997	38.81	129.36
Quercetin	365	10-100	y = 665.123,790 x + 1.013.845,255	1.000	1.16	3.88

a - y = peak area; x = concentration in μg/mL. b - LOD, limit of detection. c - LOQ, limit of quantification.

Appendix D. Zero-order kinetic data for orange juice

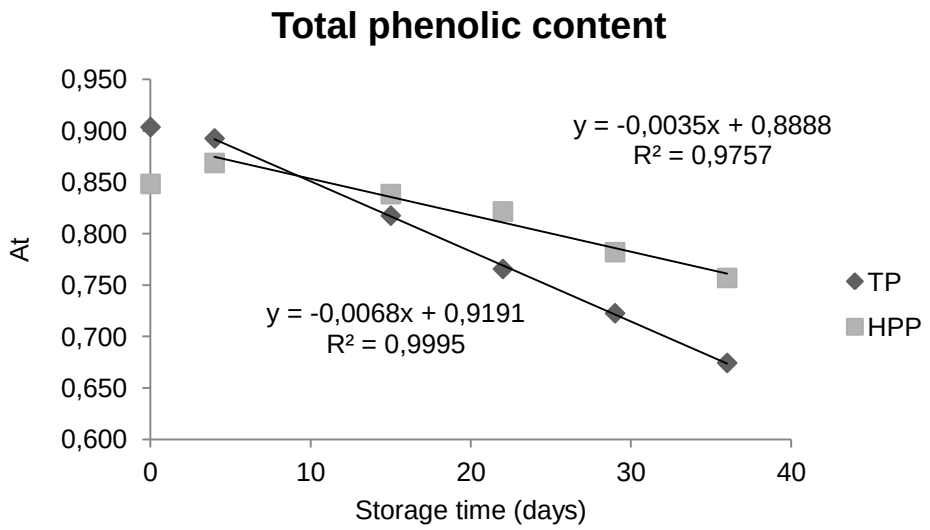

Figure D1: Effect of thermal (TP) and pressure (HPP) treatments on total phenolic content (*At*) as a function of storage time.

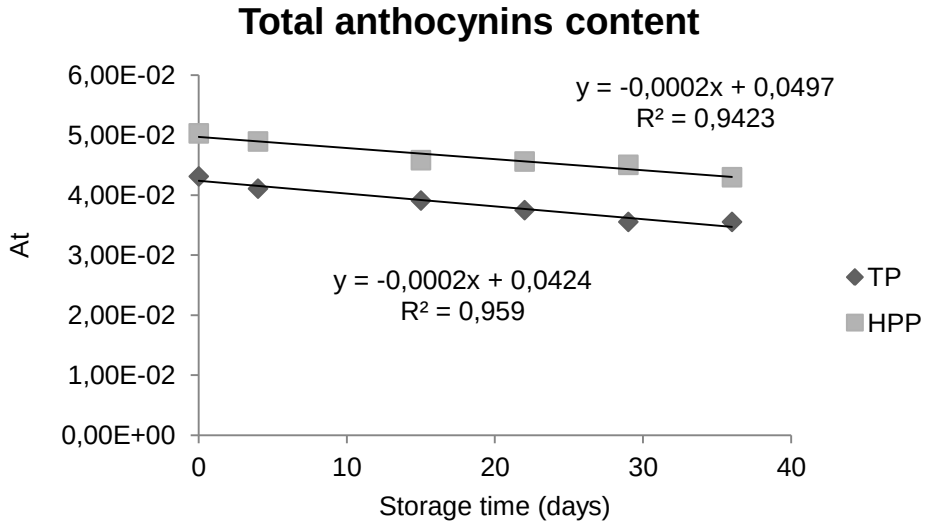

Figure D2: Effect of thermal (TP) and pressure (HPP) treatments on total anthocyanins content (*At*) as a function of storage time.

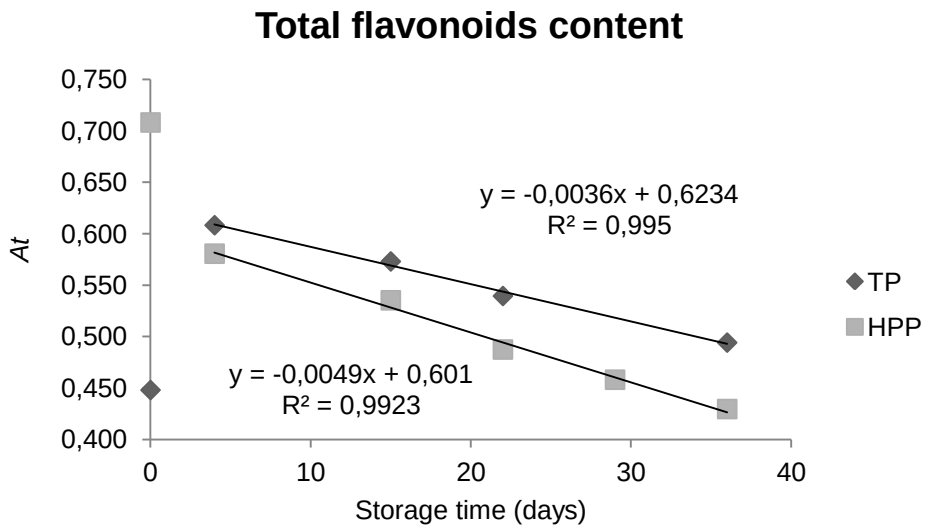

Figure D3: Effect of thermal (TP) and pressure (HPP) treatments on total flavonoids content (*At*) as a function of storage time.

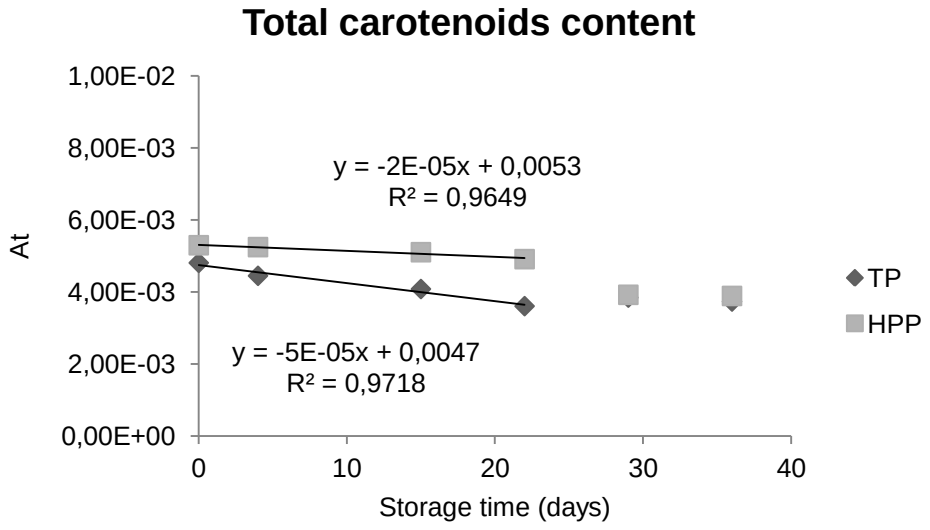

Figure D4: Effect of thermal (TP) and pressure (HPP) treatments on total carotenoids content (*At*) as a function of storage time.

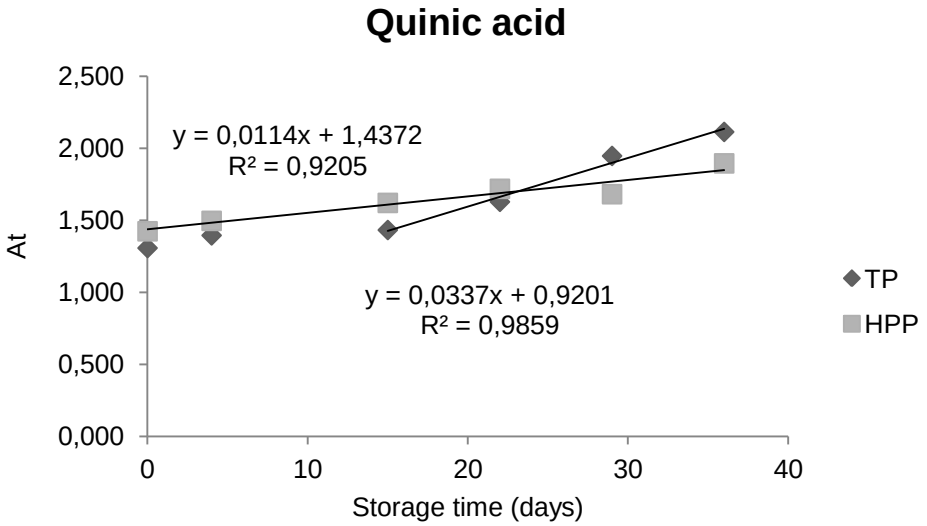

Figure D5: Effect of thermal (TP) and pressure (HPP) treatments on quinic acid concentration (*At*) as a function of storage time.

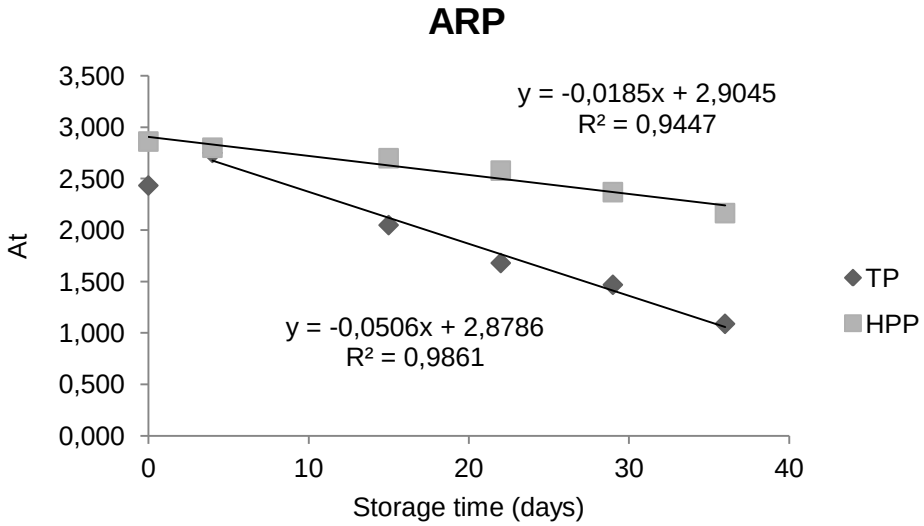

Figure D6: Effect of thermal (TP) and pressure (HPP) treatments on ARP (*At*) as a function of storage time.