INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

Carotenoid-based solutions for the replacement of artificial colorants in pastry products

Pedro Miguel Mota Martins

Dissertation submitted to Escola Superior Agrária de Bragança
to obtain the Degree of Master in Biotechnological Engineering

Supervisors
Doutora Maria Inês Moreira Figueiredo Dias
Doutora Carla Susana Correia Pereira

Bragança
2021

This work has been financed by the FEDER-Interreg España-Portugal programme for financial support through the project TRANSCoLAB 0612_TRANS_CO_LAB_2_P; to the European Regional Development Fund (ERDF) through the Regional Operational Program North 2020, within the scope of Project Mobilizador Norte-01-0247-FEDER-024479: ValorNatural®.

I. ACKNOWLEDGMENTS

I would like to first express my special thanks of gratitude to my supervisors Dr. Maria Inês Moreira Figueiredo Dias and Dr. Carla Susana Correia Pereira for their warm welcome, guidance of this project and for being excellent people to talk to.

My thanks also go towards the coordinator of my master's degree Professor Paula Rodrigues for being an exceptional teacher and showing me the scientific path of life.

I am fortunate to have been a part of the CIMO group for working with all kinds of people with different nationality making it a unique experience.

I want to thank the scientific team for helping me a lot through the experimental work of this project, namely Adriana Molina and Leonardo Corrêa for their huge help, without them I would have been overwhelmed of lab work.

A huge thanks towards a great friend and former pharmacy and chemistry of natural products master's colleague Luís Palmeira who changed me to be more responsible and more mature, helping me throughout the academic life and teaching me how to stand up for myself whenever unfortunate life events happen.

All the people that have been with me since the beginning of my life in Bragança, namely Bruno Nogueira, Rosalina Gomes and Pedro Pinheiro. Friends of heart met in Bragança whom I believe will be a lifetime friendship.

Most importantly, I am very grateful for my family's unconditional and loving support namely my mother Maria Gracinda and my step-father Edmundo Lopes, without him I would have never gone to Bragança to begin with.

II. ABSTRACT

Colour has a great importance in the first consumers' impression, allowing to infer about the overall quality, the taste, the smell, the texture, and even the safety of foodstuff. For these reasons, there is a massive use of colorants in food products. Among the numerous natural matrices potentially used for the extraction of colouring compounds, the fruits from the genus *Solanum* represent promising sources of pigments, namely carotenoids. This reason, together with the fact that large amounts of fresh tomato wastes (resulting from crop growing, packaging, processing, storage, and sale) are discarded worldwide, make the recovery of valuable colorant biomolecules from agri-food wastes a crucial step of the circular economy by re-introducing them into the food chain as ingredients.

In the present work, ultrasound-assisted methodologies were applied to obtain carotenoid-rich colouring extracts from three varieties of tomato (traditional red tomato - *Solanum lycopersicum* L., cherry tomato – *S. lycopersicum* var. *cerasiforme*, and yellow pear tomato – *S. lycopersum* 'Beam's Yellow Pear), using green solvents, and HLPC-DAD-ESI/MS to establish the carotenoid profile of each sample. Furthermore, the most promising and bioactive extracts were applied in a commercialized pastry cream to substitute the currently used artificial colorants, and finally an in-depth nutritional, chemical, and bioactive characterization of the obtained products was performed, complemented with their physical attributes, studied at Time 0 and 3 days of storage.

Lycopene and β-carotene were the major carotenoids found in tomato extracts. None of the studied samples showed hepatotoxicity (including the artificial colorant), and cherry tomato extract was the one with the highest antioxidant and antimicrobial activity.

All the creams coloured with the three tomato extracts revealed a uniform aspect and were stable in terms of structure and colour after the heat resistance test. The colour of the creams revealed no significant change over time; however, the pH levels suffered a growing tendency, except for the cream coloured with yellow pear tomato extract, which presented the most stable pH levels. As expected, the cream with the artificial colorant showed the best texture attributes, since the high quantity of carotenoid-rich extracts needed to achieved the final color lead to a weaker texture. Nevertheless, the cream coloured with cherry tomato extract presented the strongest cohesiveness and work of cohesion, while cream with yellow pear tomato presented the best firmness and consistency.

Cherry tomato coloured cream presented the highest amounts of protein, fat, and ash, as also higher energetic value. Three soluble sugars were found, fructose, glucose, and trehalose; and citric acid was the major organic acid detected. Saturated fatty acids were found in the highest percentages in all samples, mainly due to the presence of palmitic acid, being oleic acid the second most abundant fatty acid.

Finally, all creams revealed antioxidant properties, similar to the respective extracts, and no toxicity was observed for the VERO cell line. A significant improvement in the antimicrobial activity of the creams was verified, comparing with the extract, especially in the cream coloured with cherry tomato extract.

The colouring capacity of these molecules makes them a very attractive target for the industrial sector, since carotenoid-based colorants appear as a valid solution for application in the pastry sector, that greatly relies on yellow/orange artificial colorants.

Keywords: *Solanum lycopersicum* L., ultrasound-assisted extraction, HLPC-DAD-ESI/MS, carotenoid-rich extracts, pastry products.

III. RESUMO

A cor tem uma grande importância na primeira impressão do consumidor, permitindo-lhes inferior sobre a qualidade geral, o sabor, o cheiro e até mesmo sobre a segurança do alimento. Por estas razões, os corantes são massivamente utilizados em produtos alimentares. Entre as inúmeras matrizes naturais potencialmente utilizadas para a extração de compostos corantes, os frutos do género *Solanum* representam fontes promissoras de pigmentos como os carotenoides. Esta razão, juntamente com o facto de que grandes quantidades de resíduos de tomate fresco (resultantes da produção, embalamento, processamento, armazenamento e venda) são descartadas em todo o mundo, tornam a recuperação de biomoléculas corantes de alto valor acrescentado a partir de resíduos agroalimentares, uma etapa crucial da economia circular através da sua reintrodução na cadeia alimentar como ingredientes.

Neste trabalho, foram aplicadas metodologias de extração assistidas por ultrassons para obter extratos corantes ricos em carotenoides a partir de três variedades de tomate (tomate vermelho tradicional - *Solanum lycopersicum* L., tomate cereja – *S. lycopersicum* var. Cerasiforme e tomate pêra amarelo – *S. Lycopersum* 'Beam's Yellow Pear), usando solventes verdes, e HLPC-DAD-ESI/MS para estabelecer o perfil de carotenoides de cada amostra. Posteriormente, os extratos mais promissores e bioativos foram aplicados num creme de pasteleiro comercial de forma a substituir os corantes artificias atualmente usados. Finalmente, foi efetuado um estudo profundo de caracterização nutricional, química e bioativa, bem como dos parâmetros físicos dos cremes, avaliados no tempo 0 e após 3 dias de armazenamento.

O licopeno e o β-caroteno foram os principais carotenoides detetados nos extratos de tomate. Nenhuma das amostras estudadas apresentou hepatotoxicidade (incluindo o corante artificial), sendo o extrato de tomate cereja o que apresentou maior atividade antioxidante e antimicrobiana.

Todos os cremes corados com os três extratos de tomate revelaram um aspeto uniforme e estabilidade em termos de estrutura e de cor após o teste de resistência ao calor. A cor dos cremes não revelou nenhuma alteração significativa ao longo do tempo; no entanto, os níveis de pH sofreram tendência crescente, exceto para o creme corado com extrato de tomate pêra amarelo, que apresentou os níveis de pH mais estáveis. Como esperado, o creme preparado com o corante artificial apresentou os melhores atributos de textura, uma vez que a grande quantidade de extrato, rico em carotenoides, necessário

para atingir a cor final provoca alterações na textura. No entanto, o creme corado com extrato de tomate cereja apresentou maior coesividade e trabalho de coesão, enquanto o creme com tomate pêra amarelo apresentou melhor firmeza e consistência.

O creme com extrato de tomate cereja apresentou os teores mais elevados de proteína, gordura e cinzas, além de um valor energético superior. Três açúcares solúveis foram encontrados, frutose, glicose e trealose; e o ácido cítrico foi o principal ácido orgânico detetado. Os ácidos gordos saturados foram os mais abundantes em todas as amostras, principalmente devido à presença do ácido palmítico, sendo o ácido oleico o segundo ácido gordo mais abundante.

Por fim, todos os cremes revelaram propriedades antioxidantes semelhantes aos respetivos extratos, não sendo observada toxicidade para a linha celular VERO. Verificou-se uma melhoria significativa na atividade antimicrobiana dos cremes, em comparação com o extrato, principalmente no creme corado com extrato de tomate cereja.

A capacidade corante destas moléculas torna-as um alvo muito atrativo para o setor industrial, surgindo os corantes à base de carotenoides como uma solução válida para aplicação no setor da pastelaria, que depende muito dos corantes artificiais amarelo/laranja.

Palavras-chave: *Solanum lycopersicum* L., extração assistida por ultrassons, HLPC-DAD-ESI/MS, extratos ricos em carotenoides, produtos de pastelaria.

IV. INDEX

V. TABLE INDEX

VI. FIGURE INDEX

VII. LIST OF ABBREVIATIONS

AAPH	2,2'-azobis (2-amidinopropano)
AAS	Atomic absorption spectroscopy
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATCC	American type culture collection
CFU	Colony-forming unit
DAD	Diode array detector
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DPPH	2,2-difenil-1-picril-hidrazilo/2,2-Diphenyl-1-picrylhydrazyl
dw	Dry weight
IC_{50}	Effective concentration achieving 50% of antioxidant activity
ESI	Electrospray ionization
ex.	Example
FAME	Fatty acids methyl ester
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FID	Flame ionization detector
FL	Fluorescence
GC	Gas-chromatography
GI_{50}	Sample concentration that inhibited 50% of the net cell growth
HBSS	Hank’s balanced salt solution
HPLC	High-performance liquid chromatography
INT	*p*-Iodonitrotetrazolium chloride
IS	Internal standard
KOH	Potassium hydroxide
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass-to-charge ratio
MA	Malt agar

MBC	Minimum bactericidal concentration
MDA-TBA	Malondialdehyde-thiobarbituric acid
ME	Maceration extraction
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MTBE	Methyl tert-butyl ether
MS	Mass spectrometry
MS^2	Second stage of mass spectrometry
mu	Mass unit
MUFA	Monounsaturated fatty acids
NaOH	Sodium hydroxide
PDA	Photodiode array detector
PUFA	Polyunsaturated fatty acids
R^2	Coefficient of determination
RI	Refraction index
Rt	Retention time
OxHLIA	Oxidative haemolysis inhibition assay
SD	Standard deviation
SFA	Saturated fatty acids
SPSS	Statistical package for the social sciences
SRB	Sulphorhodamine B
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
tr	Traces
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSB	Tryptic soy broth
UAE	Ultrasound assisted extraction
UFLC	Ultra-fast liquid chromatography
UV	Ultraviolet radiation
v/v	Volumetric percentage
w/w	Percentage solution
λ_{max}	Wavelength of maximum absorption

INTRODUCTION

1 Introduction

1.1 Food colorants

The success or failure of a novel food product in the market can be highly conditioned by the first impression on the consumer's point of view, being the colour of that product the main criteria when it comes to accepting or rejecting it, since it can influence the idea of the composition of the product, but also the taste or even the smell (Lakshmi, 2014).

According to the FDA (Food and Drug Administration), a food colorant is "*any dye, pigment or substance which when added or applied to a food, drug or cosmetic, or to the human body, is capable (alone or through reactions with other substances) of imparting colour*" (Food and Drug Administration (FDA), 2018). The food industry offers the market a wide variety of colours, provided by artificial or natural pigments. In food products, these compounds find even more application precisely because the consumer will prefer to buy an enhanced brighter food than a darker natural one (due to oxygenation, air temperature, moisture, and storage conditions), considering both can be optimal for consumption (Spence, 2015).

The market of food additives has been greatly affected by major events like the progressive growth of processed food after World War II, which also led to an increase in the use of artificial colorants, either by the immense amount of new food products that have been emerging or as a marketing strategy to appeal to consumption (Solymosi, Latruffe, Morant-Manceau, & Schoefs, 2015).

However, the application of large amounts of these artificial pigments in foodstuff has been related to long-term health effects in the consumer, justifying the increasing awareness worldwide for the preference of natural-based food colorants. This tendency has directed the academia and the food industry for the development of novel natural-based colorant solutions to be applied in food (Martins, Roriz, Morales, Barros, & Ferreira, 2016). Food colour can be considered one of the most impressive and delightful attributes of foodstuff, being diverse colorants available as solid powders, liquid, gelatinous, or paste solutions (Martins et al., 2016).

In this sense, the present work aims to develop a novel carotenoid-based colorant extracted from tomato bio-residues, to be applied in a pastry product.

1.2 Artificial colorants versus natural colorants

Synthetic or artificial food colorants are among the most commonly used in food industry and, therefore, also the most studied in terms of health security. An artificial colorant is produced by chemical synthesis and/or chemical modification of several precursor compounds, being usually named after the main colour provided (König, 2015). There are six main classifications of artificial colorants (**Figure 1**), the azo, azo-pyrazolone, triarylmethane, canthene, quinolone, and indigoid (König, 2015). These colorants were developed after deep, time-consuming, and difficult scientific studies, along with marketing strategies to attract consumers. However, these colorants are also gaining the attention of food councils like EFSA (European Food Safety Authority) in Europe and FDA in the United States, for their hazardous health effects in humans, mainly in children (largest consumers of artificially coloured products), such as itching, urticaria, anaphylaxis, hypersensitivity intolerance reactions, but also mutagenic, carcinogenic, genotoxic, and cytotoxic effect (Amchova, Kotolova, & Ruda-Kucerova, 2015; Gautam, 2016).

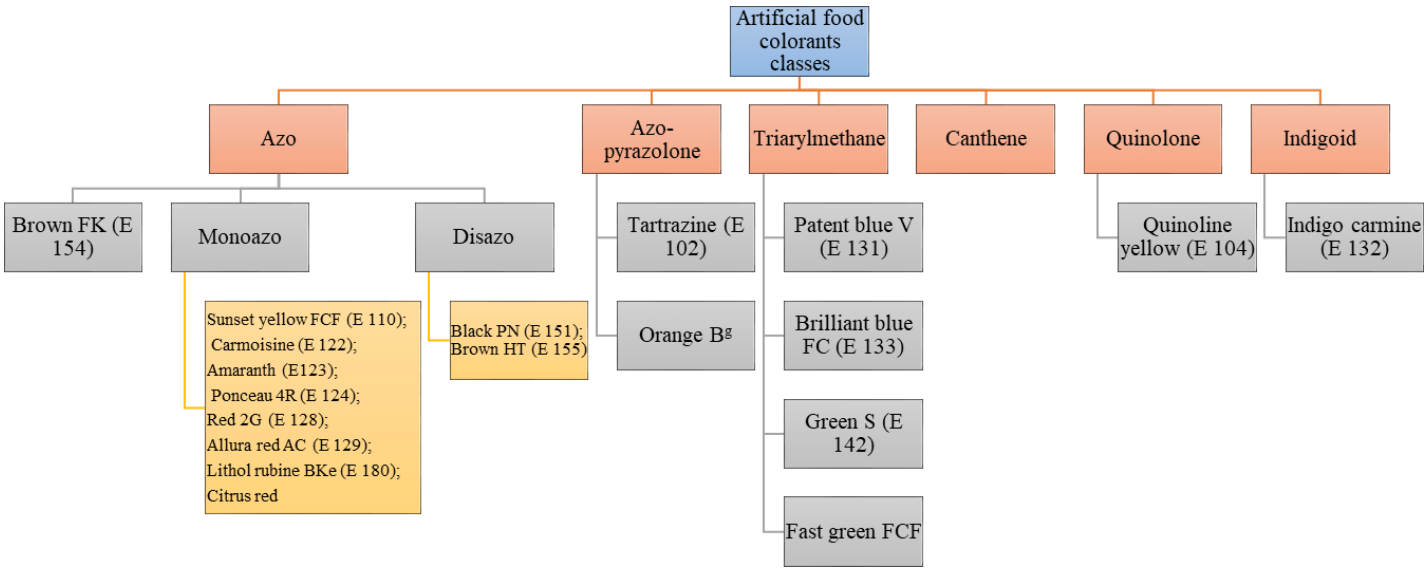


Figure 1. Six main classifications of artificial colorants approved for consumption in Europe (König, 2015).

On the other hand, a natural colorant is defined as a substance used for pigmentation, derived from a natural source and its obtaining does not involve chemical reactions/modifications (Solymosi et al., 2015). There are already natural colorants approved by the regulatory agencies to be used in several industrial fields, such as anthocyanins (E163, (EFSA, 2014), betalains (E162, (EFSA, 2015), caramel (E150, 150a,

E150b, E150c, and E150d, (EFSA, 2011), carminic acid (E120, (EFSA, 2016), carotenoids and astaxanthin (E160 and E161, (EFSA, 2010, 2012), and chlorophylls and their derivatives (E140 and E141, (EFSA, 2015c, 2015a) (**Table 1**) (Solymosi et al., 2015). These colorants have shown to be as effective as those derived from chemical synthesis, with the additional benefits of being safer for the consumer (without toxicity and long-term hazardous health effects), but also providing some bioactive properties, such as antioxidant, antimicrobial and anti-inflammatory, among others (Martins et al., 2016).

The underlying research for approval and regulation of the use of natural-based colorants for the cosmetic industry is reviewed every two years, while in the food industry 50% of the processed food recipes with natural colouring compounds are reviewed each year (Solymosi et al., 2015). The increasing demand for these type of natural products, combined with the consumer's concern and deeper knowledge, have been pressuring the industry and regulatory agencies to further investigate the beneficial and hazardous effects of these products (Salinas Moreno, Rubio Hernández, & Díaz Velázquez, 2010).

Carotenoids are among the natural pigments with greater potential to be used in several industrial fields. These colouring compounds present prominent colouring attributes, but also health-related benefits, which make them a subject of great interest, being the focus of this thesis.

1.3 Carotenoids

The name “Carotenoid” is derived from the name “carrots” (*Daucus carota* L.). They consist of a group of tetraterpenoid pigments that exist mainly in plants, fruits, and vegetables. Animals cannot synthesize carotenoids, being only acquired from their dietary intake as for the pink salmon flesh and some bird’s plumage reddish-yellow colour (Mortensen, 2006).

These compounds have been applied industrially in food, cosmetics, and pharmaceutical products and proved to have health benefits, such as cancer and macular degradation prevention (Young & Lowe, 2018). More than 600 different types of carotenoids have been identified and characterized, with the vast majority presenting the capacity to protect cells against oxidative damage and improving immunity and reactions against burns (Mezzomo & Ferreira, 2016; Young & Lowe, 2018).

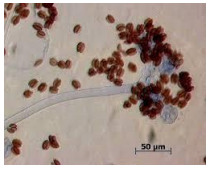
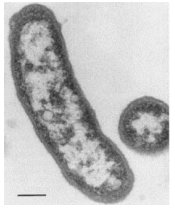
Carotenoids first description dates back to the early nineteenth century, in paprika,

saffron, annatto, and carrots (Maoka, 2020) where Karrer and Khun were the first to provide a structure elucidation description of *β*-carotene and lycopene in the 1930s (Maoka, 2020).

Their scientific research also led to the discovery of lutein, zeaxanthin, and astaxanthin (Britton, 2020). Throughout the century, the improvements performed in the techniques for structural elucidation and identification, such as High-Performance Liquid Chromatography (HPLC) coupled to mass spectrometry (MS), allowed the validation of the previous studies and the identification of new carotenoid structures (Maoka, 2020). The FDA (Food and Drug Administration) and FAO (Food and Agricultural Organization) permits the usage of carotenoids in food, such as *β*-carotene extracted from the vegetables *Blakeslea trispora* and *Dunaliella salina* (60a(i), 160a(ii), respectively) and carrot oil (160a(i)), lycopene from tomato extract (160d), capsanthin and capsorubin from paprika and paprika oleoresin (160c), and lutein from Tagetes (Aztec marigold) meal and extract (161b) (Cardoso et al., 2017).

Structurally, carotenoids are polyisoprenoid compounds synthesized by tail-to-tail linkage of two C_{20} geranylgenaryl molecules and are produced by variation of the parent C_{40} skeleton (**Figure 2**). It is possible to distinguish between the hydrocarbon carotenoids, named carotenes made only of C and H, and the oxidized carotenoids, named xanthophylls (oxycarotenoids) that present some *O*-substituent groups such as hydroxy, keto, and epoxy groups (Mortensen, 2006). Carotenoids present linear or cyclic groups, cyclohexane, and cyclopentane at the extremes of the molecule. These end-groups combining with the oxygen- containing functional groups and changes in the hydrogenation level allows the panoply of structures existing in this family of natural colorants (Oliver & Palou, 2000).

Table 1. Examples of natural colouring compounds approved to be used in Europe by EFSA.

Natural compounds	**Anthocyanins**	**Betalains**	**Caramel**	**Carminic acid**	**Carotenoids and astaxanthin**	**Chlorophylls and its derivatives**
E-code	E163	E162	E150, E150a, E150b, E150c, E150d	E120	E160 and E161	E140 and E141
Source	Pericarp of *Cyanococcus*	*Beta vulgaris L.* var. *ruba* roots	Heating of carbohydrates	Dried body of *Dactylopius coccus*	Mixed culture of two sexual mating types (+) and (-) of natural strains of the fungus *Blakeslea trispora* and dried cells of *Paracoccus carotinifaciens*	*Spinacia oleracea* leaves and copper salts added chlorophylls
					 	

β-carotene is mostly found in carrots, presents an orange-red colour and, since it is one of the most consumed carotenoids, it can be found in all human tissues, including blood. Due to its high bioactivity and relation to vitamin A it is widely used in medicine as a colouring pigment. It has been applied in non-alcoholic beverages, edible fats, cheese pastry products, and ice cream (Bogacz-Radomska & Harasym, 2018). However, more recent trials have not been conclusive in the evaluation of *β*-carotene supplementation effect on cardiovascular diseases and cancers, although presenting the capacity to decrease lung cancer incidents in smoker patients (Oliver & Palou, 2000).

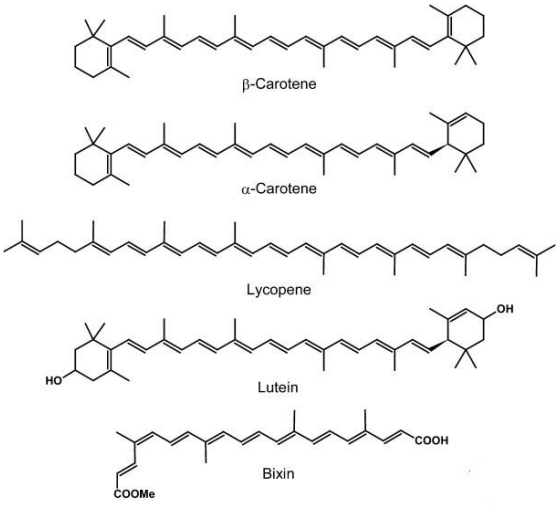


Figure 2. Chemical structure of the most common carotenoids in natural matrices (Mortensen, 2006)).

Lutein is a member of the xanthophyll family of carotenoids, mostly present through diet intake in human blood serum and macula (small area of the retina of the eye responsible for central visual and high visual acuity) (Abdel-Aal, Akhtar, Zaheer, & Ali, 2013). It has been proved that lutein can be a high efficient protective carotenoid against ultraviolet wavelengths, since it can delay a potential damage caused by light in the macula portion of the eye (Roberts, Green, & Lewis, 2009). Similar effects were also observed with zeaxanthin (Roberts et al., 2009). These two compounds, lutein and zeaxanthin, possess two hydroxyl groups, one on each side of the molecule, which are believed to play a critical role in their biological function. Similarly, these carotenoid compounds play a protective function in plants against photo- induced free radical damage (Alves-Rodrigues & Shao, 2004). Leafy green vegetables, such as spinach and kale, are rich in luteolin, presenting 12 mg of lutein in 100 g of spinach and up to 40 mg in 100 g of kale. Other foods with yellow colour, corn and egg yolks, also have these compounds in their

composition, but in lower amounts (Alves-Rodrigues & Shao, 2004).

Lycopene is another carotenoid compound that also presents antioxidant properties, being responsible for the majority of the red-coloured pigmentation in fruits and vegetables, such as tomato (Suwanaruang, 2016). In fact, combining these two factors: i) their strong antioxidant activity (oxidative stress is considered to be an important factor in the appearance of chronic diseases,) and ii) having tomato as their primary source (one of the largest bio- residues production industries in the world), lycopene has become one of the molecules with greatest interest in the scientific community for extraction and application in the food industry (Rao & Shen, 2002).

In **Table 2,** the content of the three previously mentioned carotenoids in kales, spinaches, and tomatoes are presented. As expected, tomato presents the highest content in lycopene, while kale and spinaches are richer in lutein and *β*-carotene.

Table 2. Carotenoid content in kale, spinach, and tomato (Alves-Rodrigues & Shao, 2004).

	Content (µg/100 g dry matter)		
Plant	***β*-carotene**	**Lycopene**	**Lutein**
Kale	9226	Not detected	39550
Spinach	5597	Not detected	11938
Tomato	393	3025	130

The pharmaceutical industry is also a frequent user of natural-based carotenoid solutions, mainly due to its anti-inflammatory, antioxidant, immunomodulatory, anticancer, and anti-obesity properties (Cardoso et al., 2017). The transportation of the carotenoids in the human organism is performed by lipoproteins in the intestinal mucosa to the blood vessels, and as such its functional properties are related to reactions like oxidation, reduction, and hydrogen abstraction (Cardoso et al., 2017).

However, there is a great disadvantage in terms of production and extraction yield of carotenoids. Regarding production, it is very dependent on edaphoclimatic conditions and seasonality of the raw material, involving high production costs. In terms of extraction yield, the mean value is around 0.004%, for example, through the processing of 50 kg of carrots, only 2 g of *β*-carotene in crystalline form is obtained. For these reasons, great efforts are being made to increase these compounds *in situ* (during the production of the raw plant material), but also new and more efficient extraction processes are being implemented to optimize the extraction conditions and increase the yield (Bogacz-

Radomska & Harasym, 2018).

1.4 Tomato: Botanical description and view as a source of carotenoids

The traditional red tomato (*Solanum lycopersicum* L.) is one of the most well-known and most important fruit vegetable in the world, having an annual production of 170 million Tons, occupying a total area of 5 million hectares (Umpiérrez et al., 2017).

Tomato is a versatile vegetable for culinary purpose, being one of the most preferred garden crops, after potatoes, and ranking the seventh most important crop species, after maize, rice, wheat, potatoes, soybeans, and cassava. Today, the tomato market is dominated by China followed by India, USA, Turkey, Egypt, Iran, Italy, Brazil, Spain, and Uzbekistan but also countries from Northern Europe, although the production being mostly made in controlled greenhouse conditions (Bergougnoux, 2014). This fruit can be consumed fresh and utilized in the manufacture of a wide range of processed products such as paste, powder, ketchup, sauce, soup, and canned whole fruits (Salim, Rashid, Hossain, & Zakaria, 2020).

From a botanical point of view, tomato is considered a fruit for being a part of a plant that develops from a flower's ovary. The flower, after fully growing, becomes the fleshy-fruit corresponding to the ovary and it is composed of an epidermis, a thick pericarp, and a placental tissue surrounding the seeds (**Figure 3A**). Usually, it takes around 2 to 3 weeks for the flower to develop, followed by an intensive mitotic division activity lasting for 2 weeks approximately, then going through a process of cell expansion and finally the phase of ripening and maturation (**Figure 3B**).

Tomato is predominantly rich in lycopene, presenting also in its composition luteolin and *β*-carotene, although in much lower amount (Martí, Roselló, & Cebolla-Cornejo, 2016). The described amount of lycopene in tomatoes greatly differentiates it from other plant matrices since it ranges between 7.8 and 18.1 mg per 1 g of fresh weighted tomato, representing up to 71% of the total carotenoid content of tomato, as it can be seen in **Table 3** (Martí et al., 2016). Some studies report that the increased consumption of tomato and tomato-based products can potentially reduce the risk of certain type of cancers such as prostate and stomach cancer, due to the anti-carcinogenic activity of lycopene (Lin & Chen, 2003).

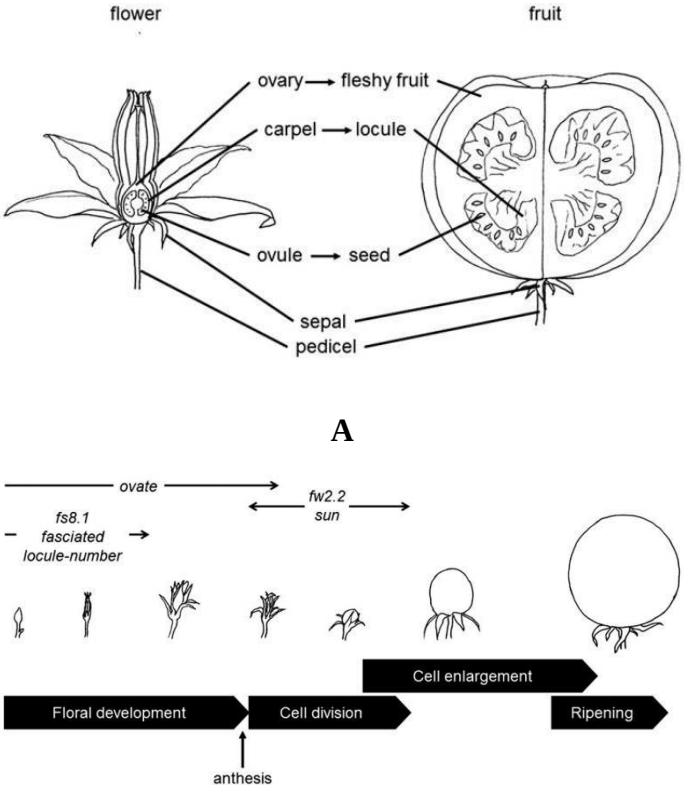


Figure 3. Botanical anatomy of tomato and its flower (**A**) and scheme of tomato flower development until the ripening of the fruit (**B**) (Bergougnoux, 2014).

The storage conditions of tomato have proven to influence the content of lycopene in the final product that goes to the market: temperatures of storage ranging from 15 to 25 °C can increase lycopene content and, consequently, also increase some biological activities, such as antioxidant (Toor & Savage, 2006).

Table 3. Carotenoid composition (mg/100 g fresh weight) in *S. lycopersicum* (Martí et al., 2016).

Carotenoid		Concentration ranges
Lycopene		7.8 - 18.1
Phytoene		1.0 - 2.9
	Phytofluene	0.2 - 1.6
	***β*-Carotene**	0.1 - 1.2
	***γ*-Carotene**	0.05 - 0.3
	***δ*-Carotene**	0 - 0.03
Lutein		0.09
	Neurosporene	0 - 0.03
	***α*-Carotene**	0 - 0.002

Not only the storage conditions influence the chemical composition of tomato, but also the edaphoclimatic, production, and processing conditions. The ripening state is also a parameter that influences the content of carotenoid compounds, since it is characterized by the softening of the fruit and the degradation of chlorophylls, that will provide the fruit with a more intense red colour and increased respiration rate and synthesis of acids, sugars, and lycopene (Hernández Suárez, Rodríguez Rodríguez, & Díaz Romero, 2008).

Tomato is not only a source of carotenoids but also nutrients, vitamin C (ascorbic acid) and minerals, such as potassium. It presents low lipid content and moderate sugar concentration (Bergougnoux, 2014).

The botanical variety of tomato is immense, existing different kinds of varieties of tomatoes, such as cherry tomato (*S. lycopersicum* var. *cerasiforme*, **Figure 4**), which is a smaller variant of the more well-known classic tomato, measuring between 1.5-3.5 cm in diameter, similar to the size of a golf ball.

Figure 4. *S. lycopersicum* var. cerasiforme (cherry tomato).

In terms of carotenoid concentration, cherry tomato is not as rich as other types of tomato, like purple tomato (*S. lycopersicum L.* cv. *Micro-Tom*). Though the abundance of lycopene is also observed in cherry tomato, it was verified that the carotenoid concentration was mostly higher in the pulp than in the peel, 11.17 mg per 1 g of dry weight and 8.47 mg per 1 g of dry weight, respectively (Campestrini et al., 2019).

Other varieties of tomato, like yellow pear tomato (*S. lycopersum* 'Beam's Yellow Pear), a yellow variant of the traditional red tomato, stand out due to its bright yellow colour and different shape. Some studies showed that the yellow pear appears to have very similar *β*- carotene and lycopene content than other red tomatoes (Emmanuel, Olugboyega, John, Adenike, & Edith, 2018). It is believed that this colour is a result of a

mutation of one of the genes of the normal tomato, where the production of lycopene is blocked, preventing the fruit from turning red (Do Rêgo, Finger, Casali, & Cardoso, 1999). Since these yellow variants are not as well studied as their red counterparts, it is of great interest to invest on experiments on yellow pear tomatoes.

1.5 Using carotenoids as a base for food colorants

1.5.1 Carotenoid extraction techniques

There are a lot of techniques that can be applied for the extraction of carotenoid molecules. All of them present advantages and disadvantages, mainly due to the special characteristics of carotenoids in terms of polarity and propensity to degradation. The Soxhlet extraction is one of the most commonly used, for being a simple and conventional method, but it can cause thermal degradation and *cis-trans* isomerization of carotenoids (Macías-Sánchez, Fernandez-Sevilla, Fernández, García, & Grima, 2010), being also very time and solvent- consuming (Campestrini et al., 2019). Microwave-assisted extraction (MAE) is a straightforward, quick, and economic method for the extraction of carotenoids, but, as the Soxhlet extraction, it can cause thermal degradation of the carotenoid molecules (Cardenas-Toro et al., 2015; Ho, Ferruzzi, Liceaga, & San Martín-González, 2015).

Pulsed electric field (PEF) extraction can also be used to obtain high extraction yields, being a non-thermal process that uses very low energy. However, the PEF parameters may differ, causing changes in the electrical conductivity of the sample (Gómez et al., 2019). As for the enzyme-assisted extraction (EAE), it represents a rapid and efficient extraction method with minimal employment of solvents, but with costs associated to enzymes use (Nadar, Rao, & Rathod, 2018). Nonetheless, this methodology has already been used for the obtaining of carotenoids from the hexane oil extract of marigold flower (*Tagetes erecta* L.) from Mexico (Barzana et al., 2002).

On the other hand, ultrasound-assisted extraction (UAE) technique has proven to be one of the most reliable methodologies to obtain carotenoid-rich extracts. It is being applied in numerous extraction procedures for different types of compounds in different types of matrices. This technique has shown to be the most cost-efficient in comparison with other techniques, some studies show that parameters such as solvent/CDW ratio, power, pulse and time are to be taken into consideration when working with carotenoid

extracts (Singh, Barrow, Mathur, Tuli, & Puri, 2015). UAE is usually applied in a solid/fluid mixture, being the fluid either a gas or a liquid, and the extraction enhancement is linked to the propagation of ultrasound pressure waves, resulting in the so called cavitation phenomena (Esclapez, García-Pérez, Mulet, & Cárcel, 2011; Vilkhu, Mawson, Simons, & Bates, 2008) The high shear forces formed in the implosion of the cavitation bubbles cause an increased mass transfer, with acceleration of the eddy and internal diffusion, and consequently surface peeling, erosion, and particle breakdown, that expose even more the inner content of the cells, further increasing the mass transfer (Vilkhu et al., 2008), and thus resulting in higher yields of extraction. Since heat generation is conducted in this method, albeit not as impactful, molecules prone to degradation in high temperatures, such as carotenoids, are easier to extract using this type of technology.

However, it is necessary to compare the results obtained from these new extraction technologies with more conventional ones (such as maceration assisted extractions – ME) in order to provide an insight of the real impact of such technologies (Esclapez et al., 2011).

ME is the most widely employed methodology for the extraction of non-volatile compounds, with simple and economic protocols. However, this technique is time-consuming, with labour-intensive procedures depending on the type of targeted compounds and extended concentration steps, also involving large volumes of hazardous solvents. These issues have been limiting its implementation at industrial scale and also rise safety and security problems if applied for the food, cosmetic, and pharmaceutical industries (Lima, Nunes, & Block, 2020; Radojković et al., 2016).

In **Table 4**, some examples of maceration and ultrasound assisted extraction studies for the obtaining of carotenoids in different plant matrices are described. As it is possible to observe in ME studies, the time used for the extraction procedures in very long. Twenty-four hours of extraction were required in *Morus nigra* L. and *Morus alba* L. leaves to obtain 22.42±0.08 g/100g and 23.40±0.09 g/100g of carotenoids, respectively (Radojković et al., 2016). In *S. lycopersicum* L. bio-residues, seven days of extraction were applied, using different oils in order to obtain a range of concentrations between 1.6±0.1 and 6.1±0.4 mg of carotenoids/Kg of dry plant (Nour, Corbu, Rotaru, Karageorgou, & Lalas, 2018). If we compare these latest results in tomato with the one obtained by the same author using UAE during 50 minutes, the concentrations obtained were ten times higher, with a concentration range of 16.7±0.5 to 50.6±2.8 of carotenoids/Kg of dry plant (Nour et al., 2018).

Table 4. Examples of maceration and ultrasound assisted extraction studies for the obtaining of carotenoids from different plant matrices.

Species	Part of the plant studied	Solvent used	Extraction conditions	Quantification	Reference
MAE					
Morus nigra L. *Morus alba* L.	Leaf	70% ethanol	T: 30°C; t: 24h	22.42±0.08 g/100g 23.40±0.09 g/100g	(Radojković et al., 2016)
Citrus sinensis cv. 'Valencia'	Endocarp	Ethyl acetate-methanol mixtures	T: room temperature; 2h	[124.68±3.32 to 140.45±4.96] µg/g	(Petry & Mercadante, 2018)
S. lycopersicum L.	Bio-residues	Different oils	T: 20°C; t: 7 days	[1.6±0.1 to 6.1±0.4] mg/Kg	(Nour et al., 2018)
Psidium guajava L. cv. 'Pedro Sato	Pulp and waste	Celite and ethyl acetate	T: 25°C; t: 1 h	[50 to 140] mg of *β*-carotene/100g	(Lima et al., 2020)
UAE					
Dacus carota L.	Root	Ethanol (100%)	T: 30°C; t: 30 min , 100 W	83.32% yield of extraction	(Purohit & Gogate, 2015)
Dacus carota L.	Root	Hexane (100%)	22.5 W/cm^2, T: 40°C; t: 20 min	334.75 mg of *β*-carotene/l	(Li, Fabiano-Tixier, Tomao, Cravotto, & Chemat, 2013)
S. lycopersicum L.	Bio-residues	Different oils	T: 20°C; t: 50 min	[16.7±0.5 to 50.6±2.8] mg/Kg	(Nour et al., 2018)
S. lycopersicum L.	Bio-residues	Hexane:ethanol:acetone (2:1:1 *v/v*), containing 0.05 % (*w/v*) BHT (butylat-edhydroxytoluene	T: 20, 40, and 60 °C; t:10, 20, 30, and 40 min	[39.5±0.04 to 90.1±0.76] mg of lycopene/kg	(Civan & Kumcuoglu, 2019)
Citrus reticulate Blanco	Epicarp flour	Sunflower oil	T: 60°C; t: 60 min; P: 240 W	140.70±2.66 mg of *β*-carotene/100 g	(Ordóñez-Santos, Esparza-Estrada, & Vanegas-Mahecha, 2020)
Passiflora edulis Curtis	Peal	Olive oil/Sunflower oil	T: 47°C; t: 39 min; P: 100 W	1235.047 and 1176.195 µg/100 g	(Chutia & Mahanta, 2020)
Mangifera indica L.	Pulp juice	-	T: 25°C; t: 15-30 min; P: 30 W	[48.92±1.32 to 89.53±1.82] µg/100 ml	(Santhirasegaram, Razali, & Somasundram, 2013)

Civan & Kumcuoglu (2019) also used UAE methodologies to obtain carotenoids from tomato, using different solvents (hexane, ethanol, and acetone) in very small extraction times, and obtained an impressive 39.5±0.04 to 90.1±0.76 mg of lycopene/kg of dry plant. These excellent results are mainly due to the type of solvents used, with very high affinity for carotenoid molecules; however, the use of these type of solvents is limited in the food industry due to the hazardous effects for human consumption, except for ethanol, which application is considered safe in specific concentrations.

1.5.2 Stability of carotenoid-based natural extracts

As already referred in this work, the use of natural-based colouring extracts has been increasingly explored in various industrial sectors. The lack of toxicity combined with the functional properties of these natural extracts make them very appealing for the industry and consumers. However, the stability issues of these natural molecules, regarding presence/absence of light, pH and temperature conditions, and oxygen concentrations, are considered a disadvantage compared to artificial counterparts. It becomes, then, essential to identify the mechanisms of carotenoid degradation in each product/process/storage/application (Boon, McClements, Weiss, & Decker, 2010a; Chen, Peng, & Chen, 1996).

The presence or absence of light is effectively a parameter that highly influences the stability of carotenoids, where 24 hours of exposure to light could decrease 90.2% of the total carotenoid content, and after 216 hours, an additional 49.6% (Kadian, Sharma, & Sood, 2013). The thermal stability is also a concern, specially to β-carotene-rich extracts, since this compound is more prone to degradation, while luteolin proved to be more resistant (Kadian et al., 2013).

Temperatures of 35°C with the presence of light are enough to reduce the lutein, α-carotene, β- carotene, and vitamin A contents on carrot juice (Chen et al., 1996). The maturity/ripening state of the plant and its geographical origin are also parameters to be taken into account, specially the maturity of the product, due to metabolic activity of ethylene (occurring during ripening) and chlorophylls and chloroplasts rupture (development of chromoplasts), that leads to a higher concentration of carotenoids and also its preservation (Carrillo-Lopez & Yahia, 2010; Dragovic-Uzelac, Levaj, Mrkic, Bursac, & Boras, 2007).

To overcome these stability limitations, many of the works developed with new

carotenoid-rich natural extracts have resorted to the use of emulsion systems. The use of emulsions protects the extracts, within a lipid capsule, physically and against oxidation processes (Boon et al., 2010a; Linke & Drusch, 2018) There are several advantages in the use of emulsions to protect carotenoid molecules, namely: i) creation of physical or electrostatic barriers for iron and other prooxidants that are common in the aqueous components of food; ii) easy to incorporate with lower costs associated; iii) retention of the bioactive properties of the molecules (Boon et al., 2010a; Linke & Drusch, 2018). Nonetheless, environmental stresses commonly found in food processing can lead to the instability of these emulsions (Boon et al., 2010a; Linke & Drusch, 2018).

1.6 Applying carotenoid-based colorants in food products

The development of new natural colouring solutions rich in carotenoids has as main objective their application in food and cosmetic products, as it has been verified with the use of *β*-carotene, astaxanthin, canthaxanthin, lycopene, and lutein (J. Singh, Jayaprakasha, & Patil, 2017). There are numerous studies being conducted over the past few years for that same purpose. In the impossibility of summarizing all of these studies in the present seminar, some examples are pointed out below:

i) in Asia, the red pigments extracted from red koji rice are being used as food colorants in wine, red soy cheddar, and meat (Dufossé et al., 2005);

ii) *Brevibacterium* carotenoids were applied in the French cheddar named "*vieux-dish*", providing it with a characteristic orange-red-brown shading, increasing its sensory quality and organoleptic acceptance by the consumers (Galaup et al., 2015);

iii) Baby formulas products are enriched with natural pigments (lutein in bosom milk) as a functionalizing agent to improve children's health in Russia (Kon, Gmoshinskaya, Safronova, Alarcon, & Vandenplas, 2014);

iv) Fucoxanthin carotenoid from the palatable ocean *Undaria pinnatifida* (wakame), in Japan, is being incorporated in pastry and pasta, with great acceptance among the local population (Prabhasankar et al., 2009);

v) Carotene derivatives and lycopene extracted from carrots and tomatoes are being applied in food products to provide them orange-red colour, namely pastry products, ice cream, processed tomato fruits, and some non-alcoholic drinks (Bogacz-Radomska & Harasym, 2018).

2 Objectives

The main objective of the present work was to explore *Lycopersum* carotenoid-rich colouring extracts to be applied in a commercialized pastry cream in order to substitute the currently used artificial colorants.

2.1 Specific objectives

i) Prospection of natural sources of carotenoids, focused on bio-residues from the production of three *Lycopersum* genus fruits.

ii) Extraction of the carotenoids through ultrasound assisted (*US*) extraction and analysis of the individual profile in carotenoids of each sample by liquid chromatography coupled to a diode array detector and mass spectrometry equipped with an electrospray ionization source (HPLC-DAD/ESI-MSn).

iii) Application of the developed colorants in a pastry cream produced by the company "TecPan" and stability assessment in the conditions to which the cream is usually submitted.

iv) Determination of the nutritional value and chemical composition of the developed products, as also evaluation of the bioactive properties of the colouring extracts and developed products.

MATERIAL AND METHODS

3 Material and Methods

3.1 Standards and reagents

The standard reference mixture of fatty acid methyl ester FAME) 37 (pattern 47885-U) was acquired in the company Sigma-Aldrich (St. Louis, MO, USA), as also sugars (D(+)-sucrose, D(+)-fructose, D(+)- trehalose), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and organic acid patterns (oxalic acid, quinic acid, malic acid, citric acid, fumaric acid, and succinic acid). For the oxidative haemolysis assays, the reagent 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was acquired in Sigma-Aldrich (St. Louis, MO, EUA). For the hepatotoxicity assays, fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640, and DMEM media were from Hyclone (Logan, UT, USA) were used. Acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA), phosphatebuffered saline (PBS), and Tris were acquired from Sigma. Methanol, ethanol and all other solvents and reagents were acquired from scientific retailers. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

3.2 Prospection of natural sources of carotenoids

As previously stated in the introduction section, the traditional red tomato (*S. lycopersicum* L.) is one of the most well-known and most consumed fruit vegetable among the *Solanum* species (Umpiérrez et al., 2017), having been selected for this study as a source of carotenoids for the further development of an innovative food product. A local tomato producer (Bragança region, Portugal) was contacted to supply the raw material and, from his production, three kinds of tomatoes were selected: the traditional red tomato (*S. lycopersicum* L.), cherry tomato (*S. lycopersicum* var. *cerasiforme*), and yellow pear tomato (*S. lycopersum* 'Beam's Yellow Pear), codified as "RT", "CT", and "YT", respectively (**Figure 5)**. The fruits were harvested in the end of September of 2020, in an advanced state of ripeness, not ideal for consumption. These fruits were considered bio-residues since they would have only been used by the producer to fertilize the land for the following year's production.

After collection, the samples were separated by genus (RT, CT, and YT), washed in tap water, weighed, frozen, and further lyophilized until complete dryness. Then, all samples were reduced to a fine powder (approximately 35 mesh) using a knife mill (model A327R1, Moulinex, Madrid, Spain), stored at room temperature, and protected from light until further use.

A **B** **C**

Figure 5. Tomatoes collected from a local producer: **A** – RT: traditional red tomato (*S. lycopersicum* L.); **B** - CT: cherry tomato (*S. lycopersicum* var. cerasiforme); and **C** – YT: yellow pear tomato (*S. lycopersum* 'Beam's Yellow Pear).

3.3 Carotenoids' extraction, identification by HPLC-DAD/ESI-MSn, and bioactive properties

*3.3.1 Ultrasound assisted extraction (*UAE*)*

For ultrasound-assisted extraction (UAE), the samples were subjected to 500 W of ultrasonic power, for 10 min at room temperature, avoiding light exposure, employing ethanol (90%) and hexane (100%) as extraction solvents. The extraction was performed using an ultrasonic system (**Figure 6A**, Ultrasonic homogenizer, model CY-500, Optic Ivymen Systen, Barcelona, Spain) equipped with a titanium probe, according to a previously described methodology (Rocha et al., 2020). Briefly, 1 g of sample was placed in a beaker with 30 mL of solvent. After extraction, the mixture was centrifuged at 4000 *g* for 10 min. The supernatant was filtered through Whatman filter paper No. 4 and evaporated for complete dryness of the solvents under vacuum at 50 °C using a rotary evaporator (**Figure 6B**, Buchi, 3000 series, Flawil, Switzerland). Of the obtained extract, 10 mg were weighed and dissolved in 1 mL of the corresponding extraction solvent (10 mg/mL) and filtered again though a 0.22 μm nylon filter to an amber injection vial (1.5

mL) for further HPLC analysis.

The hexane extracts were only used on the carotenoid identification assay, the following methodologies instead used only hydroethanolic compounds.

A **B**

Figure 6. Ultrasound chamber (**A**) and rotary evaporator (**B**).

3.3.2 Carotenoids' identification by HPLC-DAD/ESI-MSn

The extracts obtained were analyzed for their carotenoids' composition, by High Performance Liquid Chromatography (HPLC; Dionex Ultimate 3000 UPLC and Linear Ion Trap LTQ XL, Thermo Scientific, San Jose, CA, USA) coupled to a diode array detector (DAD) and an electrospray ionization source (ESI) coupled to a mass spectrometer (MS). The system was equipped with a quaternary pump, an auto-sampler (kept at 5 ºC), a degasser and an automated thermostatized column compartment. The chromatographic separation was achieved with Synergy 4u Hydro-RP (4.6 x 250 mm, 4 μm; Phenomenex, Canada) column thermostatized at 30 ºC. The solvents used were: (A) 100 % methanol:butylated hydroxytoluene (BHT, 0.1%, 10 mg/mL) and (B) 100% methyl tert-butyl ether (MTBE):BHT (0.1%, 10 mg/mL). The established elution gradient was isocratic: 5% B (5 min), 5% to 30% B (15 min), 30 to 40% B (10 min), 40 to 50% B (10 min), 50 to 60% B (10 min), 60 to 65% B (5 min), and re-equilibration of the column (5 min), using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 430, 470, and 640 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in positive mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source.

Nitrogen served as the sheath gas (50 psi) and the system was operated with a spray voltage of 5 kV, a source temperature of 350 ºC, and a capillary voltage of -15V. The tube lens offset was kept at a voltage of -6 V. The full scan covered the mass range from *m/z* 100 to 1500. The collision energy used was 35 (arbitrary units). Data acquisition was carried out with Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA).

The identification was performed by comparison of the chromatographic characteristics of the detected compounds with available authentic standards or tentatively identified comparing the obtained data with the reported in literature.

3.3.3 Evaluation of the bioactive properties of the carotenoid-rich extracts

3.3.3.1 In vitro antioxidant properties: Inhibition of lipid peroxidation through thiobarbituric acid's reactive products (TBARS) and Oxidative hemolysis inhibition (OxHLIA)

For the analysis of antioxidant activity using the TBARS method, the protocol described by Sarmento, Barros, Fernandes, Carvalho, and Ferreira (2015) was used. The lyophilized hydroethanolic extracts were re-dissolved in ethanol:water (80:20, *v/v*) to obtain a 0.5 mg/mL stock solution, diluting successively to obtain six concentrations below it. In parallel, a pig brain suspension (*Sus scrofa*) was prepared, in which a portion of the brain was added together with Tris-HCl buffer (20 mM, pH 7.4) in a ratio of 1:2 (*m/v*), centrifuging the mixture at 3500 rpm for 10 min at a temperature of 10 ºC to avoid rancidification of the mixture. In eppendorf tubes, 200 µL of each of the solutions of hydroethanolic extract were placed, to which 100 µL of ascorbic acid (0.1 mM), 100 µL of iron sulfate ($FeSO_4$ - 10 mM), and 100 µL of the supernatant homogenized from pig brain were added and incubated at 37.5 ºC for 1 hour. After incubation, 500 µL of trichloroacetic acid (28% *m/v*) was added to stop the reaction and 380 µL of thiobarbituric acid (2% *w/v*, TBA) was also added. The tubes were placed in a water bath at 80 ºC for 20 minutes, in order to promote the reaction between TBA and malondialdehyde (MDA - reactive oxygen species resulting from lipid peroxidation that occurs in pig brain tissue). Subsequently, the mixture was centrifuged at 3500 rpm for 5 min, to separate the residues from the supernatant. The colour intensity of the MDA - TBA complex was measured at 532 nm. The percentage of inhibition of lipid peroxidation was calculated using the following equation:

% inhibition of lipid peroxidation = (A - B) / A x 100

where A and B refer to the absorbance of the control (water) and the extract solution, respectively. The extract concentration corresponding to 50% inhibition of lipid peroxidation (IC_{50}) was calculated from the graph of the percentage of inhibition of TBARS formation as a function of the extract concentration. As a positive control, Trolox was used and the results were expressed in µg/ml.

The antihaemolytic activity was assessed using the OxHLIA assay previously described by Lockowandt et al. (2019). Aliquots of sheep's blood were collected from healthy animals and centrifuged at 1000 rpm for 5 min at a temperature of 10 ºC to obtain the erythrocytes (the plasma and buffy coat were discarded). The erythrocytes were first washed with NaCl (150 mM) and three times with phosphate buffered saline (PBS, pH 7.4). The erythrocyte pellet was then re-suspended in PBS 2.8% (*v/v*).

In a 48-well flat bottom plate, 200 µL of the erythrocyte solution was mixed with 400 µL of PBS solution (negative control), of antioxidant samples dissolved in PBS or water (for complete haemolysis). Trolox was used as a positive control. The plates were pre-incubated at 37 ºC for 10 min with shaking, followed by the addition of 2,2'-Azobis dihydrochloride (2-amidinopropane) (AAPH, 160 mM in PBS, 200 µL) and, then, incubated in the same conditions. The optical density was measured at 690 nm at 10 min intervals for about 400 min (Takebayashi, Iwahashi, Ishimi, & Tai, 2012). The percentage of the erythrocyte population that remains intact (P) was calculated according to the following equation:

P (%) = (St - CH0 / S0 - CH0) × 100

where St and S0 correspond to the optical density of the sample at times t and 0 min, respectively, and CH0 is the optical density of complete haemolysis at 0 min. The results were expressed as haemolysis delay time (Δt), which was calculated according to the following equation:

Δt (min) = Ht50 (sample) - Ht50 (control) (5)

where Ht50 corresponds to the haemolytic time of 50% (min) obtained graphically from the haemolysis curve of each concentration of antioxidant sample. The Δt values were subsequently linearly correlated with the different sample concentrations, from which the concentration with the ability to delay haemolysis by 60 min (IC_{50} (60 min), µg/mL) was calculated.

3.3.3.2 Hepatotoxic activity

One non-tumor cell line was tested: Vero (African green monkey kidney), The cells were maintained in DMEM medium supplemented with fetal bovine serum (10%), glutamine, and antibiotics. The culture flasks were incubated in an incubator at 37ºC and with 5% CO_2, under a humid atmosphere. The cells were used only when they had 70 to 80% confluence. A known mass of each of the extracts (8 mg) was dissolved in H_2O (1 mL), in order to obtain the stock solutions with a concentration of 8 mg/mL, from which successive dilutions were made, obtaining the concentrations to be tested (0.125 - 8 mg/mL). Each of the extract concentrations (10 µL) were incubated with the cell suspension (190 µL) of the cell lines tested in 96-well microplates for 72 hours. The microplates were incubated at 37ºC and with 5% CO_2, in a humid atmosphere, after checking the adherence of the cells. A density of 19,000 cells/well was used. After the incubation period, the cells were corrected: TCA (10% w/v; 100 µL) was previously cooled and plates were incubated for 1 hour at 4ºC, washed with water and, after drying, a SRB solution (0.057%, m/v; 100 µL) was added, left to stand at room temperature for 30 minutes. To remove non-adhered SRB, plates were washed three times with a solution of acetic acid (1% v/v) and placed to dry. Finally, an adhered SRB was solubilized with Tris (10 mM, 200 µL) and the absorbance at a wavelength of 540 nm was read in the Biotek ELX800 microplate reader. The results were expressed in terms of extract concentration with the ability to inhibit cell growth by 50% - (µg/mL) GI_{50}. Ellipticin was used as a positive control.

3.3.3.3 Antibacterial activity - Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Food contaminants (bacteria): The extracts were tested against five Gram-negative bacteria, namely *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp (ATCC 13076), and *Yersinia enterocolitica* (ATCC 8610) and three Gram-positive bacteria, namely *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), and *Staphylococcus aureus* (ATCC 25923). All these microorganisms were purchase at Frilabo, Porto, Portugal. The bacteria were incubated at 37 ºC in an appropriate fresh medium, for 24 h

before analysis to maintain the exponential growth phase.

Clinical bacteria: The bacterial strains were clinical isolates obtained from patients hospitalized in various departments at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Five Gram-negative bacteria (*Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Morganella morganii*) and three Gram-positive bacteria (*Enterococcus faecalis, Listeria monocytogenes,* and methicillin-resistant *Staphylococcus aureus (MRSA*)) were tested.

The MIC determination in all bacteria were conducted using a colorimetric assay, according to the described by Pires et al, (2018). The samples were, first of all, dissolved in 5% (v/v) dimethyl sulfoxide (DMSO) and 95% of autoclaved distilled water to give a final concentration of 20 mg/ mL for the stock solution. 90 μL of this concentration was added in the first well (96-well microplate) in duplicate with 100 μL of Tryptic Soy Broth (TSB). In the remaining wells, 90 μL of TSB medium was added. Then, the samples were serially diluted to obtain the concentration ranges from 10 to 0.03125 mg/mL. To finish, 10 μL of inoculum (standardized at 1.5×10^6 Colony Forming Unit (CFU) /mL) was added to all the wells assuring the presence of 1.5×10^5 CFU. Two negative controls were prepared, one with TSB and another one with the extract. Two positive controls were prepared: with TSB and each inoculum and another with medium, antibiotics, and bacteria. Ampicillin and Streptomicin were used for all bacteria tested and Meticilin was also used for *Staphylococcus aureus.* The microplates were incubated at 37°C for 24 h. The MIC of samples was detected following the addition (40 μl) of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT) and incubation at 37°C for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth, determinated by the change of the coloration from yellow to pink if the microorganisms are viable. For the determination of MBC, 10 μL of liquid from each well that showed no change in colour was plated on solid medium, Blood agar (7% sheep blood) and incubated at 37°C for 24 h. The lowest concentration that yielded no growth, determine the MBC. MBC was defined as the lowest concentration required to kill bacteria.

3.3.3.4 Antifungal activity - Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The antifungal activity was performed according to the described by Heleno et al., (2013). *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC

16404) were used. The organisms were obtained from Frilabo, Porto, Portugal. The micromycetes were maintained on malt agar and the cultures stored at 4 °C, being further placed in new medium and incubated at 25ºC for 72h. In order to investigate the antifungal activity, the fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µL per well. The samples were first of all dissolved in 5% (v/v) dimethyl sulfoxide (DMSO) and 95% of autoclaved distilled water to give a final concentration of 20 mg/ mL for the stock solution. Afterwards, 90 µL of this concentration was added in the first well (96-well microplate) in duplicate with 100 µL of Malt Extract Broth (MEB).

In the remaining wells, 90 µL of medium MEB were placed. Then, the samples were serially diluted to obtain the concentration range of 10 to 0.03125 mg/mL. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microplate. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentration (MFC) was determined by serial subcultivation of a 2 µL of tested compounds dissolved in medium and inoculated for 72 h, into microplates containing 100 µL of MEB per well and further incubation 72 h at 26 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Commercial fungicide ketoconazole (Frilabo, Porto, Portugal), was used as positive control.

3.4 Development of the pastry cream

One of the most important objectives of this work was the development of a new natural colouring solution rich in carotenoids to replace artificial colorants used by the food industry. To fulfill this objective, a semi-complete powder product for the preparation of non-coloured pastry creams (commercial name CREMIBOM extra) was kindly provided by Tecpan - Tecnologia e Produtos P/ Pastelaria E Panificação Lda. company (located in Mirandela, district of Bragança, Portugal). This product is usually sold with artificial colorants (patented mixture) to companies dedicated to pastry confection, which only have to add water and sugar (if necessary) to obtain a yellow cream, mimicking the more traditional egg cream. The ingredients, method of use, and recipe are described in **Table 4.**

Five different creams were developed: i) without colorant (C-wc); ii) with

artificial colorants mixture used by the company (C-ac); iii) with traditional red yellow tomato extract (C-RTe); iv) with cherry tomato extract (C-CTe); v) and with yellow tomato extract (C-YTe). The extracts selected were the hydroethanolic extracts obtained in **section 3.3.1** of this thesis. For the cream preparation (~ 900 g), the base recipe was followed (without the sugar) as well as the method of use. The concentration of the artificial colorant was the same used by the company (4 g/Kg of dried powder). Regarding the concentration of the carotenoid-rich solutions, initial tests were performed to try to achieve a similar colour to the artificial colorant. This was not possible since the brightness presented by the artificial colorant could not by achieved by the natural counterparts.

Table 4. Label description of the semi-complete powder product used for the preparation of non-coloured pastry creams.

Ingredients	Sugar Modified starch Dairy solids Vegetable fat Thickener (E401) Stabilizers (E450 and E516) Salt Preservatives (E202) Flavour Colorant Mix (4 g/Kg of dried powder) Allergens (Dairy derivatives) It may also contain traces of gluten, egg powder or sesame seeds-based products, sulfur dioxide, and sulfites.
Recipe	CREMIBOM extra 1Kg Sugar 1 Kg Water 3L
Method of use	Mix the powder with the sugar. Pour the mixture over the water and beat at fast speed for approximately 3 minutes. Wait some time before using.

Therefore, extract concentrations were tested to resemble the colour of a traditional egg cream as close as possible, reaching a maximum concentration (from which there were no significant differences in the cream) of 80 g/Kg of dried powder. Additional extractions

were performed as described in **section 3.3.1** to obtain the necessary quantity of extract. The dissolution of the extract was made in the water used for cream preparation. Immediately after the preparation, the cream samples were divided into four groups (**Figure 8**):

i) the first group was used for the heat resistance test, carried out in the fresh cream immediately after preparation, placing a small sample of the creams in an oven at 100ºC for 5 minutes to check its cohesiveness and colour maintenance after baking;

ii) the second group, also consisting of fresh cream immediately after preparation, was used to evaluate the physical properties of colour, pH, and texture;

iii) in the third group, the sample was weighed, frozen, lyophilized, and reduced to a fine powder (35 mesh) for further analysis of its nutritional, chemical, and bioactive properties (this group corresponded to Time 0); and finally

iv) in the fourth group the sample was stored in polyethylene bags, suitable for food storage, and placed in a refrigeration chamber at 4ºC for three days, after which the sample was evaluated for its physical properties (colour, pH, and texture).

3.4.1 Physical properties

As described above and outlined in **Figure 8**, all physical properties tests were carried out on the fresh cream immediately after preparation (Time 0 days) and after storage under refrigeration at 4°C (Time 3 days).

3.4.1.1 Colour

To measure the colour of the five pastry creams prepared, a colorimeter (model CR-400; Konica Minolta Sensing, Inc., Tokyo, Japan) attached to an adapter for granular materials (model CRA50) was used, following the methodology previously described by Roriz, Barros, Prieto, Morales, & Ferreira (2017) (**Figure 7**). The value of the three-dimensional coordinates CIE *L**, *a**, and *b**, were obtained in a computerized system with a type C illuminant and an 8 mm diameter diaphragm, for data processing, Spectra Magic Nx software (version CMS100W 2.03.0006, Konica Minolta, Japan) was used. Regarding the coordinates obtained, *L** represents luminosity, *a** represents chromaticity in an axis from green (-) to red (+), and *b** represents chromaticity on an axis from blue (-) to yellow (+). For colour evaluation of the creams, the readings were made at 3

different points for each portion (the cream was placed on a watch glass in order to obtain a uniform and wide surface with three possible reading points), considering the average values. Before and after analysis, the instrument used was calibrated with a white standard (Spectra Magic NX Instruction Manual, Konica Minolta Sensing, Inc., 2009, Japan).

Figure 7. Colorimeter (model CR-400; Konica Minolta Sensing, Inc., Tokyo, Japan).

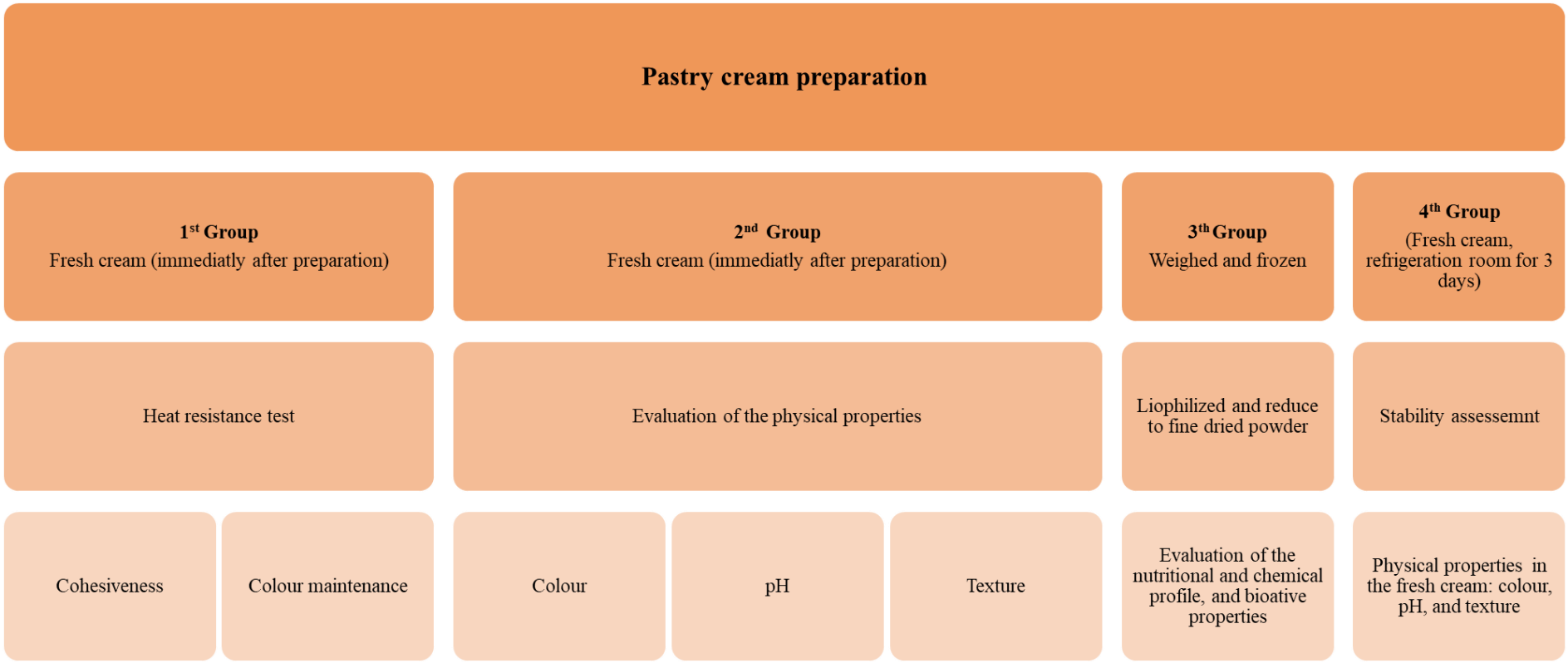
3.4.1.2 pH

The pH was measured using a calibrated digital pH meter (portable food and dairy pH meter HI 99161, Hanna Instruments, Woonsocket, RI, USA). The pH measurement was carried out in three different points of each fresh cream.

3.4.1.3 Texture

The texture analysis of the pastry cream was performed by a "backward extrusion" with the use of a TA.TXplus texture analyser (Stable Microsystems, Surrey, UK) running the "Texture Expert" software, also using a pressure cell of 50 kg. Penetration tests were made in *Measure Force in Compression* mode at 20 mm of depth, using a 45 mm diameter perspex disc and a 45 mm high cup. The type of test was compression moved at a steady speed of 4 mm/s, between the lowering of the cell (1^{st} cycle) and the rising of the cell (2^{nd} cycle) there was a pause for 5 s. After the analysis, a macro was used to measure various parameters of texture, namely firmness, consistency, cohesiveness, and work of cohesion The texture results were reached through the Exponent program, proprietary of Stable Micro Systems.

Figure 8. Schematic representation of the analyses carried out in all cream formulations developed at Time 0 days and Time 3 days.



3.4.2 Nutritional composition of the pastry creams

As described above and outlined in **Figure 8**, the nutritional composition analysis was carried out on the fresh cream immediately after preparation (Time 0 days) and after storage under refrigeration at 4°C (Time 3 days). For this, the protein, total fat, ash, and carbohydrates content, and energy value were determined using the official methods of analyse described by AOAC (2016).

3.4.2.1 Protein and moisture content

The protein content (P (%)) was determined by using the macro-Kjeldahl technique (AOAC 978.04), which allows the quantification of the crude protein content based on the nitrogen content present in the sample. Using 1 g of lyophilized pastry cream sample, 15 mL of concentrated sulphuric acid and two Kjetabs tablets (5 g of potassium sulfate and 0.5 g of copper II sulfate, catalysts) were added for this process. The tubes were placed in the digester (FossTM Digester) at a temperature of 400 °C for 70 minutes in vacuum. Subsequently, after cooling down the sample, 25 mL of water was added and it was transferred to the macro-Kjeldahl equipment (Pro-Nitro A, JP Selecta, Barcelona, Spain), which automatically carried out a distillation. The nitrogen content (mg) measured by the equipment was multiplied by the factor of correction (6,25) allowing for the calculation of the protein content in g/100 g of dry sample. The moisture content was obtained by subtracting the weight of the non-lyophilized sample with the weight of the same sample lyophilized, then dividing by the former weight.

3.4.2.2 Total fat

The total fat was determined by extracting a known mass from the sample (3g), using a Soxhlet apparatus, with petroleum ether, for approximately 8 hours (between 16 to 20 turns in the Soxhlet apparatus, **Figure 9**), at a temperature of about 60 ºC (AOAC 920.85). The volatile fraction was evaporated and the lipid fraction was placed in an oven until constant weight. The results were expressed in g/100 g dry weight.

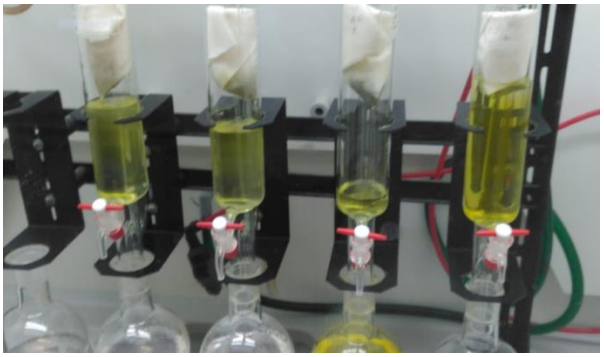


Figure 9. Soxhlet apparatus used for extraction of the total fat.

3.4.2.3 Ash and carbohydrate content and energy value

The ash content was determined by incinerating about 0.5 g of sample in a muffle at 600 ºC (AOAC 923.03) and, finally, the carbohydrate content was calculated by difference of all the parameters obtained previously. In both, the results were expressed in g/100 g dry weight. The energy value was calculated as described by Regulation (EC) No 1169/2011, (2011) and according to the equation:

Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat)

3.4.3 Chemical composition of the pastry creams

As described above and outlined in **Figure 8**, the chemical composition analysis was carried out on the fresh cream immediately after preparation (Time 0 days) and after storage under refrigeration at 4°C (Time 3 days). Different chromatographic methodologies were performed in order to evaluate the content in free sugars and organic acids, and fatty acid profile.

3.4.3.1 Free sugars

From the sample used for Soxhlet extraction (in which the lipid fraction was removed), approximately 1 g of mass was weighed and 1 ml of internal standard solution (melezitose, 25 mg /ml) was added. Subsequently, extraction was carried out, in which 40 mL of 80% ethanol was added to the sample, in a water bath at 80 ºC for 1 hour and 30 min, with alternating agitation every 15 minutes. Subsequently, the samples were centrifuged (K24OR refrigerated centrifuge, Centurion, West Sussex, United Kingdom) for 10 min at 3500 rpm, filtered, and the supernatant transferred to a glass flask in which

the ethanol was evaporated at 50 ºC under reduced pressure (Büchi R-210 rotary evaporator, Flawil, Switzerland). The water obtained after evaporation of the supernatant, was then subjected to a process to remove the remaining lipids, where it was washed three times with 10 ml of ethyl ether. The samples were, then, placed at 40 ºC to remove residual ethyl ether, and the residue was dissolved in distilled water to be measured at 5 mL and, later, filtered through 0.2 μm nylon filters for vials for later analysis of the sugar profile in the HPLC system.

Chromatographic determination of free sugars was performed by high-performance liquid chromatography coupled to a refractive index detector (HPLC-RI, Knauer, Smartline 1000 system, Berlin, Germany), as described by the authors Barros et al. (2013). The HPLC system (Knauer, Smartline system) used is equipped with an IR detector (Knauer Smartline 2300) and a 100-5 NH_2 Eurospher column (4.6 × 250 mm, 5 μm, Knauer). The mobile phase used was acetonitrile/deionized water, 70:30 (*v/v*) with a flow rate of 1 mL/min in isocratic mode. The identification of sugars was performed by comparing the relative retention times of the sample peaks with authentic standards (fructose (y = 1.04*x*. R^2 = 0.999; LOD = 0.05 mg/mL; LOQ = 0.18 mg/mL); glucose (y = 0.935*x*. R^2 = 0.999; LOD = 0.08 mg/mL; LOQ = 0.25 mg/mL); and trehalose (y = 0.991*x*. R^2 = 0.999; LOD = 0.07 mg/mL; LOQ = 0.24 mg/mL) and the quantification performed by internal normalization of the peak chromatographic area using the melezitose peak (PI) as a standard. The results were expressed in g per 100 g of dry sample weight.

3.4.3.2 Organic acids

Approximately 1g of the lyophilized creams was weighed into a glass beaker protected from light (previously wrapped in aluminium foil) and 25 ml of metaphosphoric acid (4.5%, *v/v*) was added in a concentration of 10 mg/mL, at a room temperature of about 25 ° C. The mixture was placed under magnetic stirring for 20 minutes and then filtered into a 20 ml test tube. For chromatographic analysis, the samples were filtered into an amber vial (1.5 mL) through a 0.2 μm nylon filter for further analysis on HPLC.

The chromatographic determination of the profile in organic acids was performed by ultra-fast liquid chromatography coupled to a diode detector (UFLC-PDA; Shimadzu Coperation, Kyoto, Japan) as previously described by Barros et al. (2013). The separation of the compounds was carried out through a C_{18} reverse phase column (250 mm x 4.6 mm, 5 μm, Phenomenex), heated at 35 ºC. The detection occurred through a diode array

detector (DAD) programmed to acquire the predefined wavelengths of 215 nm and 245 nm. The elution solvent used in isocratic mode was sulphuric acid (3.6 mM, H_2SO_4). The identification of organic acids and their quantification was determined by comparing retention times and UV spectra with commercial standards. Quantification was performed by comparing the peak area at the programmed wavelength with the corresponding commercial standard, using 7-level calibration lines of: oxalic acid ($y = 1\times10^7x + 231891$; $R^2 = 0.9999$; LOD = 6.3 µg/mL; LOQ = 20.8 µg/mL); quinic acid ($y = 671557x + 14583$; $R^2 = 0.9998$; LOD = 11.3 µg/mL; LOQ = 37.6 µg/mL); malic acid ($y = 950041x + 6255.6$; $R^2 = 0.9999$; LOD = 15.9 µg/mL; LOQ = 52.9 µg/mL); citric acid ($y = 1\times10^6x - 10277$; $R^2 = 0.9997$; LOD = 4.4 µg/mL; LOQ = 14.5 µg/mL); fumaric acid ($y = 154862x + 1\times10^6$; $R^2 = 0.9977$; LOD = 42.5 µg/mL; LOQ = 141.7 µg/mL); succinic acid ($y = 640365x - 17602$; $R^2 = 0.9995$; LOD = 6.3 µg/mL; LOQ = 20.8 µg/mL). The results were expressed in g per 100 g of dry sample weight.

3.4.3.3 Fatty acids

To determine the fatty acid profile, the lipid extract obtained after Soxhlet extraction (**see section 3.4.3.3)** was subjected to a derivatization process to obtain the volatile fatty acids, called FAME (Fatty acid methyl ester). For this, to the lipid extract was added 5 mL of a 2:1:1 (*v/v/v*) methanol/sulphuric acid/toluene solution, and placed in a water bath at 50 °C for 12 hours with stirring 160 revolutions per minute (rpm). After which, in order to enhance the phase separation, 3 ml of distilled water and 3 ml of ethyl ether were added in which the FAME was dissolved. To remove any water residue, the supernatant was placed in contact with a small portion of anhydrous sodium sulphate, recovering the sample and filtering it (0.2 µm nylon filter) into a vial with a Teflon membrane cover. Fatty acids were determined by gas chromatography coupled to a flame ionization detector (GC-FID, instrument DANI model GC 1000, Milan, Italy), as described by Barros et al. (2013).

The identification of the fatty acid profile was done through a GC system (Model DANI GC 1000) equipped with a split/splitless injector, flame ionization detector (FID, 260 °C) and a Macherey Nagel column (30 m × 0.32 mm × 0.25 µm). The oven was programmed with the following parameters: the initial temperature of the column was 100 ºC, for 2 min; then, the temperature was increased at 10 ºC/min up to 140 ºC, 3 ºC/min up to 190 ºC, 30 ºC/min up to 260 ºC for 2 min. Hydrogen (carrier gas) had a flow rate of

4.0 ml/min (0.61 bar), measured at 50 °C. The split injection (1:50) was performed at 250 ºC. For each analysis, 1 µL of the sample was injected. The identification of fatty acids was made based on the relative retention times of the peaks of the standard mixture of 37 FAMEs and the samples. For the processing of the results, the software program Clarity 4.0.1.7 (DataApex, Podohradska, Czech Republic) was used, and the results were expressed as a relative percentage (%) of each fatty acid detected.

3.4.4 Bioactive properties of the pastry creams

As described above and outlined in **Figure 8**, the bioactive properties analysis was carried out on the fresh cream immediately after preparation (Time 0 days) and after storage under refrigeration at 4°C (Time 3 days). Hydroethanolic extracts of the pastry cream samples were prepared, using 1 g of the lyophilized sample powder and stirring with 30 mL of ethanol/water (80:20 *v/v*) at room temperature and 150 rpm, for 1 h. The extract was filtered through Whatman filter paper no. 4. The residue was re-extracted once more under the same conditions. Afterwards, the combined extracts were evaporated under vacuum (rotary evaporator Büchi R-210, Flawil, Switzerland) and further lyophilized. The lyophilized extracts were then stored protected from light and humidity for further analysis. The antioxidant, hepatotoxic, antibacterial, and antifungal activities tests were performed accordingly with the protocols described in **sub-topic 3.3.3** (**3.3.3.1**, **3.3.3.2**, **3.3.3.3**, and **3.3.3.4**, respectively).

3.5 Statistical analysis

For each analysis with the dry samples and the respective hydroethanolic extracts, all tests were performed in triplicate, the results being expressed as mean values and standard deviation (SD). Significant differences between samples were analysed using Student's t-test with $\alpha = 0.05$, using IBM SPSS Statistics for Windows, Version 23.0. (IBM Corp., Armonk, New York, USA).

RESULTS AND DISCUSSION

.

4 Results and discussion

4.1 Profile of the carotenoid-rich extracts and corresponding bioactive properties

4.1.1 Chromatographic identification of carotenoids by HPLC-DAD-ESI/MSn

The carotenoid profile of the hydroethanolic and hexane extracts from red tomato, cherry tomato, and yellow tomato is presented in **Table 5**. An exemplificative carotenoid profile from red tomato hexane extract is represented in **Figure 10**. Two carotenoids were found in the samples, lycopene ($[H]^+$ at *m/z* 537) and β-carotene ($[H]^+$ at *m/z* 537), and identified by comparing their retention time, wavelengths of maximum absorption, and protonated ion with available standard compounds.

There are several studies regarding the identification of these two compounds in *S. lycopersum* samples, being also described as the major carotenoids found in tomato samples (Daood et al., 2021; Gentili et al., 2015; Van Meulebroek, Vanden Bussche, Steppe, & Vanhaecke, 2014).

Unfortunately, it was not possible to present the quantification results of the samples due to the restrictions caused by the Covid-19 pandemic, which limited the time available in the laboratory, but also to serious technical problems with the HPLC-MS equipment, therefore not allowing the presentation of concentrations in carotenoids in the extracts under study.

For the application in pastry creams (**section 4.2.**), hydroethanolic extracts were used, as this work aimed to find a substitute for the commonly used hexane solvent for carotenoids extraction, and also because ethanol is allowed for food purposes in the European Union.

Table 5.Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectrometric data in positive mode, and identification of the carotenoids present in the hydroethanolic and hexane extracts of tomato samples.

Peak	Rt (min)	λmax (nm)	[H]+ (m/z)	Identification
1	8.2	420/441/470	537	Lycopene
2	32.06	423/450/476	537	β-carotene

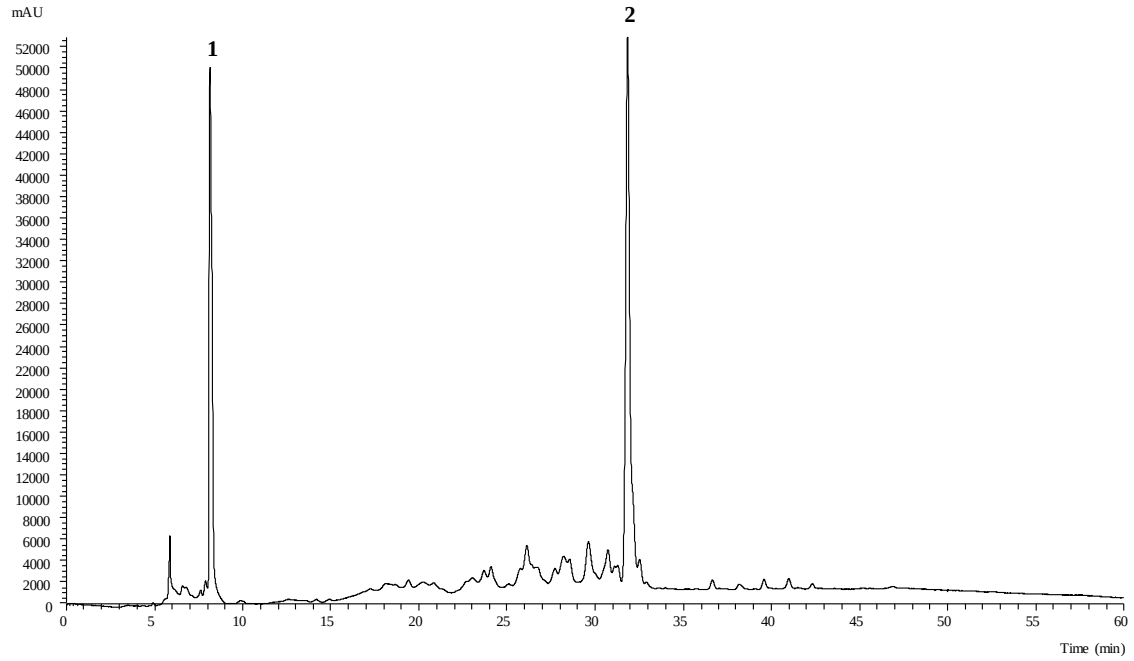


Figure 10. Exemplificative carotenoid profile obtained in the hexane extract of RT sample recorded at 430 nm.

4.1.2 Bioactive properties of the carotenoid-rich extracts

4.1.2.1 Antioxidant and hepatotoxic activities

Data regarding the antioxidant activity of the carotenoid-rich extracts is described in **Table 6**. The IC_{50} value reveals the amount of extract capable to inhibit 50% of the lipid peroxidation, in the case of TBARS assay, or the haemolysis of the blood cells, in OxHLIA case. As such, the lowest the concentration, the highest the antioxidant capacity of the extracts. As it is possible to observe, CT sample revealed the lowest IC_{50} values for both assays, 0.31±0.01 mg/mL and 0.18±0.01 mg/mL for TBARS and OxHLIA, respectively. On the other hand, the artificial colorant, despite presenting activity, revealed the highest values for TBARS and OxHLIA assays (7.9±0.1 and 0.62±0.05, respectively), which can lead to a first conclusion, that its use on food products does not infer an interesting functionality to the final product. The results obtained for the three species of tomato under study in TBARS assay, are in accordance with the previously obtained by Pinela et al., (2019), in eighteen different Portuguese species and varieties of tomato conserved *ex vitro* in a germplasm bank. Also, for OxHLIA assay with the obtained in *Lycopersicon esculentum* Mill bio-residues from different origins (Pinela et al., 2017).

When compared to the positive control, Trolox, the results are not very encouraging. However, non-purified natural extracts could have a series of compounds that may be influencing their bioactive action, or even have another type of synergistic behaviour when incorporated into a food product.

None of the studied samples showed activity at the maximum tested concentration for VERO cell lines, thus not presenting hepatotoxicity (including the artificial colorant), which reveals their potential to be incorporated in a food product. However, it is important to state that the toxicity of artificial food colorants is not limited to the liver, but has a wide range of cellular targets, such as skin cells or nervous system cells (Sunday N. Okafor et al., 2016), and therefore, with only one assay it is not safe to validate the total and absolute safety of the artificial colorant under study.

Table 6. Antioxidant activity of the hydroethanolic carotenoid-rich extracts (mean ± SD).

	Samples				**Controls**	
	AC	**RT**	**CT**	**YT**	**Trolox**	**Ellipticine**
Antioxidant activity (IC_{50} values, mg/mL)						
TBARS	7.9±0.1[a]	1.61±0.01[c]	0.31±0.01[d]	1.9±0.3[b]	0.14±0.01	-
OxHLIA Δ*t* = 60 min	0.62±0.05[a]	0.401±0.027[b]	0.18±0.01[d]	0.36±0.01[c]	0.0218±0.0002	-

AC – artificial colorant; RT - traditional red tomato (*S. lycopersicum* L.); CT - cherry tomato (*S. lycopersicum* var. cerasiforme); and YT - yellow pear tomato (*S. lycopersum* 'Beam's Yellow Pear). In each row, different letters mean significant differences between species ($p < 0.05$).

4.1.2.2 Antibacterial and antifungal activities

The results of the antimicrobial activity are described in **Figure 11** and expressed in minimal inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC). It is noticeable that the artificial colorant present antimicrobial activity against all Gram-negative bacteria and fungal strains, and two Gram-positive bacteria (*L. monocytogenes* and *S. aureus*). Since it is not possible to reveal the chemical composition of the artificial colorant, no cause-effect statements will be made on the results obtained for this sample.

As for the tomato extracts, RT and YT got very similar MIC results against *S. enterocolitica* (5 mg/mL), *Y. enterocolitica* (2.5 mg/mL), *S. aureus* (2.5 mg/mL), *A. brasiliensis*, and *A. fumigatus* (10 mg/mL). CT sample also presented similar results against the same microbial strains, except for *Y. enterocolitica* (1.25 mg/mL); but most

importantly, it presented activity against all microbial strains tested (despite in a relatively high concentration).

None of the samples presented bactericidal or fungicidal activity, which are typical results in clinically isolated bacteria, as they have a long history of susceptibility and resistance to antibiotics, and for this reason are not susceptible to low concentrations of non-purified compounds.

Szabo et al. (2019) revealed that methanolic extracts of a variety of tomato peels have an effectiveness against Gram-positive bacteria such as *S. aureus*, where MIC values showed to be between 2.5 and 5 mg/mL, which is in accordance with the present case study.

Results and Discussion

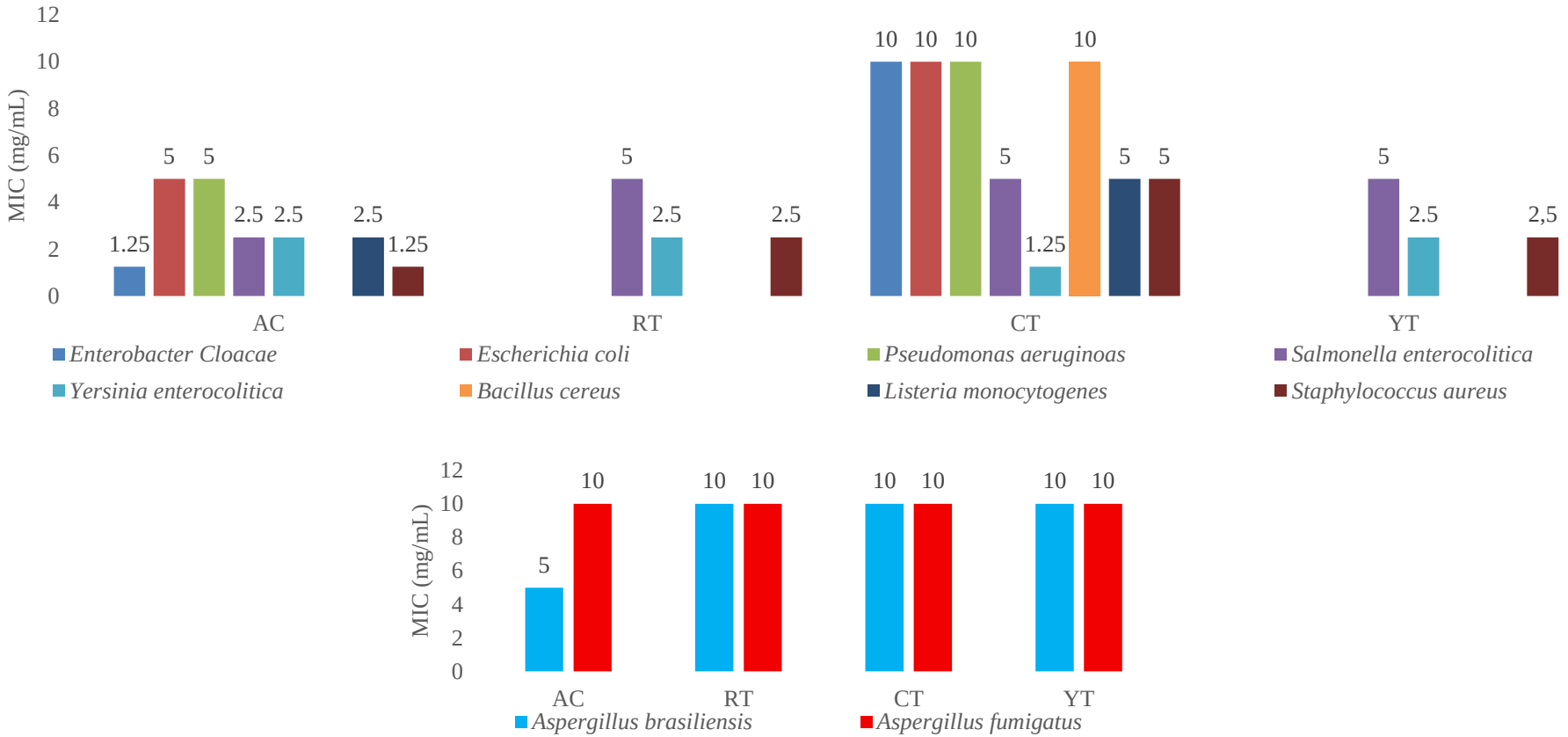


Figure 11. Antibacterial and antifungal MIC values of carotenoid-rich extracts and AC. MIC- minimum inhibitory concentration; AC – Artificial colorant; RT - traditional red tomato (*S. lycopersicum* L.); CT - cherry tomato (*S. lycopersicum* var. cerasiforme); and YT - yellow pear tomato (*S. lycopersum* 'Beam's Yellow Pear). Missing columns represent MIC values > 10.

4.2 Pastry cream physical characteristics, nutritional and chemical composition, and bioactive properties

In this section, all the results obtained for the developed pastry creams will be presented. As previously described in the ”Material and Methods” section, the brightness of the artificial colorant could not be achieved by the natural extracts. As such, the pastry creams were developed to resemble the colour of a traditional egg cream as closely as possible.

In **Figure 12**, it is possible to observe the five pastry creams developed, being noticeable the brightness of the artificial colorant in **Figure 12B**. The pastry cream developed with yellow tomato extract (C-YTe, **Figure 12E**) presented, to the naked eye, a much lighter and more beige colour than the intended, even at the highest concentration tested. However, the creams developed with traditional red yellow tomato extract (C-RTe, **Figure 12C**) and cherry tomato extract (C-CTe, **Figure 12D**), again to the naked eye, presented a soft yellow egg yolk colour, very similar to egg creams marketed in traditional pastries. It should be noted, however, that all the creams developed with the carotenoid-rich solutions were very uniform, showing no traces of undissolved extract. This means that despite the more polar nature of the molecules, it is possible to obtain an extract that is soluble in aqueous-base matrices, which is a determining factor for the formulation of new colorant solutions for marketing purposes.

After the cream preparation, a heat resistance test was carried out, placing a small amount of the creams in an oven at 100^{o}C for 5 minutes. The structure of the cream was not affected by the heat, remaining cohesive and with the structure intact after cooking. The cream colour also did not change after baking in all samples. This fact is extremely important, as the well-known instability or thermal degradation of carotenoids (Boon, McClements, Weiss, & Decker, 2010b), but their incorporation in the pastry cream somehow protected them from degradation, functioning as a kind of encapsulation of the carotenoid molecules.

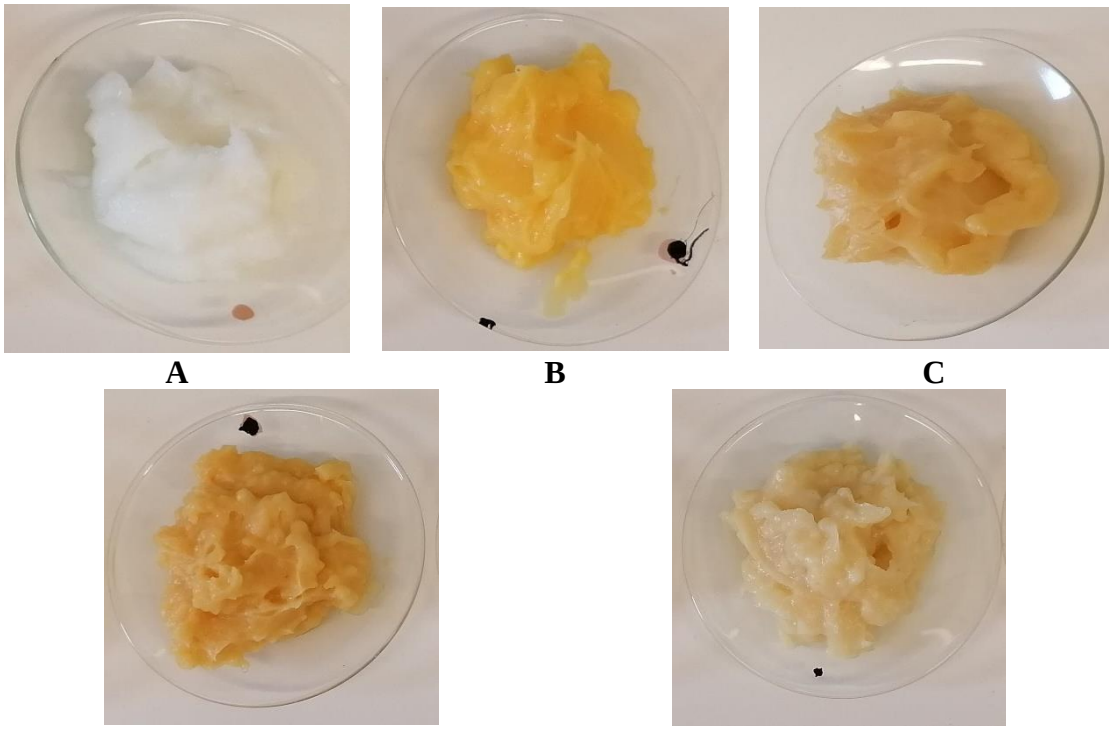


Figure 12. Pastry cream developed: **A**- without colouring (C-wc); **B** - with artificial colorant mixture used by the company (C-ac); **C** - with traditional red yellow tomato extract (C-RTe); **D** - with cherry tomato extract (C-CTe); and **E** - with yellow tomato extract (C-YTe).

4.2.1 Physical properties

The colour of the pastry cream is a very important aspect for the scope of this thesis, as previously mentioned in **Section 1.1**. The results obtained in the colorimeter, expressed in *L**, *a**, and *b** at Time 0 and Time 3 days, are represented in **Table 7**. For a better visualization of the colour changes over the storage time, **Table 8** shows the colours obtained after converting the *L**, *a**, and *b** parameters, into RGB code using the online converter: https://www.easyrgb.com/en/convert.php#inputFORM.

The C-wc sample presented the highest *L** and the lowest *a** and *b** values (95.29±0.5, -2.02±0.04, and 9.87±0.029, respectively). These results were expected since it is the cream with no colorant added, and so the values are very close to the ones obtained in the calibration of the equipment. After 3 days of storage (**Table 8**), there were no significant changes in the parameters, although the brightness of the cream changed, with a decrease of *L** (93.09±0.598).

The C-ac cream presented the highest *b** value (80.09±0.830), increasing after 3 days (83.13±0.549), making it the most yellowish cream of the five. This was an expected result since it is composed by chemical molecules not so susceptible to degradation as the

natural counterparts. Analysing C-RTe sample, it was possible to observe that, after three days, the majority of the parameters stayed the same except for the *L** values, which raised slightly, making it appear clearer.

All creams with natural colorants tend to lose some of their coloration after being stored, which is possibly due to the nature of carotenoids that tend to degrade over time (Boon et al., 2010b); nevertheless, to the naked eye, there were no significant changes in the creams colours.

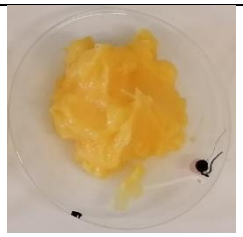
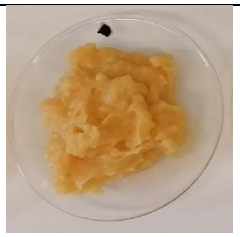
Table 7. Colour parameters of *b*:* blueness (–) to yellowness (+); *a*:* greenness (–) to redness (+); and *L*:* darkness (0) to lightness (100) in the five pastry creams developed at Time 0 days and Time 3 days.

	Time 0 days			**Time 3 days**		
	L*	***a****	***b****	***L****	***a****	***b****
C-wc	95.29±0.5^{a}	-2.02±0.04^{e}	9.87±0.029^{e}	93.09±0.598^{a}	-2.48±0.067^{e}	11.65±0.345^{e}
C-ar	84.76±0.494^{c}	5.26±0.136^{b}	80.09±0.830^{a}	85.14±0.234^{b}	5.61±0.112^{b}	83.13±0.549^{a}
C-RTe	85.34±0.129^{b}	5.33±0.290^{c}	63.93±2.178^{c}	83.7±0.05^{d}	3.66±0.079^{c}	60.31±0.688^{c}
C-CTe	80.27±0.667^{d}	11.09±0.380^{a}	71.84±2.398^{b}	80.15±0.378^{e}	7.59±0.384^{a}	68.7±1.315^{b}
C-YTe	81.28±0.431^{e}	-0.67±0.285^{d}	42.64±2.701^{d}	84.43±0.832^{c}	-1.05±0.413^{d}	42.92±0.75^{d}

Cream without colorant (C-wc); Cream with artificial colorants mixture used by the company (C-ac); Cream with traditional red yellow tomato extract (C-RTe); Cream with cherry tomato extract (C-CTe); Cream with yellow tomato extract (C-YTe). In each column, different letters mean significant differences between species ($p < 0.05$).

The pH level was determined before and after the heat resistance test, and after 3 days of storage (**Table 9**). The initial pH of C-wc was 6.87 and it lowered to 6.73 after the oven testing; then, it raised until it almost reached the original value. C-ac had the same behaviour, except that it preserved the same pH value even after heat treatment. Both C-RTe and C-CTe had lower pH than C-wc and C-ac with 5.1 until the heat treatment, where it lowered very slightly. After storage, a difference was observed, C-RTe raised its pH more than C-CTe, which made the latter have a similar behaviour to C-ac.

Table 8. Colour representation of the creams at Time 0 days and Time 3 days, using RGB scale from 0 to 255.

Samples		Time 0 days	Time 3 days
C-wc		**R: 246** **G: 242** **B: 222**	**R: 240** **G: 236** **B: 213**
C-ac		**R: 255** **G: 205** **B:39**	**R: 248** **G:203** **B:91**
C-RTe		**R: 255** **G:207** **B:87**	**R:248** **G: 203** **B: 91**
C-CTe		**R: 252** **G:189** **B:53**	**R: 245** **G:191** **B:61**
C-YTe		**R: 227** **G:200** **B:121**	**R: 236** **G:209** **B:129**

Cream without colouring (C-wc); Cream with artificial colorant mixture used by the company (C-ac); Cream with traditional red yellow tomato extract (C-RTe); Cream with cherry tomato extract (C-CTe); Cream with yellow tomato extract (C-YTe).

As for C-YTe, it has a higher pH level than the other two natural colorants, but it had the same behaviour with heat treatment, as well as with storage treatment, as C-RTe and C-CTe.

The fact that C-RTe, C-CTe, and C-YTe have lower pH than C-wc and C-ac, demonstrates that organic acids lower the overall pH of the cream when added to it due to their low pH nature (Pérez-gálvez, Viera, & Roca, 2020).

The difference of pH between C-wc and C-ac is slight but more noticeable after 3 days, which can mean that the artificial colorant added to C-ac has little to no impact on the pH of the cream. After being in the oven for 2 minutes at 100 ºC, the creams suffered very little change in their pH, which can be due to the fact that water tends to lower its pH as the temperature increases (Kulthanan, Nuchkull, & Varothai, 2013).

The most interesting aspect of these results is the fact that C-ac kept its pH intact after 3 days of storage, thus demonstrating the ability of artificial colorants to preserve food products, as previously mentioned on the introduction of this thesis. As for the creams prepared with natural extracts, after 3 days of storage, their pH slightly raised, with C-YTe being the closest to pH=7. C-YTe was the one with the most stable pH levels, raising only 0.01 pH after 3 days.

Table 9. pH levels of the creams measured before and after heat resistance test, after coming out of the oven, and after being 3 days in storage at 4ºC.

	Before heat resistance test	**After heat resistance test**	**Time 3 days**
C-wc	6.87	6.73	6.81
C-ac	6.8	6.72	6.72
C-RTe	5.1	5.05	5.21
C-CTe	5.1	5.07	5.08
C-YTe	5.82	5.72	5.76

Cream without colouring (C-wc); Cream with artificial colorant mixture used by the company (C-ac); Cream with traditional red yellow tomato extract (C-RTe); Cream with cherry tomato extract (C-CTe); Cream with yellow tomato extract (C-YTe).

Texture analysis of the creams was achieved by analysing the rheological properties of the prepared creams, by assessing the graphs resulting from the texturometer. Rheology can be referred as the science behind material deformation when under mechanical forces, where the section of the graph between 1 and 2 represented by the vertical lines, is the constant lowering of the probe (positive values), whereas the section represented between 2 and 3 is the rising of the probe (negative values) then the probe stops moving after 17.5 seconds. Between these sections, 4 parameters are measured: firmness, consistency, cohesiveness, and work of cohesion, where the maximum positive and negative forces applied are taken as firmness and cohesiveness, respectively, and the areas represented by the positive and negative plots are taken as consistency and work of cohesion,

respectively (**Figure 13**).

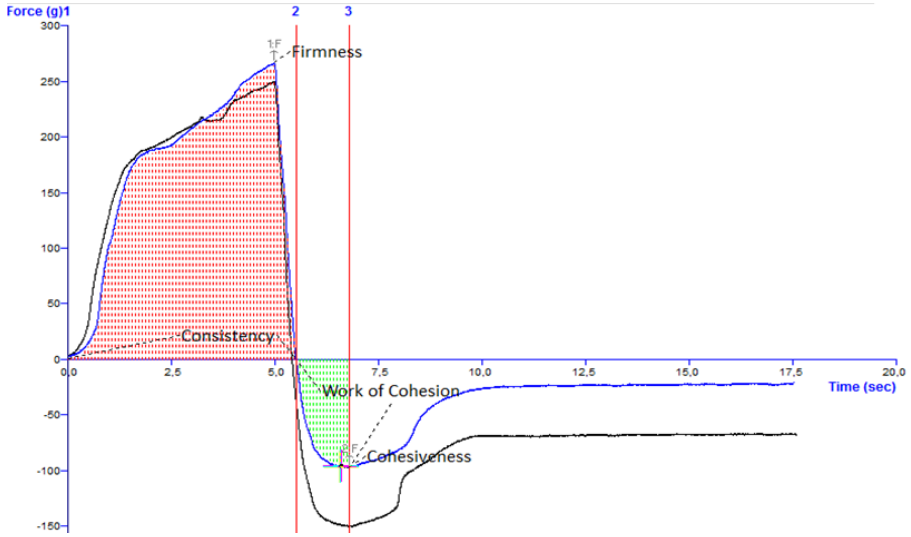


Figure 13. Texturometer graph taken directly from the device software, where firmness, consistency, cohesiveness, and work of cohesion are represented.

In the case of firmness, this parameter represents the necessary force to deform the cream sample, meaning the sample's maximum resistance to the force applied by the texturometer. Consistency represents the strength of the internal bonds making up the body of the food sample. Cohesiveness can be represented by the inner strength of a food sample to withstand tearing, as the probe rises, the material that sticks to the probe will eventually break from the bottom of the cup, the moment it breaks is the negative value which represents the cohesiveness. The higher the cohesiveness of a sample, the harder it is to tear it. The fourth and last parameter to be assessed is the work of cohesion, which can be measured by the time needed for the sample to tear itself when being pulled from the bottom of the cup until it inevitably breaks.

The results from the pastry creams analysis obtained from the texturometer were assessed and converted into column charts, where each column represents one of each parameter previously mentioned in relation to *y* value of force (g or g/sec). Values of firmness, consistency, cohesiveness, and work of cohesion of C-ac, C-RTe, C-CTe, and C-YTe, as well as the control (C-wc), for comparing purposes, are represented in **Figure 14**.

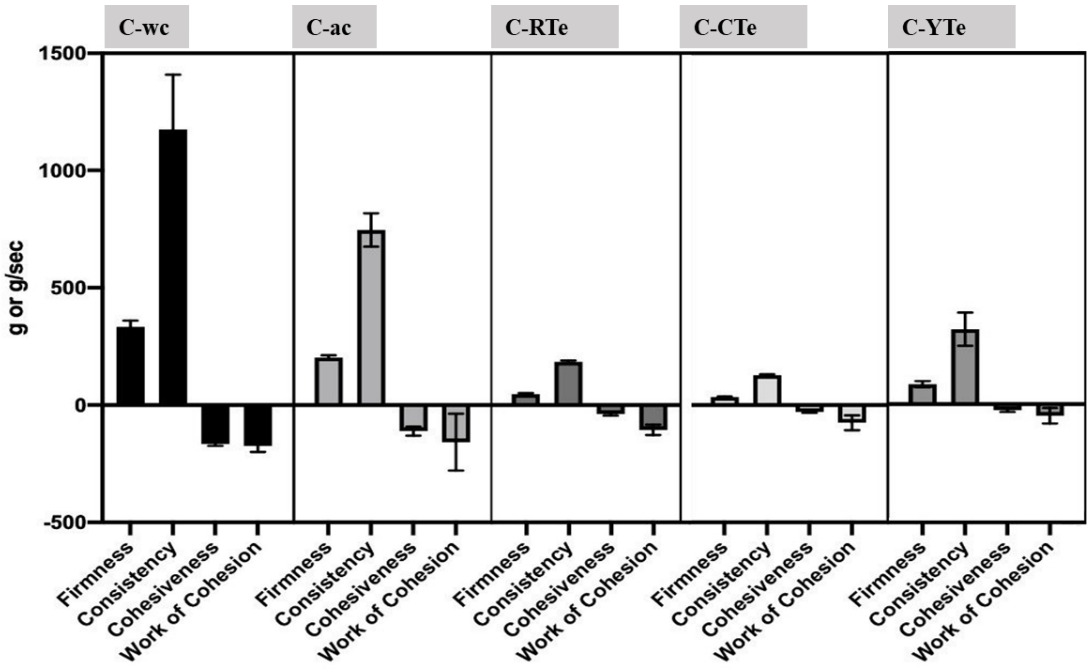


Figure 14. Dimensions of texture, namely firmness, consistency, cohesiveness, and work of cohesion, measured in the five pastry creams developed.

C-ac had the best values of all the creams, being harder to break under pressure (firmness), being capable of withstanding a constant pressure without breaking for the most time (consistency), being the hardest to tear (cohesiveness), and having the most time needed to tear itself under a constant counter-pressure (work of cohesion).

Since all pastry creams with carotenoids incorporated (C-RTe, C-CTe, and C-YTe) showed weaker texture properties, it can be concluded that the vast amount of carotenoid content present in C-RTe, C-CTe and C-YTe (8% of extract used in comparison with 0.4% of AC extract used for C-ac) alters the inner bonds of the pastry cream which results in a change on the four parameters previously mentioned, namely the reduction of its properties.

Particularly C-CTe has the strongest cohesiveness and work of cohesion from the three naturally colored creams, although very slightly, and C-YTe has the best firmness and consistency of all three pastry creams with natural colorants.

C-CTe has shown to have the weakest results, being the least similar to C-ac texture wise. One possibility of this observations in the texture can be due to the high amount of extract used for the naturally colored creams when compared to the quantity of artificial colorant added to C-ac.

In a previous study, carotenoids derived from carrots were incorporated in cake and

it was shown that cake loses its firmness when adding carotenoids, in comparison with its control medium (Zaki, Sheir, & Sakr, 2018). In cohesiveness studies, one in particular showed that mango bars have low cohesiveness when incorporated with low acyl gellan, which shows that a higher concentration of carotenoids from mango in a single bar has shown to be the least cohesive (Danalache, Beirão-da-Costa, Mata, Alves, & Moldão-Martins, 2015). Another study concluded that orange-fleshed sweet potato bread, enriched with carotenoids, showed lower values of firmness and cohesiveness in comparison with white bread (Wanjuu et al., 2018).

4.2.2 Nutritional composition

Data on the nutritional composition of the five pastry creams developed are described in **Table 10**. Although pastry creams are consumed in fresh, the results are expressed in dry weight (dw, are more reliable since it excludes the fluctuation of water), and moisture content is given for conversion purposes. Overall, carbohydrates are the major macronutrient found in the samples, as expected, being C-YTe the sample with the highest amounts (95.91±0.01 g/100 g dw). Ash, fat, and proteins follow, by this order, the representativeness in the samples. Overall, it was the C-CTe sample that presented the highest amounts of proteins, fat, and ash (1.7±0.1 mg/100g dw, 3.2±0.1, and 3.7±0.1 g/100 dw, respectively), as also higher energetic value (401.1±0.3 kcal/100g dw).

Table 10. Moisture content and nutritional profile of the five pastry creams developed in Time 0 days (Mean±SD).

	C-Wc	**C-ac**	**C-RTe**	**C-CTe**	**C-YTe**
Moisture (%)	78.2±1.2^{a}	75±1^{c}	76.9±1.4^{b}	74.1±0.1^{d}	73.6±0.3^{e}
Proteins (mg/100 g dw)	1.3±0.1^{b}	1.34±0.04^{b}	1.7±0.1^{a}	1.7±0.1^{a}	1.7±0.1^{a}
Fat (g/100 g dw)	1.11±0.03^{d}	1.12±0.03^{d}	1.5±0.1^{c}	3.2±0.1^{a}	1.61±0.02^{b}
Ash (g/100 g dw)	3.4±0.1^{b}	2.07±0.03^{d}	2.1±0.1^{d}	3.7±0.1^{a}	2.581±0.003^{c}
Total available carbohydrates (g/100 g dw)	95.5±0.1^{d}	96.8±0.2^{b}	96.5±0.1^{c}	93.1±0.2^{e}	95.91±0.01^{a}
Energy contribution (kcal/100 g dw)	391.8±0.6^{d}	397.2±0.4^{c}	399.1±0.5^{b}	401.1±0.3^{a}	397.2±0.1^{c}

dw: dry weight basis; Cream without colouring (C-wc); Cream with artificial colorant mixture used by the company (C-ac); Cream with traditional red yellow tomato extract (C-RTe); Cream with cherry tomato extract (C-CTe); Cream with yellow tomato extract (C-YTe). In each row, different letters mean significant differences between species ($p < 0.05$).

A significant observation that could be made was on the lipid content of C-CTe,

which can be more or less the triple amount than the lipid content of C-ac and C-wc, and the double amount for the other two naturally coloured creams C-RTe and C-YTe. This can be due to the lipid content of tomatoes, which depending on the variety or the soil used on the production of tomato can result in a higher amount of lipids (Fernandes et al., 2021).

The protein content was very low in all creams, but the highest values were obtained in those incorporated with tomato extracts, as expected (Bashir et al., 2016).

4.2.3 Chemical composition

For the chemical composition of the five pastry creams developed, soluble sugars, organic acids, and fatty acids content were studied, and are described in **Table 11**. Three soluble sugars were found: fructose, glucose, and trehalose. The sample presenting the highest amounts was C-RTe with a total concentration of 43.2±2.3 g/100g dw, mainly due to the presence of glucose (36±2 g/100g dw). Overall, the creams samples incorporated with carotenoid-rich solutions presented the highest content in sugars. Fructose was only detected in those samples (C-wc and C-ac did not present fructose), leading to the conclusion that it may be a sugar that comes specifically from tomato samples, as confirmed by Vogel et al., (2010), who states that the main sugars of tomato are glucose and fructose.

Regarding organic acids (**Table 11**), once again, the creams incorporated with carotenoid-rich solutions revealed the highest amounts. A total of six organic acids were found, being C-RTe and C-CTe the ones presenting the highest total organic acids content (2.178±0.004 g/100g dw), mainly due to the presence of citric acid (1.32±0.01 g/100g dw). As it happened with sugars, C-wc and C-ac did not present some of the organic acids found (malic, fumaric, and succinic), leading to the conclusion that this compounds can be naturally present in the tomato samples, as reported by Ordóñez-Santos, Vázquez-Odériz, & Romero-Rodríguez, (2011) in conventional and organically grown tomatoes.

Table 11. Soluble sugars, organic acids, and fatty acid composition of the five pastry creams developed in Time 0 days (Mean±SD).

	C-wc	C-ac	C-RTe	C-CTe	C-YTe
Soluble sugars (g/100 g dw)					
Fructose	nd	nd	2.07±0.04^{a}	1.5±0.1^{c}	1.7±0.1^{b}
Glucose	30.5±1.6^{d}	31.2±0.02^{c}	36±2^{a}	34.8±0.9^{b}	30.6±0.2^{d}
Trehalose	5.5±0.3^{c}	5.74±0.03^{ab}	5.6±0.3^{b}	5.8±0.1^{a}	5.03±0.31^{d}
Total	**36.04±1.92^{d}**	**37±1^{c}**	**43.2±2.3^{a}**	**42±1^{ab}**	**37.3±0.7^{c}**
Organic acids (g/100 g dw)					
Oxalic	0.1747±0.0004^{a}	0.1565±0.0001^{b}	0.1304±0.002^{c}	0.130±0.002^{c}	0.114±0.002^{d}
Quinic	0.4244±0.0003^{b}	0.262±0.005^{c}	0.448±0.002^{a}	0.447±0.002^{ab}	0.126±0.002^{d}
Malic	nd	nd	0.144±0.001^{a}	0.144±0.001^{a}	0.051±0.002^{b}
Citric	0.38±0.01^{d}	0.486±0.004^{c}	1.32±0.01^{a}	1.32±0.01^{a}	0.93±0.01^{b}
Fumaric	nd	nd	0.0000082±0.0000001^{b}	0.0000082±0.0000001^{b}	0.0000096±0.0000002^{a}
Succinic	nd	nd	0.134±0.004^{a}	0.134±0.004^{a}	0.128±0.01^{b}
Total	**0.98±0.01^{c}**	**0.9037±0.0004^{d}**	**2.178±0.004^{a}**	**2.178±0.004^{a}**	**1.351±0.005^{b}**
Fatty acid (relative percentage (%)					
Lauric acid (C12:0)	0.98±0.01^{a}	0.82±0.04^{b}	0.38±0.01^{e}	0.78±0.01^{c}	0.66±0.03^{d}
Tridecylic acid (C13:0)	nd	nd	0.16±0.01	nd	nd
Myristic acid (C14:0)	4.1±0.1^{a}	3.9±0.1^{b}	3.47±0.03^{cd}	3.72±0.01^{c}	3.1±0.1^{e}
Pentadecylic acid (C15:0)	nd	0.52±0.01^{a}	0.27±0.01^{b}	0.199±0.004^{d}	0.253±0.002^{c}
Palmitic acid (C16:0)	44.3±0.1^{b}	51±1^{a}	43±1^{c}	41±1^{e}	42±1^{d}
Palmitoleic acid (C16:1)	nd	nd	1.17±0.02	nd	nd

Results and Discussion

Heptadecanoic acid (C17:0)	0.74±0.02^{b}	1.2±0.1^{a}	0.54±0.01^{c}	0.43±0.01^{d}	0.39±0.01^{e}
Stearic acid acid (C18:0)	4.4±0.2^{b}	5.7±0.2^{a}	4.2±0.2^{c}	4.4±0.1^{b}	4.2±0.2^{c}
Oleic acid (C18:1n9c)	35.4±0.1^{a}	34.2±0.4^{b}	35±1^{a}	33.7±0.4^{c}	34±1^{c}
Linolenic acid (C18:2n6c)	6.7±0.1^{d}	nd	8.03±0.07^{c}	11.2±0.2^{a}	10.6±0.4^{b}
α-linolenic acid (C18:3n3)	1.32±0.04^{c}	1.48±0.03^{c}	0.98±0.02^{d}	1.64±0.02^{b}	3.4±0.01^{a}
Arachidic acid (C20 :0)	0.72±0.04^{c}	nd	1.39±0.05^{b}	1.55±0.01^{a}	0.39±0.02^{d}
Eicosenoic acid (C20:1)	nd	nd	0.35±0.01	nd	nd
Behenic acid (C22:0)	0.56±0.03^{b}	0.64±0.01^{a}	nd	0.37±0.01^{d}	0.47±0.01^{c}
Lignoceric acid (C24:0)	0.63±0.02^{a}	0.53±0.01^{b}	0.39±0.01^{d}	0.399±0.001^{d}	0.403±0.031^{c}
SFA	**56.5±0.2^{b}**	**64.3±0.4^{a}**	**54±1^{c}**	**53±1^{c}**	**52±1^{d}**
MUFA	**35.4±0.1^{b}**	**34.2±0.4^{c}**	**37±1^{a}**	**33.8±0.4^{d}**	**34±1^{d}**
PUFA	**8.04±0.24^{d}**	**1.48±0.03^{e}**	**9.01±0.05^{c}**	**12.8±0.2^{b}**	**13.9±0.4^{a}**

dw: dry weight basis; nd – not detected. Cream without colouring (C-wc); Cream with artificial colorant mixture used by the company (C-ac); Cream with traditional red tomato extract (C-RTe); Cream with cherry tomato extract (C-CTe); Cream with yellow tomato extract (C-YTe). In each row, different letters mean significant differences between species ($p < 0.05$).

Finally, regarding fatty acid content (**Table 11**), the division between the control and artificial colorant sample and the carotenoid-rich solutions samples is not so linear, as in the sugar and organic acid profile. Fifteen fatty acids were found and, overall, the saturated fatty acids were the main contributors in all the studied samples, especially in C-ac sample, with a relative percentage of 64.3±0.4, mainly due to the presence of palmitic acid (C16:0, 51±1%). C-RTe sample presented the highest percentage of monounsaturated fatty acid (37±1%) and C-YTe sample presented the highest percentage of polyunsaturated fatty acids (13.9±0.4%). Palmitic acid and oleic acid were the main fatty acids found in all samples, derived from the groups SFA and PUFA respectively and present very interesting *in vitro* bioactive properties (Ricchi et al., 2009).

(Cámara et al., 2001) analysed the fatty acid content of tomato pomace which showed high amounts of unsaturated fatty acids such as PUFA and MUFA in comparison with the lower amounts of SFA which can justify the unsaturated fatty acids amount content of the natural-colored creams of the present work.

4.2.4 Bioactive properties

4.2.4.1 Antioxidant, hepatotoxic, and antimicrobial activities

Data on the antioxidant activity behaviour of the five creams developed are present in **Table 12**.

Table 12. Antioxidant activity of the five creams developed (mean ± SD).

	Samples					**Controls**	
	C-wc	**C-ac**	**C-RTe**	**C-CTe**	**C-YTe**	**Trolox**	**Ellipticine**
Antioxidant activity (IC_{50} values, mg/mL)							
TBARS	9.09 ± 0.42^{b}	7.90 ± 0.21^{d}	8.10 ± 0.12^{c}	5.29 ± 0.26^{e}	10.82 ± 0.26^{a}	0.14±0.01	-
OxHLIA Δ*t* = 60 min	2.7 ± 0.1^{a}	2.6 ± 0.1^{a}	0.8 ± 0.1^{bc}	0.87 ± 0.03^{b}	1.6 ± 0.1^{d}	0.0218±0.0002	-

Cream without colouring (C-wc); Cream with artificial colorant mixture used by the company (C-ac); Cream with traditional red yellow tomato extract (C-RTe); Cream with cherry tomato extract (C-CTe); Cream with yellow tomato extract (C-YTe). In each row, different letters mean significant differences between species ($p < 0.05$).

The creams provide higher IC_{50} values compared to the control, therefore the antioxidant properties are weaker when compared to the hydroethanolic extracts (**Table**

6). Once again, the C-CTe sample revealed the lowest values for TBARS assay (5.29±0.26 mg/mL), and now C-RTe sample revealed the lowest IC_{50} values for OxHLIA assay (0.8±0.1 mg/mL). Just like the hydroethanolic extracts, the creams did not reveal hepatotoxic activity against VERO cell lines at the maximum concentration tested.

Finally, as for the antimicrobial activity of the creams developed (**Figure 15**) a significant improvement in the antimicrobial activity can be verified in relation to the extract, especially in the cream incorporated with cherry tomato extract (**Figure 11**). The hydroethanolic extract from this sample had already shown an antibacterial action against a large number of strains, and now in creams the MIC values have decreased. More studies would be needed to better understand the results, but there may be some synergistic effect between some components of the cream and the extract that increased its action. It can also be explained by the fact that the C-wc sample has antimicrobial activity and, therefore, it will have an influence on the improvement of the performance of the samples incorporated with carotenoid-rich extracts.

Results and Discussion

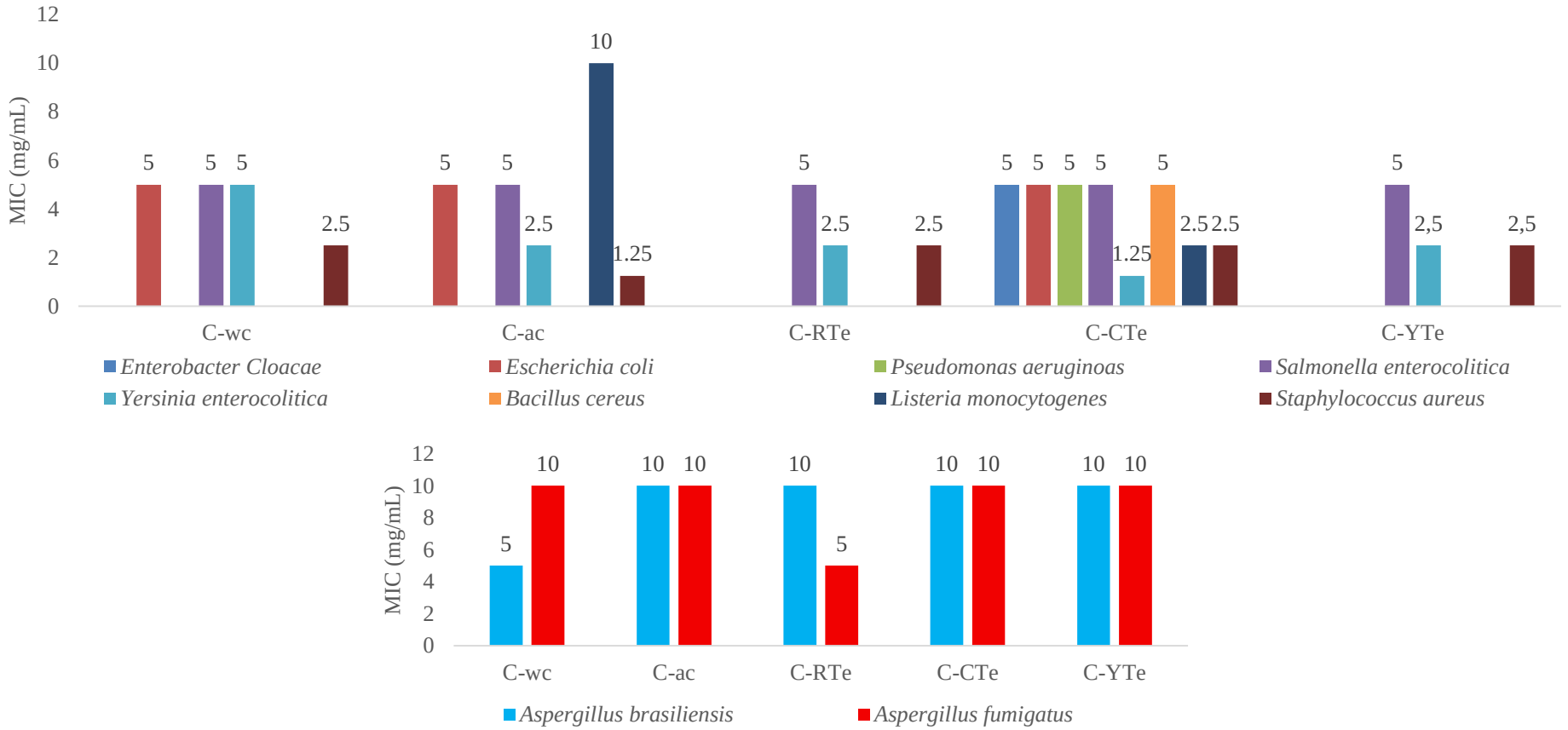


Figure 15. Antibacterial and antifungal MIC values of the five developed pastry creams. MIC-minimum inhibitory concentration; Cream without colouring (C-wc); Cream with artificial colorant mixture used by the company (C-ac); Cream with traditional red yellow tomato extract (C-RTe); Cream with cherry tomato extract (C-CTe); Cream with yellow tomato extract (C-YTe). Missing columns represent MIC values > 10.

CONCLUSION AND FUTURE PERSPECTIVES

5. Conclusion and Future Perspectives

The present work intended to extract and characterize carotenoid-rich colouring extracts obtained from *S. Lycopersum* L. bio-residues, for further application in a commercialized pastry cream in order to substitute the currently used artificial colorants.

With that in mind, a series of objectives were outlined, all successfully achieved in this thesis, beginning with prospection of natural sources of carotenoids, focused specifically on the bio-residues from the production of *Lycopersum* fruits. Further on, an ultrasound assisted (UAE) extraction was applied in order to obtain hydroethanolic and hexane extracts to be analysed for their content in carotenoids by HPLC-DAD/ESI-MSn. Finally, the hydroethanolic extracts were applied in a pastry cream, produced by the company "TecPan", and their nutritional and chemical profile was assessed, as also their physical properties at Time 0 and Time 3.

From the prospection performed, three kinds of tomatoes were selected for this study: the traditional red tomato (*S. lycopersicum* L.), cherry tomato (*S. lycopersicum* var. *cerasiforme*), and yellow pear tomato (*S. lycopersum* 'Beam's Yellow Pear). Both in their hydroethanolic and hexane extracts, two carotenoids were found, lycopene and β-carotene. But, as previously stated, unfortunately it was not possible to present the quantification results of the samples. However, for the application in pastry creams, the hydroethanolic extracts were chosen, since they are considered greener solvents than hexane and their use for food purposes is already allowed in the European Union. None of the studied samples showed hepatotoxicity (including the artificial colorant), which reveals their potential to be incorporated in a food product, and the cherry tomato was the one presenting the highest antioxidant activity, with the lowest EC_{50} values for TBARS and OxHLIA assays, and also the one presenting antimicrobial activity against a larger number of bacterial and fungal strains studied.

All the creams incorporated with the three tomato extracts were very uniform, being possible to completely dissolve the extracts in the water, and also, after the heat resistance test, the structure and colour of the cream was not affected. Regarding the physical properties, measured at Time 0 and 3 days, it is possible to state that the colour attribute of the creams suffers no significant change over time, despite the decreasing tendency observed. With the pH levels, a growing tendency was observed, except for the cream coloured with yellow pear tomato, that presented the most stable pH levels. As for the texture, and as expected, the cream with the artificial colorant showed the best texture

attributes of firmness, consistency, cohesiveness, and work of cohesion. Weaker texture properties were found in all creams developed with the natural extracts, possibly due to the quantity of carotenoid-rich extracts needed to achieved the final color. The cream coloured with cherry tomato presented the strongest cohesiveness and work of cohesion, while cream with yellow pear tomato presented the best firmness and consistency.

As for the nutritional properties of the pastry creams developed, cherry tomato incorporated cream presented the highest amounts of proteins, fat, and ash, as also higher energetic value. Three soluble sugars were found, fructose, glucose, and trehalose, being the cream incorporated with traditional red tomato the one with the highest concentrations. Both of these latest creams also presented the highest amounts of organic acids, mainly due to the presence of citric acid. In the lipid fraction, saturated fatty acids were found in highest percentages in all samples; however, the highest percentage of monounsaturated and polyunsaturated fatty acid were found in the creams incorporated with traditional red tomato extract and yellow pear tomato extract, respectively. Palmitic and oleic acid were the most prominent fatty acids found.

Finally, all creams revealed antioxidant properties, similar to the individual extracts, and also no toxicity was observed for the VERO cell line. A significant improvement in the antimicrobial activity could be verified in relation to the extract, especially in the cream incorporated with cherry tomato extract.

These results proved the potential of carotenoid-rich alternative solutions from tomato bio-residues in the replacement of artificial and probably more toxic sources, in order to provide an alternative to the worldwide artificial colorants, as also to contribute to the functionalization of the final product, adding some beneficial health effects for the consumer.

As future perspectives of this work, it would be important to:

1. Perform microbial load studies to certify the shelf life of the products developed. The microbial load studies would be performed in the final products but also in the flour powder used to prepare the pastry cream;
2. Study the influence of a thermal treatment in the carotenoid-based extracts such as pasteurization or lyophilisation (under very controlled conditions of temperature and pressure), to reduce the microbial load, without losing the colouring capacity;
3. Study the influence of the packaging of the samples for the prevention of the degradation of the final products;
4. Study the population acceptance through a sensory analysis of the prepared products;
5. Test other food matrices to apply the carotenoid-base extracts.

References

Abdel-Aal, E. S. M., Akhtar, H., Zaheer, K., & Ali, R. (2013). Dietary sources of lutein and zeaxanthin carotenoids and their role in eye health. *Nutrients*, 5(4), 1169-1185. https://doi.org/10.3390/nu5041169

Alves-Rodrigues, A., & Shao, A. (2004). The science behind lutein. *Toxicology Letters*, *150*(1), 57–83.

Amchova, P., Kotolova, H., & Ruda-Kucerova, J. (2015). Health safety issues of synthetic food colorants. *Regulatory Toxicology and Pharmacology*, *73*(3), 914–922.

AOAC. (2016). *Official Methods of Analysis of AOAC International (2016)*. (AOAC INTERNATIONAL, Ed.) (20th Editi). Rockville, MD, USA.

Barros, L., Pereira, E., Calhelha, R. C., Dueñas, M., Carvalho, A. M., Santos-Buelga, C., & Ferreira, I. C. F. R. (2013). Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of Chenopodium ambrosioides L. *Journal of Functional Foods*, *5*(4), 1732–1740.

Barzana, E., Rubio, D., Santamaria, R. I., Garcia-Correa, O., Garcia, F., Sanz, V. E. R., & López-Munguía, A. (2002). Enzyme-mediated solvent extraction of carotenoids from Marigold flower (Tagetes erecta). *Journal of Agricultural and Food Chemistry*, *50*(16), 4491–4496.

Bashir, Z., Shafique, S., Ahmad, A., Shafique, S., Yasin, N. A., Ashraf, Y., … Noreen, S. (2016). Tomato Plant Proteins Actively Responding to Fungal Applications and Their Role in Cell Physiology. *Frontiers in Physiology*, *7*, 257. https://doi.org/10.3389/FPHYS.2016.00257/BIBTEX

Bergougnoux, V. (2014). The history of tomato: From domestication to biopharming. *Biotechnology Advances*, 32(1), 170-189.

Bogacz-Radomska, L., & Harasym, J. (2018). β-Carotene-properties and production methods. *Food Quality and Safety*, *2*(2), 69–74.

Boon, C. S., McClements, D. J., Weiss, J., & Decker, E. A. (2010a). Factors influencing the chemical stability of carotenoids in foods. *Critical Reviews in Food Science and Nutrition*, *50*(6), 515–532.

Boon, C. S., McClements, D. J., Weiss, J., & Decker, E. A. (2010b). Factors influencing the chemical stability of carotenoids in foods. *Critical Reviews in Food Science and Nutrition*, *50*(6), 515–532. https://doi.org/10.1080/10408390802565889

Britton, G. (2020). Carotenoid research: History and new perspectives for chemistry in

biological systems. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids, 1865(11), 158699*. https://doi.org/10.1016/j.bbalip.2020.158699

Campestrini, L. H., Melo, P. S., Peres, L. E. P., Calhelha, R. C., Ferreira, I. C. F. R., & Alencar, S. M. (2019). A new variety of purple tomato as a rich source of bioactive carotenoids and its potential health benefits. *Heliyon*, *5*(11), e02831.

Cardenas-Toro, F. P., Alcázar-Alay, S. C., Coutinho, J. P., Godoy, H. T., Forster-Carneiro, T., & Meireles, M. A. A. (2015). Pressurized liquid extraction and low-pressure solvent extraction of carotenoids from pressed palm fiber: Experimental and economical evaluation. *Food and Bioproducts Processing*, *94*, 90–100.

Cardoso, L. A. C., Karp, S. G., Vendruscolo, F., Kanno, K. Y. F., Zoz, L. I. C., & Carvalho, J. C. (2017). Biotechnological Production of Carotenoids and Their Applications in Food and Pharmaceutical Products. *Carotenoids*, *8*, 125–141.

Carrillo-Lopez, A., & Yahia, E. M. (2010). Qualitative and quantitative changes in carotenoids and phenolic compounds in tomato fruit during ripening. In *Acta Horticulturae* (Vol. 877, pp. 1303–1308). International Society for Horticultural Science.

Chen, H. E., Peng, H. Y., & Chen, B. H. (1996). Stability of carotenoids and vitamin A during storage of carrot juice. *Food Chemistry*, *57*(4), 497–503.

Chutia, H., & Mahanta, C. L. (2020). Green ultrasound and microwave extraction of carotenoids from passion fruit peel using vegetable oils as a solvent: Optimization, comparison, kinetics, and thermodynamic studies. *Innovative Food Science and Emerging Technologies*, *67*, 102547.

Civan, M., & Kumcuoglu, S. (2019). Green ultrasound-assisted extraction of carotenoid and capsaicinoid from the pulp of hot pepper paste based on the bio-refinery concept. *LWT*, *113*, 108320.

Danalache, F., Beirão-da-Costa, S., Mata, P., Alves, V. D., & Moldão-Martins, M. (2015). Texture, microstructure and consumer preference of mango bars jellified with gellan gum. *LWT*, *62*(1), 584–591. https://doi.org/10.1016/j.lwt.2014.12.040

Daood, H. G., Ráth, S., Palotás, G., Halász, G., Hamow, K., & Helyes, L. (2021). Efficient HPLC Separation on a Core-C30 Column with MS2 Characterization of Isomers, Derivatives and Unusual Carotenoids from Tomato Products. *Journal of Chromatographic Science*. https://doi.org/10.1093/chromsci/bmab085

Do Rêgo, E. R., Finger, F. L., Casali, V. W. D., & Cardoso, A. A. (1999). Inheritance of fruit color and pigment changes in a yellow tomato (Lycopersicon esculentum Mill.)

mutant. *Genetics and Molecular Biology*, *22*(1), 101–104.

Dragovic-Uzelac, V., Levaj, B., Mrkic, V., Bursac, D., & Boras, M. (2007). The content of polyphenols and carotenoids in three apricot cultivars depending on stage of maturity and geographical region. *Food Chemistry*, *102*(3), 966–975.

Dufossé, L., Galaup, P., Yaron, A., Arad, S. M., Blanc, P., Murthy, K. N. C., & Ravishankar, G. A. (2005). Microorganisms and microalgae as sources of pigments for food use: A scientific oddity or an industrial reality? In *Trends in Food Science and Technology* (Vol. 16, pp. 389–406).

EFSA, E. P. on F. A. and N. S. added to F. (2010). Scientific Opinion on the re-evaluation of canthaxanthin (E 161 g) as a food additive. *EFSA Journal*, *8*(10), 1–42.

EFSA, E. P. on F. A. and N. S. added to F. (2011). Scientific Opinion on the re-evaluation of caramel colours (E 150 a,b,c,d) as food additives. *EFSA Journal*, *9*(3), 1–103.

EFSA, E. P. on F. A. and N. S. added to F. (2012). Scientific Opinion on the re-evaluation of mixed carotenes (E 160a (i)) and beta-carotene (E 160a (ii)) as a food additive. *EFSA Journal*, *10*(3), 1–67.

EFSA, E. P. on F. A. and N. S. added to F. (2014). Scientific Opinion on the re-evaluation of anthocyanins (E 163) as a food additive. *EFSA Journal*, *12*(4), 1–51.

EFSA, E. P. on F. A. and N. S. added to F. (2015a). Scientific Opinion on re-evaluation of copper complexes of chlorophylls (E 141(i)) and chlorophyllins (E 141(ii)) as food additives. *EFSA Journal*, *13*(6), 1–60.

EFSA, E. P. on F. A. and N. S. added to F. (2015b). Scientific Opinion on the re-evaluation of beetroot red (E 162) as a food additive. *EFSA Journal*, *13*(12), 1–56.

EFSA, E. P. on F. A. and N. S. added to F. (2015c). Scientific Opinion on the re-evaluation of chlorophylls (E 140(i)) as food additives. *EFSA Journal*, *13*(5), 1–51.

EFSA, E. P. on F. A. and N. S. added to F. (2016). Scientific Opinion on the re-evaluation of cochineal, carminic acid, carmines (E 120) as a food additive. *EFSA Journal*, *13*(11).

Emmanuel, A. O., Olugboyega, A. O., John, A. O., Adenike, S. B., & Edith, E. C. (2018). The effect of shelf life on vitamins, lycopene and sugar composition of some common Nigerian tomato varieties. *European Journal of Biotechnology and Bioscience*, *6*(5), 56–60.

Esclapez, M. D., García-Pérez, J. V., Mulet, A., & Cárcel, J. A. (2011). Ultrasound-Assisted Extraction of Natural Products. *Food Engineering Reviews*, *3*(2), 108–120.

Fernandes, I., Leça, J. M., Aguiar, R., Fernandes, T., Marques, J. C., & Cordeiro, N.

(2021). Influence of Crop System Fruit Quality, Carotenoids, Fatty Acids and Phenolic Compounds in Cherry Tomatoes. *Agricultural Research*, *10*(1), 56–65. https://doi.org/10.1007/s40003-020-00478-z

Food and Drug Administration (FDA). (2018). Overview of Food Ingredients, Additives & Colors | FDA.

Galaup, P., Sutthiwong, N., Leclercq-Perlat, M. N., Valla, A., Caro, Y., Fouillaud, M., … Dufossé, L. (2015). First isolation of Brevibacterium sp. pigments in the rind of an industrial red-smear-ripened soft cheese. *International Journal of Dairy Technology*, *68*(1), 144–147.

Gautam, N. (2016). Food Colorants and their Toxicology: An Overview. *Sunsari Technical College Journal*, *2*(1), 69–75.

Gentili, A., Caretti, F., Ventura, S., Pérez-Fernández, V., Venditti, A., & Curini, R. (2015). Screening of carotenoids in tomato fruits by using liquid chromatography with diode array-linear ion trap mass spectrometry detection. *Journal of Agricultural and Food Chemistry*, *63*(33), 7428–7439. https://doi.org/10.1021/acs.jafc.5b02910

Gómez, B., Munekata, P. E. S., Gavahian, M., Barba, F. J., Martí-Quijal, F. J., Bolumar, T., … Lorenzo, J. M. (2019). Application of pulsed electric fields in meat and fish processing industries: An overview. *Food Research International*, *123*, 95-105.

Heleno, S. A., Ferreira, I. C. F. R., Esteves, A. P., Ćirić, A., Glamočlija, J., Martins, A., … Queiroz, M. J. R. P. (2013). Antimicrobial and demelanizing activity of Ganoderma lucidum extract, p-hydroxybenzoic and cinnamic acids and their synthetic acetylated glucuronide methyl esters. *Food and Chemical Toxicology*, *58*, 95–100. https://doi.org/10.1016/j.fct.2013.04.025

Hernández Suárez, M., Rodríguez Rodríguez, E. M., & Díaz Romero, C. (2008). Chemical composition of tomato (Lycopersicon esculentum) from Tenerife, the Canary Islands. *Food Chemistry*, *106*(3), 1046–1056.

Ho, K. K. H. Y., Ferruzzi, M. G., Liceaga, A. M., & San Martín-González, M. F. (2015). Microwave-assisted extraction of lycopene in tomato peels: Effect of extraction conditions on all-trans and cis-isomer yields. *LWT - Food Science and Technology*, *62*(1), 160–168.

Kadian, S. S., Sharma, A., & Sood, D. R. (2013). Effect of light and heat on stability of crude carotenoid extract from natural sources. *International Journal of Pharmaceutical Sciences and Research*, *4*(6), 2415. Retrieved from www.ijpsr.com

Kon, I. Y., Gmoshinskaya, M. V., Safronova, A. I., Alarcon, P., & Vandenplas, Y. (2014).

Growth and tolerance assessment of a lutein-fortified infant formula. *Pediatric Gastroenterology, Hepatology and Nutrition*, *17*(2), 104–111.

König, J. (2015). Food colour additives of synthetic origin. In *Colour Additives for Foods and Beverages* (pp. 36–60).

Kulthanan, K., Nuchkull, P., & Varothai, S. (2013). The pH of water from various sources: an overview for recommendation for patients with atopic dermatitis. *Asia Pacific Allergy*, *3*(3), 155. https://doi.org/10.5415/apallergy.2013.3.3.155

Lakshmi, C. (2014). Food Coloring: The Natural Way. *Research Journal of Chemical Sciences*, *4*(2), 87–96.

Li, Y., Fabiano-Tixier, A. S., Tomao, V., Cravotto, G., & Chemat, F. (2013). Green ultrasound-assisted extraction of carotenoids based on the bio-refinery concept using sunflower oil as an alternative solvent. *Ultrasonics Sonochemistry*, *20*(1), 12–18.

Lima, R. da S., Nunes, I. L., & Block, J. M. (2020). Ultrasound-Assisted Extraction for the Recovery of Carotenoids from Guava's Pulp and Waste Powders. *Plant Foods for Human Nutrition*, *75*(1), 63–69.

Lin, C. H., & Chen, B. H. (2003). Determination of carotenoids in tomato juice by liquid chromatography. *Journal of Chromatography A*, *1012*(1), 103–109.

Linke, C., & Drusch, S. (2018). Pickering emulsions in foods - opportunities and limitations. *Critical Reviews in Food Science and Nutrition*, *58*(12), 1971–1985.

Lockowandt, L., Pinela, J., Roriz, C. L., Pereira, C., Abreu, R. M. V., Calhelha, R. C., … Ferreira, I. C. F. R. (2019). Chemical features and bioactivities of cornflower (Centaurea cyanus L.) capitula: The blue flowers and the unexplored non-edible part. *Industrial Crops and Products*, *128*, 496–503.

Macías-Sánchez, M. D., Fernandez-Sevilla, J. M., Fernández, F. G. A., García, M. C. C., & Grima, E. M. (2010). Supercritical fluid extraction of carotenoids from Scenedesmus almeriensis. *Food Chemistry*, *123*(3), 928–935.

Maoka, T. (2020). Carotenoids as natural functional pigments. *Journal of Natural Medicines*, *74*(1).

Martí, R., Roselló, S., & Cebolla-Cornejo, J. (2016). Tomato as a source of carotenoids and polyphenols targeted to cancer prevention. *Cancers*. https://doi.org/10.3390/cancers8060058

Martins, N., Roriz, C. L., Morales, P., Barros, L., & Ferreira, I. C. F. R. (2016, June 1). Food colorants: Challenges, opportunities and current desires of agro-industries to ensure consumer expectations and regulatory practices. *Trends in Food Science and*

Technology. Elsevier. https://doi.org/10.1016/j.tifs.2016.03.009

Mezzomo, N., & Ferreira, S. R. S. (2016). Carotenoids functionality, sources, and processing by supercritical technology: A review. *Journal of Chemistry*, *2016*, 16.

Mortensen, A. (2006). Carotenoids and other pigments as natural colorants. In *Pure and Applied Chemistry* (Vol. 78, pp. 1477–1491).

Nadar, S. S., Rao, P., & Rathod, V. K. (2018). Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review. *Food Research International*.

Nour, V., Corbu, A. R., Rotaru, P., Karageorgou, I., & Lalas, S. (2018). Effect of carotenoids, extracted from dry tomato waste, on the stability and characteristics of various vegetable oils. *Grasas y Aceites*, *69*(1).

Oliver, J., & Palou, A. (2000). Chromatographic determination of carotenoids in foods. *Journal of Chromatography A*, *881*(1–2), 543–555.

Ordóñez-Santos, L. E., Esparza-Estrada, J., & Vanegas-Mahecha, P. (2020). Ultrasound-assisted extraction of total carotenoids from mandarin epicarp and application as natural colorant in bakery products. *LWT*, 110598.

Ordóñez-Santos, L. E., Vázquez-Odériz, M. L., & Romero-Rodríguez, M. A. (2011). Micronutrient contents in organic and conventional tomatoes (Solanum lycopersicum L.). *International Journal of Food Science and Technology*, *46*(8), 1561–1568. https://doi.org/10.1111/j.1365-2621.2011.02648.x

Pérez-gálvez, A., Viera, I., & Roca, M. (2020, June 1). Carotenoids and chlorophylls as antioxidants. *Antioxidants*. Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/antiox9060505

Petry, F. C., & Mercadante, A. Z. (2018). New method for carotenoid extraction and analysis by HPLC-DAD-MS/MS in freeze-dried citrus and mango pulps. *Journal of the Brazilian Chemical Society*, *29*(1), 205–215.

Pinela, J., Montoya, C., Carvalho, A. M., Martins, V., Rocha, F., Barata, A. M., … Ferreira, I. C. F. R. (2019). Phenolic composition and antioxidant properties of ex-situ conserved tomato (Solanum lycopersicum L.) germplasm. *Food Research International*, *125*, 108545. https://doi.org/10.1016/j.foodres.2019.108545

Pinela, J., Prieto, M. A., Barreiro, M. F., Carvalho, A. M., Oliveira, M. B. P. P., Curran, T. P., & Ferreira, I. C. F. R. (2017). Valorisation of tomato wastes for development of nutrient-rich antioxidant ingredients: A sustainable approach towards the needs of the today's society. *Innovative Food Science and Emerging Technologies*, *41*,

160–171. https://doi.org/10.1016/j.ifset.2017.02.004

Pires, T. C. S. P., Dias, M. I., Barros, L., Alves, M. J., Oliveira, M. B. P. P., Santos-Buelga, C., & Ferreira, I. C. F. R. (2018). Antioxidant and antimicrobial properties of dried Portuguese apple variety (*Malus domestica* Borkh. cv Bravo de Esmolfe). *Food Chemistry*, *240*(August 2017), 701–706.

Prabhasankar, P., Ganesan, P., Bhaskar, N., Hirose, A., Stephen, N., Gowda, L. R., … Miyashita, K. (2009). Edible Japanese seaweed, wakame (Undaria pinnatifida) as an ingredient in pasta: Chemical, functional and structural evaluation. *Food Chemistry*, *115*(2), 501–508.

Purohit, A. J., & Gogate, P. R. (2015). Ultrasound-Assisted Extraction of β-Carotene from Waste Carrot Residue: Effect of Operating Parameters and Type of Ultrasonic Irradiation. *Separation Science and Technology (Philadelphia)*, *50*(10), 1507–1517.

Radojković, M., Zeković, Z., Mašković, P., Vidović, S., Mandić, A., Mišan, A., & Đurović, S. (2016). Biological activities and chemical composition of Morus leaves extracts obtained by maceration and supercritical fluid extraction. *Journal of Supercritical Fluids*, *117*, 50–58.

Rao, A. V., & Shen, H. (2002). Effect of low dose lycopene intake on lycopene bioavailability and oxidative stress. *Nutrition Research*, *22*(10), 1125–1131.

Regulation (EC) No 1169/2011. (2011). Regulation (EC) No 1169/2011 of the European Parliament and of the Council, of 25 October 2011, on the provision of food information to consumers. *Official Journal of the European Union*, (1169), 18–63.

Ricchi, M., Odoardi, M. R., Carulli, L., Anzivino, C., Ballestri, S., Pinetti, A., … Loria, P. (2009). Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *Journal of Gastroenterology and Hepatology*, *24*(5), 830–840. https://doi.org/10.1111/J.1440-1746.2008.05733.X

Roberts, R. L., Green, J., & Lewis, B. (2009). Lutein and zeaxanthin in eye and skin health. *Clinics in Dermatology*, *27*(2), 195–201.

Rocha, R., Pinela, J., Abreu, R. M. V., Añibarro-Ortega, M., Pires, T. C. S. P., Saldanha, A. L., … Barros, L. (2020). Extraction of Anthocyanins from Red Raspberry for Natural Food Colorants Development: Processes Optimization and In Vitro Bioactivity. *Processes 2020, Vol. 8, Page 1447*, *8*(11), 1447. https://doi.org/10.3390/PR8111447

Roriz, C. L., Barros, L., Prieto, M. A., Morales, P., & Ferreira, I. C. F. R. (2017). Floral parts of Gomphrena globosa L. as a novel alternative source of betacyanins:

Optimization of the extraction using response surface methodology. *Food Chemistry*, *229*, 223–234.

Salim, M. M. R., Rashid, M. H., Hossain, M. M., & Zakaria, M. (2020). Morphological characterization of tomato (Solanum lycopersicum L.) genotypes. *Journal of the Saudi Society of Agricultural Sciences*, *19*(3), 233–240.

Salinas Moreno, Y., Rubio Hernández, D., & Díaz Velázquez, A. (2010). Extracción y uso de pigmentos del grano de maíz (ZEA MAYS l.) como colorantes en yogur. *Archivos Latinoamericanos de Nutrición*, *55*(3). Retrieved from http://ve.scielo.org/scielo.php?script=sci_arttext&pid=S0004-06222005000300011

Santhirasegaram, V., Razali, Z., & Somasundram, C. (2013). Effects of thermal treatment and sonication on quality attributes of Chokanan mango (*Mangifera indica* L.) juice. *Ultrasonics Sonochemistry*, *20*(5), 1276–1282.

Sarmento, A., Barros, L., Fernandes, Â., Carvalho, A. M., & Ferreira, I. C. (2015). Valorization of traditional foods: Nutritional and bioactive properties of *Cicer arietinum* L. and Lathyrus sativus L. pulses. *Journal of the Science of Food and Agriculture*, *95*(1), 179–185.

Singh, D., Barrow, C. J., Mathur, A. S., Tuli, D. K., & Puri, M. (2015). Optimization of zeaxanthin and β-carotene extraction from Chlorella saccharophila isolated from New Zealand marine waters. *Biocatalysis and Agricultural Biotechnology*, *4*(2), 166–173.

Singh, J., Jayaprakasha, G. K., & Patil, B. S. (2017). Rapid ultra-high-performance liquid chromatography/quadrupole time-of-flight tandem mass spectrometry and selected reaction monitoring strategy for the identification and quantification of minor spinacetin derivatives in spinach. *Rapid Communications in Mass Spectrometry*, *31*(21), 1803–1812.

Solymosi, K., Latruffe, N., Morant-Manceau, A., & Schoefs, B. (2015). Food colour additives of natural origin. In *Colour Additives for Foods and Beverages* (pp. 4–34). https://doi.org/10.1016/B978-1-78242-011-8.00001-5

Spence, C. (2015). On the psychological impact of food colour. *Flavour*, *4*(1), 1–16. https://doi.org/10.1186/s13411-015-0031-3

Sunday N. Okafor, Wilfred Obonga, Mercy A. Ezeokonkwo, Jamiu Nurudeen, Ufoma Orovwigho, & Joshua Ahiabuike. (2016). Assessment of the Health implications of Synthetic and Natural Food Colourants – A Critical Review. *Pharmaceutical and Biosciences Journal*, *4*(4), 01–11. https://doi.org/10.20510/ukjpb/4/i4/110639

Suwanaruang, T. (2016). Analyzing Lycopene Content in Fruits. *Agriculture and Agricultural Science Procedia*, *11*, 46–48.

Szabo, K., Diaconeasa, Z., Cătoi, A., Vodnar, D., C. (2019). Screening of ten tomato varieties processing waste for bioactive components and their related antioxidant and antimicrobial activities. *Antioxidants*, 8(8), 292.

Takebayashi, J., Iwahashi, N., Ishimi, Y., & Tai, A. (2012). Development of a simple 96-well plate method for evaluation of antioxidant activity based on the oxidative haemolysis inhibition assay (OxHLIA). *Food Chemistry*, *134*(1), 606–610.

Toor, R. K., & Savage, G. P. (2006). Changes in major antioxidant components of tomatoes during post-harvest storage. *Food Chemistry*, *99*(4), 724–727.

Umpiérrez, M. L., Paullier, J., Porrini, M., Garrido, M., Santos, E., & Rossini, C. (2017). Potential botanical pesticides from Asteraceae essential oils for tomato production: Activity against whiteflies, plants and bees. *Industrial Crops and Products*, *109*, 686–692.

Van Meulebroek, L., Vanden Bussche, J., Steppe, K., & Vanhaecke, L. (2014). High-resolution Orbitrap mass spectrometry for the analysis of carotenoids in tomato fruit: Validation and comparative evaluation towards UV-VIS and tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, *406*(11), 2613–2626. https://doi.org/10.1007/s00216-014-7654-6

Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry - A review. *Innovative Food Science and Emerging Technologies*, *9*(2), 161–169.

Vogel, J. T., Tieman, D. M., Sims, C. A., Odabasi, A. Z., Clark, D. G., & Klee, H. J. (2010). Carotenoid content impacts flavor acceptability in tomato (Solanum lycopersicum). *Journal of the Science of Food and Agriculture*, *90*(13), 2233–2240. https://doi.org/10.1002/JSFA.4076/FORMAT/PDF

Wanjuu, C., Abong, G., Mbogo, D., Heck, S., Low, J., & Muzhingi, T. (2018). The physiochemical properties and shelf-life of orange-fleshed sweet potato puree composite bread. *Food Science and Nutrition*, *6*(6), 1555–1563. https://doi.org/10.1002/fsn3.710

Young, A. J., & Lowe, G. L. (2018). Carotenoids—antioxidant properties. *Antioxidants*.

Zaki, N. L., Sheir, M. A., & Sakr, A. M. (2018). The efficiency of some natural beta-carotene sources in improving the quality of the cake. *Middle East Journal of Applied Sciences*, *08*(04), 1437–1450. Retrieved from

http://www.curresweb.com/mejas/mejas/2018/1437-1450.pdf