Universidade de Aveiro Departamento de Química
2015

Liliana Cristina Vieira da Silva

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica do Dr. João Manuel da Costa Araújo Pereira Coutinho, Professor Catedrático do Departamento de Química da Universidade de Aveiro e coorientação da Dr.ª Sónia Patrícia Marques Ventura, Estagiária de Pós-Doutoramento do Departamento de Química da Universidade de Aveiro.

À mãe, pai e irmã…

o júri

presidente

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palavras-chave

Licopeno, β-Caroteno, *Solanum lycopersicum L.*, Extração sólido-líquido, Fracionamento, Solventes orgânicos

resumo

O principal objetivo do presente trabalho recai no estudo de um processo eficiente para a extração e fracionamento de licopeno e β-caroteno presentes no tomate, bem como na aplicação do processo a resíduos provenientes da indústria alimentar. Esta é uma das indústrias que produz das maiores quantidades de resíduos ricos em biomoléculas com valor acrescentado e com um elevado potencial económico. No entanto, os métodos convencionais para a extração deste tipo de compostos tornam-se dispendiosos, o que inviabiliza a sua aplicação em larga escala. O licopeno e o β-caroteno são carotenóides com elevado valor comercial, conhecidos pela sua atividade antioxidante e efeitos benéficos para a saúde humana. A sua maior fonte é o tomate, um dos frutos mundialmente mais consumidos, razão pela qual as quantidades de resíduos produzidos são consideráveis. Este trabalho centra-se no desenvolvimento de um processo que permita a extração e fracionamento eficientes destes carotenóides a partir do tomate, considerando o uso de solventes mais benignos que os estudados até ao momento. Adicionalmente, foi igualmente desenvolvido o processo de fracionamento em contínuo, considerando a futura aplicação industrial do mesmo.

Assim, iniciou-se o presente trabalho com a extração destes dois carotenóides utilizando um conjunto de solventes comuns e alternativos, nomeadamente, solventes orgânicos, sais convencionais, líquidos iónicos, polímeros e surfatantes. Nesta etapa avaliou-se a capacidade de extração de cada um dos solventes. Os resultados obtidos demonstraram que uma seleção adequada do solvente pode conduzir à extração completa dos dois carotenóides numa única etapa de extração, sendo que a acetona e o tetrahidrofurano se revelaram os mais eficazes, sendo os sais, líquidos iónicos, polímeros e surfatantes pouco eficazes no processo de extração sólido-líquido, pela sua geral baixa capacidade de penetração na biomassa.

Após demonstrar a elevada capacidade dos solventes orgânicos na extração do licopeno e β-caroteno, nomeadamente tetrahidrofurano e acetona, este último solvente foi usado no desenvolvimento de processo de fracionamento, recorrendo-se para isso ao uso de solventes estratégicos. Este passo foi desenvolvido com sucesso a partir da manipulação das solubilidades de cada um dos compostos de interesse em etanol e *n*-hexano. Os resultados obtidos confirmaram a possibilidade de fracionamento dos compostos alvo, pela adição ordenada dos solventes. Cerca de 39% do β-caroteno ficou dissolvido no etanol e cerca de 64% de licopeno encontrava-se dissolvido no *n*-hexano, indicando assim a sua separação para dois solventes distintos o que demonstra o caráter seletivo do processo desenvolvido, sem qualquer etapa prévia de otimização. Este estudo revelou que a utilização de solventes orgânicos conduz à extração seletiva de licopeno e β-caroteno, permitindo a eliminação de inúmeras etapas descritas pelos métodos convencionais. Por fim, foi possível idealizar e desenvolver um processo integrado sustentável e de relevância industrial.

keywords

Lycopene, β-Carotene, *Solanum lycopersicum L*., Solid-liquid extraction, Fractionation, Organic solvents

abstract

The main objective of the present work is the study of a profitable process not only in the extraction and selective separation of lycopene and β-carotene, two compounds present in tomato, but also in its potential application to food industry wastes. This is one of the industries that produce larger amounts of wastes, which are rich in high value biomolecules with great economic interest. However, the conventional methods used to extract this kind of compounds are expensive which limits their application at large scale. Lycopene and β-carotene are carotenoids with high commercial value, known for their antioxidant activity and benefits to human health. Their biggest source is tomato, one of the world's most consumed fruits, reason for which large quantities of waste is produced. This work focuses on the study of diverse solvents with a high potential to extract carotenoids from tomato, as well as the search for more environmentally benign solvents than those currently used to extract lycopene and β-carotene from biomass. Additionally, special attention was paid to the creation of a continuous process that would allow the fractionation of the compounds for further purification.

Thus, the present work started with the extraction of both carotenoids using a wide range of solvents, namely, organic solvents, conventional salts, ionic liquids, polymers and surfactants. In this stage, each solvent was evaluated in what regards their capacity of extraction as well as their penetration ability in biomass. The results collected showed that an adequate selection of the solvents may lead to the complete extraction of both carotenoids in one single step, particularly acetone and tetrahydrofuran were the most effective ones. However, the general low penetration capacity of salts, ionic liquids, polymers and surfactants makes these solvents ineffective in the solid-liquid extraction process.

As the organic solvents showed the highest capacity to extract lycopene and β-carotene, in particular tetrahydrofuran and acetone, the latter solvent used in the development process of fractionation, using to this by strategic use of solvents. This step was only successfully developed through the manipulation of the solubility of each compound in ethanol and *n*-hexane. The results confirmed the possibility of fractionating the target compounds using the correct addition order of the solvents. Approximately, 39 % of the β-carotene was dissolved in ethanol and about 64 % of lycopene was dissolved in *n*-hexane, thus indicating their separation for two different solvents which shows the selective character of the developed process without any prior stage optimization. This study revealed that the use of organic solvents leads to selective extraction of lycopene and β-carotene, allowing diminishing the numerous stages involved in conventional methods. At the end, it was possible to idealize a sustainable and of high industrial relevance integrated process, nevertheless existing the need for additional optimization studies in the future.

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List of symbols

λ - wavelength (nm);

Abs - absorbance;

$[C]^{Ac}$ - concentration of pigment in the initial extract of acetone in µg/mL;

$[C]^{Acetone}$ - concentration of carotenoid in µg g^{-1} in acetone

$[C]^{s}$ - concentration of pigment in the solvent in µg mL^{-1};

$[C]^{Solvent}$ - concentration of carotenoid in µg g^{-1};

EE % - extraction efficiency of carotenoid;

REE % - relative extraction efficiency of the carotenoid;

V_i - volume of the initial crude extract;

V_s - volume of ethanol or n-Hexane added;

wt% - weight percentage (%).

List of abbreviations

$Al_2(SO_4)_3$ - aluminium sulphate;

CTAB - Hexadecyltrimethylammonium bromide

EtOH - ethanol;

IL - ionic liquid;

PEG - polyethylene glycol;

PPG - polypropylene glycol;

Na_2CO_3 - sodium carbonate;

SDS - sodium dodecylsulfate.

THF - Tetrahydrofuran;

UV-vis - Visible ultraviolet;

[C_4mim][DMP] - 1-butyl-3-methylimidazolium dimethylphosphate;

[C_4mim]Cl - 1-butyl-3-methylimidazolium chloride;

[C_{12}mim]Cl - 1-dodecyl-3-methylimidazolium chloride;

[Ch]Cl - cholinium chloride

[Ch][Pro] - (2-hydroxyethyl)trimethylammonium (cholinium) propionate;

[P_{44414}]Cl - tributyltetradecylphosphonium chloride;

[N_{16111}] Br - hexadecyltrimethylammonium bromide;

[N_{4444}]Cl - tetrabutylammonium chloride;

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1. GENERAL INTRODUCTION

1.1 Waste value in the food industry

Over the last years, one of the major societal concerns regards the environmental pollution and the emission of greenhouse gases, which strongly affects the ecosystems and human health. In this context, the United Nations Environment Program *(1)* has identified the *21 issues for the 21st Century* that cover a wide range of environment-related subjects and discusses them towards a more sustainable development. This scenario has also changed the way how the academic and industrial communities nowadays develop their products, processes and technologies. Green Chemistry and its 12 principles *(2)* are ruling the strategy for the design of chemical products and processes at the level of chemistry-related domains as they encourage, for instance, the prevention of wastes (1st principle), the use of safer chemicals (5th principle) and/or renewable feedstock (7th principle). In fact, the 7th Green Chemistry principle gives rise to hot topics and related ideas are consistent across the entire globe, like in the case of Horizon 2020 Program where *"Waste is a resource to recycle, reuse and recover raw materials"*. With the massive generation of wastes from distinct sources worldwide, there is an urgent demand for their valorization, for example as new raw materials *(3)*.

Since the 90s, concerns about the generation of waste by the food industry have started in the scientific community *(4)* and, more recently, the interest on such a thematic has been intensified; although the recovery and valorization of this type of waste possesses diverse sustainable applications, it still remains underexplored *(5)*. Food wastes are generated along the entire lifecycle of food, being estimated that *circa* 89 million tons of wastes *per* year are generated in the Europe Union (EU) *(6)*. These are a result of distinct types of activities, being the household responsible for the major part of the wastes formed (*circa* 42%) accompanied by the manufacturing sector (39%), while the remaining 20% are spread through the food service and retail sectors (data from 2006) *(7,8)*. In this context, one of the goals proposed by the European Commission is to reduce food waste by half until 2020 *(9)*. Indeed, the reduction of food waste is an important social issue with considerable ethical, ecological and economic implications *(10)*.

In the food industry, the generation of waste is huge and consists on a direct result of raw materials' processing, originating compounds of low nutritional value or that is unfit for consumption which are discarded as unwanted materials *(8)*; thus consequently categorized as wastes *(11)*. The food sectors that produce the highest amounts of waste

are the fruit and vegetable ones. An estimation of the amount of waste produced by these industrial sectors is shown in Table 1 *(8)*.

Table 1: Estimate of waste in the food industry [adapted from *(8)*].

Industrial Sector	Amount of waste (ton)	Waste (%)
Production, processing, and preserving of meat and meat products	150000	2.5
Production and preserving of fish and fish products	8000	3.5
Production and preserving of fruits and vegetables	279000	4.5
Manufacture of vegetables and animal oils and fats	73000	1.5
Dairy products and ice cream industry	404000	3
Production of grain and starch products	245000	1.5
Manufacture of other food products	239000	2
Industry of drinks	492000	2
Total	1890000	2.6

However, the management and treatment of these wastes is complex due to strict restrictions imposed by the actual legislation. Although the current recognition of the food wastes' valuable status, these are usually treated as garbage, disposed into landfills, used for animal feeding or sent for composting *(8,12)*. Nonetheless, the EU legislation established that landfills are not sustainable and proposed to reduce biodegradable waste in landfills (including food waste) *(13)*. Other alternatives for the management of food wastes such as bioremediation *(14–18)*, energy production and recovery of high added-value compounds are nowadays being considered. With the exception of this last option, there are several compounds of interest (*e.g.* phenolic compounds, proteins, polysaccharides, fibers, flavor compounds and phytochemicals) existing in the food wastes being underused or even completely destroyed *(8)*.

In fact, the valorization of wastes through the recovery of valuable compounds has been the matter of considerable interest over the past years as a way to decrease the negative impact of the wastes in the environment, while increasing its economic value. Thus, the term "food by-products" arises to stress out this idea within the framework of Green Chemistry, Sustainability and "Bioeconomy" concepts. This is indeed in accordance with the European legislation regarding the encouragement and promotion of the investment in research and development of biologically based markets *(19)*; yet, there is a vital need for developing new, more efficient and if possible, more sustainable recovery technologies.

1.2 Food wastes as a natural source of valuable compounds

Nowadays, there is a public demand for the preferential consumption of natural compounds over the ones produced by synthetic routes due to safety and legal issues *(20,21)*. Also from here arises the opportunity to extract several compounds from food wastes as these consist of natural sources. Several efforts have been made on developing new strategies to achieve this purpose *(22,23)*. In order to develop suitable approaches, it is of utmost importance a careful understanding on the composition of each type of waste in terms of the target compounds, especially on determining their concentration in each part of the food *(24)*. Moreover, the technology adopted to carry out the extraction must be as much efficient as possible from the operational point of view *(25)*, while simultaneously of low environmental footprint.

The processing of fruits and vegetables, as the principal generator of food wastes, originates food by-products that have already been shown to be potential sources of fibers (pectin), phenolic compounds and carotenoids *(26)*. Table 2 provides a survey of some examples regarding the extraction of valuable compounds from food wastes and the main extraction approaches used. An interesting example regards the citrus beverage industry which produces large amounts of peels and seeds that are typically discarded, of which (mostly the peels) could be recovered for the extraction of a set of valuable compounds such as phenolic compounds, carotenoids and chlorophylls *(27–29)*. Another relevant example regards the grape skins that are a natural source of anthocyanins, valuable natural antioxidants and pigments with proven benefits to human health *(30)*. The extraction of this natural pigment can be successfully achieved. The valorization of cauliflower byproducts through the recovery of phenolic compounds was also attempted *(31)*. Regarding the tomato industry wastes, some studies have shown that peels and seeds are more worthwhile in terms of nutritional compounds (*e.g.* phenolic compounds and carotenoids) than the pulp *(32)*. Industrially, the pulp is the most appealing part of this fruit, being the remaining parts treated as waste. In this context, using the tomatoes' peels and seeds for the extraction of these compounds seems to be an excellent option *(33)*. Most of the extraction approaches used for the valorization of food wastes use organic solvents or their mixtures *(27,28,31)*, while others are already starting to apply more sustainable technologies based on water, aqueous solutions of organic solvents, surfactants, also supercritical fluids and, more recently ionic liquids (ILs) *(29,31,34,35)*.

Table 2: Summary of some examples of techniques regarding the extraction of valuable compounds from food wastes and extraction method employed [adapted from *(8)*].

Extractable Biomolecule	Substrate	Extraction Method	Reference
Pectin	Apple pomace, Citrus peel, Sugar beet, Sunflower heads, wastes from tropical fruits	Solid-liquid extraction	*(36)*
Flavanones	Citrus peels and residues from segments and seeds after pressing	Solid-liquid extraction	*(36)*
Total and soluble dietary fibres	Apple pomace	Solid-liquid extraction	*(37)*
Phenolic compounds	Apple pomace	Solid-liquid extraction	*(38)*
Lycopene and β-carotene	Tomato pomace	Supercritical CO_2	*(39)*
Anthocyanins	Grape skins	Heat treatment at 70 °C, Ultrasonics, High hydrostatic pressure, Pulsed electric fields	*(40)*
Caffeine	Green tea leaves	Supercritical fluid extraction	*(41)*
Essential oils (matricine, chamazulene and α–bisabolol	Chamomile	Supercritical fluid extraction	*(42)*
Capsaicinoids and colour components	Chilli pepper	Supercritical fluid extraction	*(43)*
Oil	Rice bran	Supercritical fluid extraction	*(44)*
γ-oryzanol	Rice bran	Solid-liquid extraction	*(45)*
β-glucans	Barley bran	Solid-liquid extraction	*(46)*

Lignans	Flaxseeds	Solid-liquid extraction	*(47)*
Phenolic acids	Wheat brans	Solid-liquid extraction, ultrasound assisted extraction, microwave-assisted extraction	*(48)*
Tocopherols, tocotrienols, sterols, and squalene	Palm fatty acid distillate	Liquid-liquid extraction	*(49)*
Phenolic antioxidants	Aqueous by-products from the palm oil extraction	Separation techniques through membranes	*(49,50)*
Tocopherols and tocotrienols	Palm fatty acid distillate	Treatment with alkyl alcohol and sodium methoxide; distillation under reduced pressure; a cooling step; passage of the filtrate through an ion-exchange column with anionic exchange resin; removal of the solvent; molecular distillation	6*(51)*
Phenolic antioxidants	Aqueous by-products from the palm oil extraction	Without solvent; based on simple separation principles	*(50)*
Pepsin	Cod stomach silage	Ultrafiltration together with concentration, and spray-drying	*(52)*
Peptone	Cod stomach and viscera silage	Ultrafiltration together with concentration, and spray-drying	*(52)*
Anthocyanins	cauliflower byproducts	Solid-liquid extraction	*(31)*
Lycopene	Tomato peel	Surfactants	*(22)*
Oil	Orange peel	Ionic liquids	*(34)*

1.3 Physical and chemical properties of tomato

The tomato (botanical name *Solanum lycopersicum L.*) is a well-known important food, being the second most consumed vegetable and counting with an annual production of 100 million tones worldwide *(53-55)*. The value of tomato was disregarded until about a century ago, but nowadays its value is universally recognized due to its rich content on bioactive molecules *(53)*. Its composition is depicted in Table 3, being majorly composed of water (*circa* 94%) and its nutritional value is underlined by the presence of fibers, proteins, lipids and carbohydrates as well as micronutrients like potassium, folate and vitamins A and C. It should be highlighted that the presence of other bioactive phytochemicals like organic acids (*e.g.* citric and malic acids), phenolic compounds (*e.g.* chlorogenic and caffeic acids), flavonoids (*e.g.* quercetin and kaempferol), amino-acids (*e.g.* glutamic, aspartic, γ-aminobutyric acids and glutamine) and finally carotenoids (mainly lycopene and β-carotene) *(54,55)*. Some of these compounds possess a huge antioxidant power, being the vitamin C and the polyphenols part of the main hydrophilic antioxidants, while the vitamin E and carotenoids the major fraction of lipophilic antioxidants *(56,57)*. The relative concentrations of such chemicals in tomatoes are important to assess the tomato quality in what concerns color, texture, appearance, nutritional value, flavor and aroma *(53)*. These are highly dependent on factors such as maturity, light, temperature and climate conditions, seasonality, soil fertility, irrigation and cultural practices *(53)*. The tomatoes' maturation has an important role on the composition, since it comprises a wide set of chemical and biological phenomena, namely by softening of the tissues, chlorophyll degradation, increased respiration rate, ethylene production, synthesis of acids, sugars and lycopene. Comparing fresh tomatoes and by-products of industrial origin (peels, seeds and inedible pulp), it is verified that the last contain significant quantities of bioactive phytochemicals (*e.g.* sterols, carotenoids, terpenes). These compounds present well-known properties that enable their application at the level of the food industry (*e.g.* formulation of functional foods and as food preservatives) *(54)* and of the human health sector (*e.g.* prevention of heart diseases, cerebrovascular accidents and cancers) *(56,58)*. This is a sign of the high potential of valorizing such type of wastes.

Table 3: Nutritional value of ripe fresh tomato *(11)*.

Nutrient	Value per 100 g
Proximates	
Water (g)	94.75
Energy (Kcal)	16
Protein (g)	0.79
Total lipid (fat) (g)	0.25
Carbohydrate (g)	3.47
Fiber, total dietary (g)	1.9
Sugars, total (g)	2.55
Minerals	
Calcium (mg)	33
Iron (mg)	0.57
Magnesium (mg)	10
Phosphorus (mg)	17
Potassium (mg)	191
Sodium (mg)	115
Zinc (mg)	0.12
Vitamins	
Vitamin C, total ascorbic acid (mg)	12.6
Thiamin (mg)	0.575
Riboflavin (mg)	0.055
Niacin (mg)	0.712
Vitamin B-6 (mg)	0.111
Folate (µg)	8
Vitamin B-12 (µg)	0.00
Vitamin A (µg)	20
Vitamin E (α-tocopherol) (mg)	0.59

1.4 Carotenoids and the specific cases of lycopene and β-carotene

Carotenoids are lipophilic organic pigments that belong to the class of tetraterpenic compounds *(59)* as they are constituted by forty atoms of carbon as a result of the condensation of eight isoprene units. Their long chains of conjugated double bonds possess bilateral symmetry around the central double bond and this set of conjugated double bonds is the main responsible for their typical light absorption in the visible region of the electromagnetic spectrum *(60)*. Carotenoids have different levels of antioxidant properties and distinct colorations that are essentially a result of two main

types of modifications at the level of their typical structures; they can be the introduction of oxygen atoms or the cyclisation of terminal groups *(61)*. Thus, the carotenoids can belong to two distinct classes, namely the carotenes which are exclusively composed of carbon and hydrogen atoms and the xanthophylls which are their oxygenated counterparts *(60,62,63)*. Within the more than seven hundred carotenoids identified up to date, only few could potentially be absorbed, metabolized and used by the human body; still, considering those actually detected in blood plasma with proven health benefits the set is reduced to six (α-carotene, β-carotene, lycopene, lutein, zeaxanthin and β-cryptoxanthin – their chemical structures are represented in Figure 1) *(64)*. Moreover, carotenoids can suffer isomerization to *cis-trans* conformations due to the presence of double bonds. Even though the *trans* isomer is the most common in food (due to its higher thermodynamic stability), there are some evidences on the presence of *cis* isomers in some natural and processed vegetables and fruits *(60)*.

From the carotenoids present in tomato, the β-carotene and lycopene are the most abundant, demanding major attention in the studies reported *(22,54,65)*. According to the United States Department of Agriculture database, the lycopene and β-carotene contents of tomato can spread from 0.88 to 4.2 mg *per* 100 g and from 0.1 to 0.7 mg *per* 100 g of fresh tomato, respectively *(66)*. Lycopene is a red colored carotene, being the main responsible for the distinctive red color appearance of tomatoes, being its content directly related with the stage of maturity. The ripe tomatoes have more than 90% of lycopene concentrated on their peels and, therefore, are the richest and most available source of lycopene in nature. On the other hand, the β-carotene has an orange color and its main natural source regards carrots *(65)*. Compared with lycopene, this carotenoid is present in lower concentration and represents only 5 to 10 % of the tomatoes' total carotenoids content *(67)*.

Lycopene and β-carotene have the same molecular formula of $C_{40}H_{56}$ and an average molar mass of *circa* 537 $g.mol^{-1}$ *(68)*. As shown in Figure 1, lycopene is a polyunsaturated aliphatic compound and its linear shape comes from the thirteen carbon-carbon double bonds, of which eleven are conjugated. Moreover, it has two central methyl groups (1, 6-position to each other), while the remaining ones are in 1, 5-position between them. In nature, lycopene (as well as β-carotene) is found predominantly as a *trans* isomer, like aforementioned for the generality of carotenoids. By its side, β-carotene is composed of a symmetrical chain of 11 double bonds

conjugated with methyl branches, placed along the main chain. Its terminal groups are linked to cyclohexenyl rings with 1,1,5-trimethyl substitution (see Figure 1) *(69)*. Comparatively with lycopene, the stability of β-carotene is more significantly affected during heat treatments*(70)*.

The lycopene and β-carotene are hydrophobic compounds and quite soluble in organic solvents like hexane, acetonitrile, acetone, tetrahydrofuran and petroleum ether *(71)(72)*. Currently, there are studies about lycopene and β-carotene on several areas of expertise, since these are among the most important commercial and medicinal plant pigments found in nature as indicated by the number of various species that possesses a characteristic red/orange color – Table 4 *(62,68,73)*. Also, the interesting biological properties and high value of lycopene (10 mg of lycopene from tomato with purity ≥ 90% cost 1,022.50 euros in Sigma-Aldrich® company) and of β-carotene (5g of synthetic β-carotene with purity ≥ 93% cost 84.60 euros in Sigma-Aldrich® company) are relevant for this crescent interest. The biological activities of this compounds include antioxidant activity *(74)(75)*, induction of intercellular communication, promotion of the immune system, growth control and modulation of hormones *(76)*, highlighting their benefits for the human health *(59)(77)(78)*. For such properties, tomato consumption is associated with the prevention of various diseases, particularly cardiovascular diseases and cancer *(62,79)*. These carotenoids applications are well beyond the medical domain, having also potential at the level of the cosmetics and food industries as a natural additive or colorant alternatively to the synthetic compounds normally used *(80,81)*.

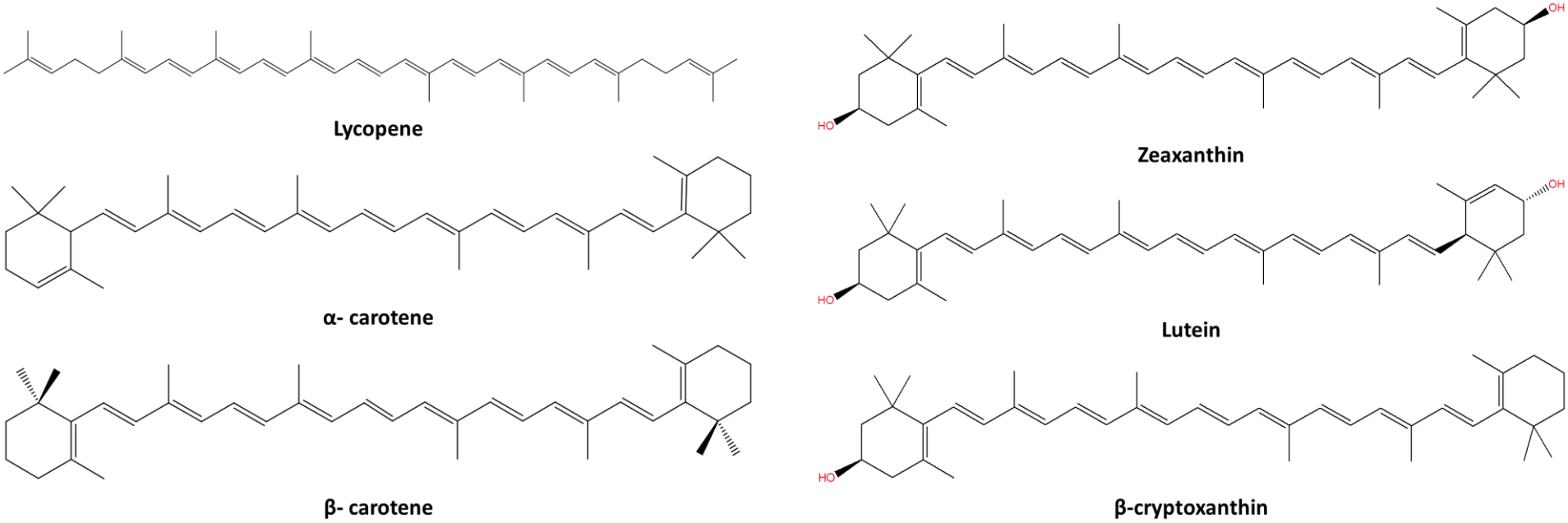


Figure 1: Chemical structures and names of the most common carotenoids *(82)*.

Table 4: Lycopene and β-carotene typical content in diverse species of fruits and vegetables [adapted from *(83,84)*].

Material	Lycopene content (mg *per* 100 g on a wet basis)	β-carotene content (mg *per* 100 g on a wet basis
Fresh tomato fruit	0.72–20	0.45
Watermelon	2.3–7.2	0.30
Guava (pink)	5.23–5.50	---
Grapefruit (pink)	0.35–3.36	0.68
Papaya	0.11–5.3	0.27
Rosehip puree	0.68–0.71	---
Carrot	0.65–0.78	8.2
Pumpkin	0.38–0.46	6.9
Sweet potato	0.02–0.11	11.5
Apple pulp	0.11–0.18	0.02
Apricot	0.01-0.05	1.1

1.4.1 Extraction of lycopene from tomato

Lycopene, as a lipophilic molecule, is usually extracted with highly toxic organic solvents such as chloroform, hexane, and petroleum ether due to its narrow solubility in water *(85)*. The extraction processing is generally performed with either fresh or wet samples *(86)*. The fresh tomato is the most preferred type of biomass used for the extraction of this natural pigment, although dehydrated biomass can also be used with water immiscible organic solvents (a prior moistening step is needed to obtain complete extraction). Different extraction systems can be used: the more conventional ones, such as solid-liquid and soxhlet extractions; as well as some alternative approaches that have gained increased attention during the last years, namely the ones using supercritical fluids, ultrasound-assisted, microwave-assisted and enzyme-assisted methods and surfactant solutions *(87,88)*. Some representative approaches created for the extraction of lycopene from tomato are represented in Table 5, while a more descriptive view of each one is provided below. In order to evaluate the success of the extraction process created, it is necessary to develop analytical methods for the quantification of the target compound. Lycopene analysis may be carried out by different methods, namely

colorimetric assays, UV-Vis spectroscopy or high performance liquid chromatography. Conventionally, the concentration of lycopene in tomatoes is determined by spectrophotometric measurement at a wavelength between 460 and 470 nm *(62,73)*. Although spectrophotometric or colorimetric approaches can be used to rapidly assess the lycopene content present in tomato-derived products, High Pressure Liquid Chromatography (HPLC) is needed for reliable analysis of food samples, since this is a more versatile, sensitive and selective method *(88)*. Most studies have focused primarily on methods that involve extraction with organic solvents, followed by quantification through spectrophotometric or HPLC-based assays *(68)*.

Table 5: Description of works reporting the extraction of lycopene from tomato matrices by using distinct extraction methods.

Substrate	Extration method	Solvent	Quantification method	Reference
Conventional methods				
Tomato paste (pulp + peel + seeds)	Solid-liquid	Hexane and metanol	HPLC	*(89)*
tomato peel	Solid-liquid	Hexane:acetone:ethanol	HPLC	*(90)*
Tomato paste (pulp + peel+ seeds)	Solid-liquid	Organic solventes	UV-Vis	*(66)*
Industrial tomato waste	Solid-liquid	Acetone, methanol, acetonitrile, chloroform, dichloromethane, hexane	HPLC	*(91)*
Tomato peel and seeds	Soxhlet	Ethanol	HPLC	*(92)*
Alternative methods				
Tomato paste (pulp + peel+ seed)	Supercritical carbon dioxide	Ethanol	HPLC	*(93)*
Industrial tomato waste	Supercritical carbon dioxide	Acetone, methanol, acetonitrile and hexane	HPLC	*(94)*
Tomato peel and seeds	Supercritical carbon dioxide	Methanol	HPLC	*(92)*
Tomato paste (pulp + peel+ seeds)	Ultrasound- and microwave-assisted	Ethyl acetate	UV-Vis	*(76)*
Tomato paste (pulp + peel + seeds)	Ultrasound-assisted	Ethyl acetate	UV-Vis	*(76)*
Tomato peel	Ultrasound- and enzyme assisted	Petroleum ether	FTIR UV-Vis	*(95)*
Industrial tomato waste	Enzyme-assisted	Hexane	UV-Vis	*(96)*
Tomato peel and industrial tomato waste	Enzyme-assisted	Petroleum ether:acetone	UV-Vis	*(97)*
Tomato peel	Surfactants and/or Enzyme-assisted	*Span20, 40, 60, 85* *Tween 80, 85* *Triton X-100*	UV-vis	*(22)*

1.4.2 Extraction of β-Carotene from tomato

β-carotene is a hydrophobic carotenoid that possesses the highest provitamin A activity *(98)*, about two-fold more efficient than the remaining carotenoids displaying this activity. Recent studies have proven the effectiveness of β-carotene in preventing cancer and cardiovascular disease *(99)*. It has considerable solubility in benzene, chloroform, ethanol, carbon disulfide and moderate solubility in ether, petroleum ether, *n*-hexane and oils *(100)*. The processes used for β-carotene extraction are typically the same used for lycopene (see Table 6). As they are normally associated to the same sources, a purification step is needed.

Table 6: Examples of works that report the extraction of β-carotene from diverse matrices by using distinct extraction methods.

Substrate	Extration method	Solvent	Quantification method	Reference
Conventional methods				
Tomato paste (pulp + peel+ seeds)	Solid-liquid	*n*-hexane, etanol, acetone	HPLC	*(101)*
Tomatoes	Solid-liquid	*n*-hexane, acetone	Uv-Vis	*(102)*
Blakeslea trispora cells.	Solid-liquid	Ethanol, 2-propanol, ethyl ether	HPLC	*(103)*
Palm Mesocarp	Soxhlet	Water, metanol, *n*-hexane, petroleum ether	HPLC	*(104)*
Alternative methods				
Palm Mesocarp	Supercritical carbon dioxide	-	HPLC	*(104)*
Tomatoes	Supercritical carbon dioxide	Acetone, water	HPLC	*(105)*
Ripe tomatoes	Supercritical carbon dioxide	Methanol, THF, water	HPLC	*(106)*
Tomatoes	Supercritical anti solvent			
Carrot	Supercritical fluid	Ethanol	HPLC	*(107)*
Carrot	Pressurized	Ethanol	HPLC	*(108)*
Cape Gooseberry	High hydrostatic pressure	Acetonitrile, ethyl acetate, ascorbic acid	HPLC	*(76)*

1.4.3 Methods used for the extraction of carotenoids

Solid–liquid extraction using organic solvents is an operation which appears in many industrial processes as the most frequently used for vegetable and fruit matrices. In this sense, the extraction of lycopene and β-carotene from tomato is not an exception (Tables 5 and 6) *(8)*. The appropriate choice of the solvent and the determination of the optimal conditions (pH, temperature, time, solid-liquid ratio, particle size, stirring rate) are crucial to determine the success of the extraction process *(8)*. However, this generally requires long extraction times, large amounts of solvents and high temperatures, yet leading to some losses and chemical degradation *(66,90)*. Moreover, low extraction efficiencies are sometimes attained, due to the difficulty of solvent molecules to penetrate the compact tomato peel matrix and to solubilize the pigment, which is deeply embedded within the chromoplasts *(22)*. Soxhlet extraction is another industrially well-established method that is also widely applied to food matrices *(8)* and repeatedly used as a reference by comparison terms and when other methods have to be created *(15,93)*. For instance, this traditional approach was applied by Cadoni *et al. (92)* and further compared to the application of a supercritical fluid-based method on the extraction of lycopene from ripe tomatoes. Compared to solid-liquid extraction, the advantages include the enhanced ability to solubilize the target chemicals due to the use of higher temperatures and the repeated washing of the matrix with fresh solvent. This is a time consuming method, requires larger amounts of solvents, being very limited for extracting thermolabile compounds *(15)*.

Some alternative methods for the recovery of carotenoids from tomatoes have been developed with the aim of reducing the environmental footprint and/or increasing the extractive performance of the conventional extraction techniques used. One example regards the supercritical fluid extraction of which supercritical CO_2 is the most common, that was already applied on extracts of a massive number of different species of plants *(109)* and intensively applied to the extraction of lycopene and β-carotene from tomatoes *(85,92,94)*. This method has a significant advantage in thermodynamic terms, since it is easy to separate the extracted compound by simply modifying the operating conditions of either pressure or temperature. Compared to other conventional technologies, the use of supercritical CO_2 becomes more appealing since it is safe, easy to recycle and the extracts obtained are cleaner. However, this is a very expensive technique, and an effective extraction often requires the addition of co-solvents

(109,110). The assisted extraction by ultrasound is also used in the extraction of bioactive compounds with added value *(111)*, namely of carotenoids from tomato industry wastes. Ultrasound allows the use of milder conditions of pressure and temperature *(8)*. This technique exhibits major disadvantages at the level of the reuse of the solvent during the process, the mandatory need for a filtration stage and cleaning steps after extraction. This leads to time consuming extractions, high solvent consumption and loss and/or low purity levels of the extracted species *(15)*. Another technique that can be used for the extraction of bioactive molecules from food wastes is the microwave-assisted extraction. Compared to solid-liquid extraction, this technique is less time consuming, uses lower amounts of solvents and allows the achievement of higher yields *(8,76)*. If compared to the supercritical fluids extraction, this is a simpler and cheaper process, but relatively to ultrasound-assisted extraction it has higher costs *(8)*. Microwave-assisted extraction of lycopene and β-carotene from tomatoes is poorly studied *(76)(112)*. Enzyme-assisted extraction is another promising alternative to conventional extraction methods, which is based on the ability of enzymes to catalyze reactions in aqueous environments *(8)*. Only few works report its application on the extraction of lycopene from tomatoes *(97)*. If the low extraction efficiencies of conventional methods may be attributed to the difficulty of solvent molecules to penetrate the compact tomato peel tissue, due to the presence of cellulose, hemicelluloses and pectin's *(113)*, the addition of enzymes allows the hydrolysis of such components. This increases the permeability and the yield of the extraction of specific compounds, such as lycopene and β-carotene *(8)*. However, this technique is of difficult scale-up as enzymes are very sensitive to changes in the media conditions. The use of aqueous solutions of surfactants *(22)*, *i.e.* amphiphilic molecules that when present above a certain value of concentration are able to form self-assembling aggregates *(114)*, is another appealing alternative. Surfactants can reduce surface and interfacial tensions as they accumulate at the interface between two immiscible fluids, having the ability of stabilizing emulsions or increasing the solubility of lipophilic compounds in aqueous media. These have already been shown to be effective in extracting many biological molecules of interest from several matrices *(22,115)*. The facility to recover a large portion of lipophilic compounds from natural sources represents a major benefit *(115)*, such as the specific case of lycopene from tomato-based matrices, particularly if combined with an enzymatic pretreatment (only one work reported up to date) *(22)*. The use of ionic liquids' aqueous solutions as extractive

agents for added-value compounds from natural sources was recently reported *(116)*. Actually, when considering the set of techniques developed as cleaner alternatives to the common solid-liquid extraction methods, the use of ILs is several times focused. A promising example of ILs' application on the extraction of β-carotene relays on the use of aqueous biphasic systems composed of phosphonium-based ILs and an inorganic salt allowing the accomplishment of outstanding partition coefficients *(117)*.

2. SCOPES AND OBJECTIVES

This work falls within the domain of Green Chemistry, having in mind that *"Waste is a resource to recycle, reuse and recover raw materials"*. In this context, it intends to valorize the food processing industry, regarding the reuse of some of the generated wastes. Thus, this work will focus on the efficient extraction of lycopene and β-carotene from the tomato biomass, followed by the fractionation of both compounds, from the tomato residues (low/no cost feedstock). In a first step, an initial screening of extractive solvents to be applied in the solid-liquid extraction of both carotenoids from tomato (herein adopted as model biomass) will be performed in order to achieve a highly efficient process. With this purpose, 25 distinct solvents were tested aiming at the definition and selection of the optimal solvent extracting the target carotenoids from the tomato biomass. In a second phase, the development of a fractionation process to obtain each of the compounds separately was designed. This step is described by three main steps, the use of ethanol (step 2) and then n-hexane (step 3) after the utilization of the best solvent (most efficient solvent extracting the compounds from the biomass, selected in Chapter 1) to extract both target compounds from the biomass (step 1).

3. EXPERIMENTAL SECTION

3.1. Screening tests: the search for the best extractive solvents

3.1.1 Chemicals

25 chemical compounds were used in the search for the optimal solvents for the extraction of lycopene and β-carotene, from tomato. The organic solvents studied in this work were acetone (100 wt% pure), *n*-hexane (96.9 wt% pure) and petroleum ether (99.0 wt% pure), both from Carlo Erba, tetrahydrofuran (THF) (99.0 wt% pure) from Sigma-Aldrich and acetonitrile (99.9 wt% pure) from HiPerSolv Chromanorm. Four alcohols were investigated, namely ethanol (98.0 wt% pure) from Carlo Erba, 1-propanol (99.5 wt % pure) from Lab-Scan, 1-butanol (99.5 wt% pure) from Panreac and 1-octanol (99.0 wt% pure) from Sigma-Aldrich. The salts used were the aluminium sulphate, $Al_2(SO_4)_3$ (≥ 98.0wt % pure); sodium carbonate, Na_2CO_3 (≥ 99.0 wt% pure), and sodium citrate dihydrate, $C_6H_9Na_3O_9 \bullet 2H_2O$ (≥ 99.0 wt % pure), acquired from Himedia, Vencilab and Merck, respectively. Also, seven ILs were studied in the present work: three imidazolium-based, namely 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl (99.0 wt% pure), 1-dodecyl-3-methylimidazolium chloride, [C_{12}mim]Cl (> 98.0 wt% pure), 1-butyl-3-methylimidazolium dimethylphosphate, [C_4mim][DMP] (> 98.0 wt% pure) all from Iolitec; one phosphonium-based called tributyltetradecylphosphonium chloride, [P_{44414}]Cl (pure) which was kindly offered by Cytec; and three ammonium-based, namely tetrabutylammonium chloride, [N_{4444}]Cl (≥ 97.0 wt% pure), cholinium chloride, [Ch]Cl (≥ 98.0 wt% pure), both from Sigma-Aldrich, and cholinium propanoate, [Ch][Prop] (≥ 99.0 wt % pure), synthesized in our laboratory. Poly(alkylene glycols) from Sigma-Aldrich, such as polyethylene glycols of 1000 and 2000 g mol^{-1} of average molecular weight, PEG 1000 and PEG 2000,respectively, and polypropylene glycol of 1200 g mol^{-1} of average molecular weight, PPG 1200, were used. Pluronic L-35 which is a PEG-block-PPG-block-PEG copolymer with a PEG/PPG ratio of 50 wt% was also investigated, which was acquired at Sigma-Aldrich.

Finally, within the class of surfactants, the cationic surfactant hexadecyltrimethylammonium bromide, CTAB, [N_{16111}]Br (99.0 wt % pure) from

Sigma-Aldrich, the anionic surfactant sodium dodecylsulfate (SDS) (99.0 wt % pure) from Alfa Aesar and the non-ionic surfactant C11-C13 9 EO's were tested. The chemical structures of all solvents screened are depicted in Figure 2.

Lycopene Complex from NaturMil, containing 5 mg of lycopene and 4.5 mg of β-carotene *per* capsule, was used to determine the calibration curves. Their chemical structures are provided in Figure 1. The fresh tomatoes were periodically bought in the same local supermarket.

Ultra-pure water was employed, which is obtained with a Milli-Q plus 185 water purification equipment.

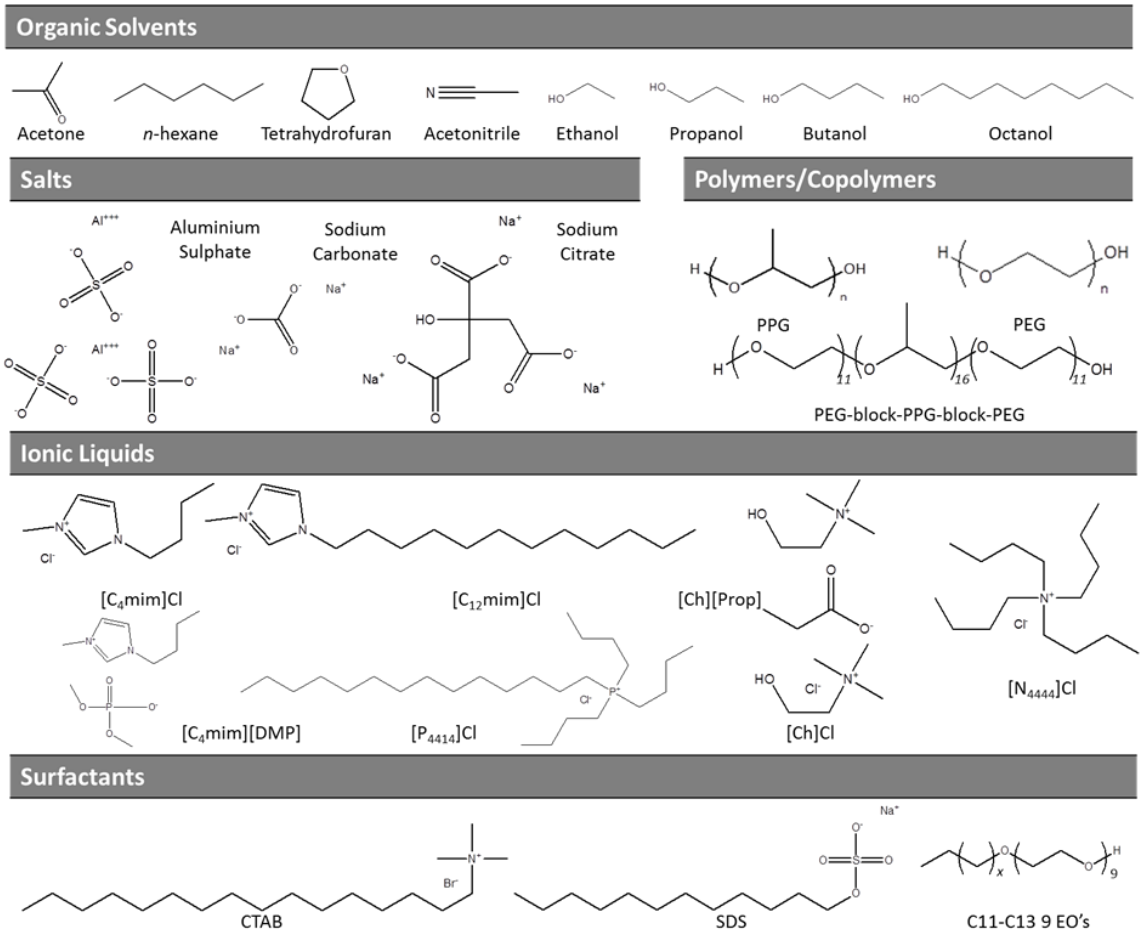


Figure 2: Chemical structure of the compounds screened for the solid-liquid extraction of lycopene and β-carotene from tomato.

3.1.2. Extraction and quantification of lycopene and β-carotene in tomato

Several studies have shown that the concentration of lycopene and β-carotene varies depending on the species, ripeness, growing site and treatment used by the producer *(56)*. Having this in mind, the tomatoes used as model biomass were always from the same species, in this case the so-called round tomatoes (with 5 to 7.5 cm of diameter) with a state of advanced maturity (uniform dark red color), as depicted in Figure 3 and always acquired in the same supermarket.

Figure 3: Tomatoes used for the extraction of carotenoids.

The tomatoes were carefully washed and the stalks and damaged parts of the fruit removed, being thus the remaining biomass stored at 4 °C. The method of extraction of lycopene and β-carotene from tomato was adapted from well-established protocols *(66,118)*. The tomatoes were meticulously triturated with a hand blender (Braun turbo, 600 Watt) aiming to obtain a uniform paste, under controlled temperature (in the freezer) and protected from the light (covered with aluminium foil). 0.5 g (Balance Accurate, ± 40SM-200A 0.0001 g, Swiss Quality +) of the resulting paste were weighed in 15 mL falcon tubes, previously covered with aluminium foil. Then, 8 mL of acetone were added (solid/liquid ratio - S/L - 1/16) and left at 4 °C for 30 minutes under constant stirring of 55 rpm on the orbital shaker. Then, the tubes were centrifuged (Kubota 2010) at 4 °C at 5000 rpm for 30 minutes, in order to separate the biomass from the supernatant. The lycopene and β-carotene content in the supernatant was spectrophotometrically assayed using calibration curves previously determined (for further details please refer to Appendix A, Figures A.1.1 and A.1.2). This procedure was adopted for the remaining solvents investigated. In the case of salts, ILs, polymers, copolymers and surfactants, these were applied as aqueous solutions. The concentration selection was based on the solubility of each compound/solvent in water. Generally, the

value adopted was 5 wt%, although some exceptions may appear as described along the discussion. In some cases, when considered advantageous, concentration optimization studies were also carried out. The amounts of either lycopene or β-carotene extracted were often determined as mass of carotene (in µg) *per* mass of biomass (in g). As fresh tomatoes were always used along the screening tests, solid-liquid extraction controls using acetone (the best solvent as it will be discussed later) were constantly applied. For that, the results obtained were always relative to those obtained using acetone as shown in Equation 1:

$$REE, \% = \frac{[C]^{Solvent}}{[C]^{Acetone}} \times 100 \quad (1)$$

where, $[C]^{Solvent}$ refers to the concentration of carotenoid in $\mu g.g^{-1}$ attained with the solid-liquid extract with the solvent of interest, $[C]^{Acetone}$ is the concentration of carotenoid in $\mu g.g^{-1}$ accomplished using acetone in the extraction process and REE is the relative extraction efficiency of the carotenoid in percentage.

3.2. Fractionation process for lycopene and β-carotene

3.2.1. Chemicals

The organic solvents studied in this section were acetone (purity of 100 wt%), *n*-hexane (purity of 96.9 wt%) and ethanol (purity of 98.0 wt%), all acquired at Carlo Erba.

3.2.2. Fractionation task: high vacuum followed by solubilization in strategical solvents

Acetone was selected as the best solvent (see section of Results) and conditions properly established as optimal (S/L ratio of 1/16, at a temperature of 4 °C and agitation 55 rpm) were fixed to carry out the solid-liquid extraction experiments further adopted in the fractionation assays. This said, the initial supernatant (coming from the solid-liquid extraction step) was centrifuged at 5000 rpm and subjected to HV, under constant agitation and at 298 ± 1 K. Since acetone is a highly volatile solvent, HV allowed the complete evaporation of acetone in approximately 10 minutes, without the compounds' degradation due to severe temperature conditions. At the end of this step, a precipitate consisting mainly of lycopene and β-carotene was obtained. Three replicates were always performed. All replicates were dried in order to avoid contamination. The precipitate obtained after HV (see Figure 4) was sequentially ressuspended in ethanol and *n*-hexane or *vice-versa*. These options were based on the discrepant solubility of lycopene and β-carotene in distinct organic solvents, in particular in ethanol and *n*-hexane – Table 7.

Table 7: Solubility of lycopene and β-carotene in acetone, *n*-hexane and ethanol *(71,119)*.

	β-Carotene	**Lycopene**
Solubility in Acetone	Soluble	Soluble
Solubility in *n*-hexane	Moderately soluble	Soluble
Solubility in Ethanol	Soluble	Moderately soluble

Thus, the fractionation process developed in this chapter follows two different routes. In Route 1, 1 mL of *n*-hexane (solvent 1) (200 µL were added up to 1 mL), followed by the micropipette aided resuspension until the saturation is achieved. Afterwards, in the same flask, 1 mL of ethanol (solvent 2) was added in the same way to dissolve the precipitate remaining on the flask. Then, in the same flask, 1 mL of *n*-hexane (solvent 2) was added in the same way to dissolve the precipitate remaining on the flask (*i.e.* that solvent 1 was not able to solubilize). In the case of Route 2, the only difference is the order in which the solvents were added (solvent 1 consists on *n*-hexane and solvent 2 represents ethanol). In both routes, the addition of the second solvent is performed after collecting the first solvent helped by the micropipette used for resuspension, avoiding further losses. Each resuspension solvent was pipetted into eppendorf tubes of 5 mL and submitted to centrifugation for 5 min at 8000 rpm, being the spectra (wavelength between 300 and 600 nm as this is the region of maximum absorbance of both flavonoids as aforementioned) further acquired in a spectrophotometer (Shimadzu UV-1700, Pharma-Spec Spectrometer).

The extraction efficiencies (EE, %) were calculated by Equation 2:

$$EE, \% = \frac{[C]_C^S \times V_i}{[C]_C^{Ac} \times V_s} \times 100 \quad (2)$$

where C_C^S is the concentration of carotenoid in the solvent (1 or 2) added during the fractionation assays in µg mL^{-1}, C_C^{Ac} is the concentration of carotenoid in the initial acetone-extract (after the solid-liquid extraction step) in µg mL^{-1}, V_i is the initial volume of acetone added in the solid-liquid extraction assays, V_s is the volume added of solvent 1 or solvent 2.

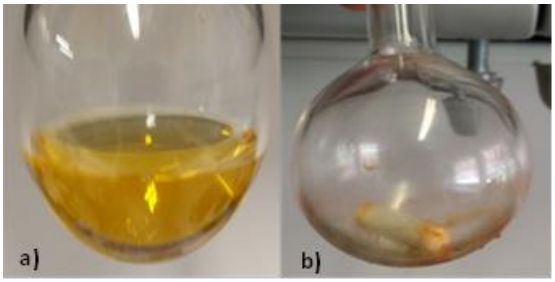


Figure 4: Initial extract of the process: a) before HV; b) after HV.

4. RESULTS AND DISCUSSION

4.1. Screening tests: the search for the best extractive solvents

The methodologies commonly used for the extraction of β-carotene and lycopene display considerable limitations as discussed above, being their resolution through the proposal of alternative methods an actual demand. Faster, cheaper, sustainable and efficient are four main characteristics desired for the new processes developed. Given the distinct extraction capabilities of different solvents for β-carotene and lycopene as already reviewed, it is worth testing several extracting agents to be applied in a solid-liquid extraction process. The objective of this part of the present thesis is to study the ability of distinct solvents to extract majorly or lycopene or β-carotene from tomatoes in a faster and effective way and attaining, if possible, some level of fractionation.

4.1.1. Establishment of the conventional solvent, storage conditions and grinding methodology

The development of more efficient, economic and environmentally-friendly processes to remove and recover lycopene and β-carotene from tomato is of high relevance. From the literature data available, it was found that the extraction of these two hydrophobic carotenoids is frequently attained by using organic solvents such as acetone, *n*-hexane, tetrahydrofuran, petroleum ether and mixtures of these solvents *(120)*. Two main methods have been reported: a method involving a first task of cell disruption using mixtures of ethanol and acetone, followed by the utilization of *n*-hexane to extract the carotenoids fraction; and the single-step utilization of mixtures of acetone and hexane or petroleum ether *(66)*. So, as an initial screening, solid-liquid extraction experiments were carried out with *n*-hexane, acetone, petroleum ether and mixture of acetone/hexane (1:3) aiming at validating the use of these two conventional solvents and finding the solvents with the best extraction capacity for β-carotene and lycopene. At the end of this process, it was found that acetone possessed a higher ability to penetrate the biomass (tomato) and consequently a higher extraction the two compounds when compared with either, *n*-hexane, petroleum ether and their mixture, as depicted in Figure 5. First of all, it was possible to obtain a visual indication of this phenomenon, since the characteristic

red color of the biomass was completely lost in contact with acetone. On the contrary, in the presence of both *n*-hexane, the petroleum ether and mixture the red color was preserved (Figure 5). These evidences are well corroborated by the amounts of both carotenoids extracted, being the acetone the solvent displaying the highest extraction ability (1348.4 μg g^{-1} for lycopene and 956.3 μg g^{-1} for β-carotene *vs.* 52.8 μg g^{-1} for lycopene and 33.9 μg g^{-1} for β-carotene with hexane, 107.2 μg g^{-1} for lycopene and 74.5 μg g^{-1} for β-carotene and 133.6 μg g^{-1} for lycopene and 89.6 μg g^{-1} for β-carotene with the acetone/*n*-hexane mixture). Moreover, the beneficial role of acetone in this extraction method is additionally proved by the higher ability of the acetone/hexane mixture when compared with the *n*-hexane.

	[Lycopene] μg g^{-1}	[β-Carotene] μg g^{-1}
Acetone	1348.4	956.3
***n*-Hexane**	52.8	33.9
Petroleum ether	107.2	74.5
Mixture (1:3)	133.6	89.6

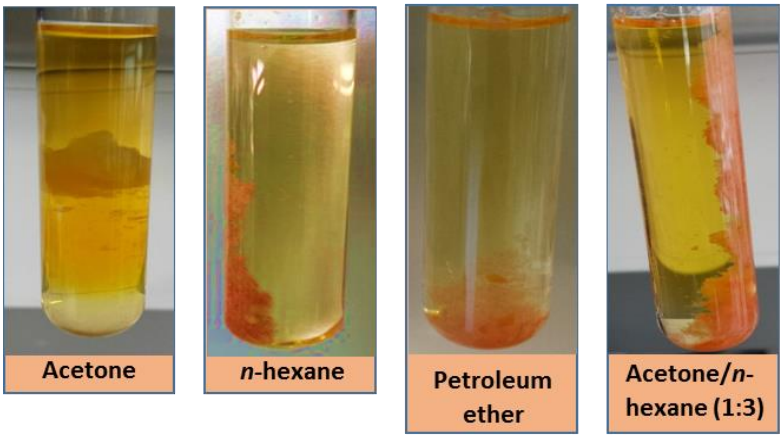


Figure 5: Concentration of lycopene and β-carotene at the end of the solid-liquid extraction using acetone, n-hexane and acetone/n-hexane mixture (1:3). The visual aspect of the extracts obtained from the solid-liquid extraction experiments: a) with acetone; b) with n-hexane; c) with acetone/hexane mixture (1:3) are also presented.

These results can be discussed on the basis of the cells' structure as these possess a plasma membrane, a protein-lipid bilayer that forms a barrier which separates cell contents from the extracellular environment. In plants cells, it can be found multiple layers of cellulose that constitutes the cell walls, which are conferring shape and rigidity to the cells. Plant cell walls are particularly resistant, preventing mechanical or chemical disruption and it is here that lays the difficulty of extracting natural pigments present in some fruits or vegetables. The cell wall is the biggest barrier to environmental aggressions and its formation is complex. The presence of palmitic, stearic, oleic, linoleic acids increases the hydrophobicity of this protective layer and inhibits the penetration of polar solutions *(121)*. Moreover, lycopene and β-carotene are usually found in chromoplasts present in the cell membrane of several plants and ripe fruits as the specific case of tomatoes *(122)*. It seems, in this context, that acetone is more efficient at disrupting the cell membranes and the chromoplasts allowing enhanced extraction yields.

Given these results, acetone is the optimal extractive agent, being from now on adopted in the remaining preliminary screening as well as a comparison solvent (or more specifically as the control). The use of fresh biomass would induce huge variability in the results due to the variability imposed by seasonality and pre-treatment (grinding and storage) among other factors. So, the pre-treatment given to the biomass was also here matter of investigation. In this context, two parallel factors were compared: the use of hand blender *vs.* the use of ultra turrax and the use of fresh *vs.* lyophilized biomass (Table 8).

Table 8: Concentration of lycopene and β-carotene at the end of the solid-liquid extraction using acetone and considering the study of different biomass pretreatments.

	[Lycopene] μg g^{-1}	**[β-Carotene] μg g^{-1}**
Hand blender	1270.81	711.94
Lyophilized	885.88	494.88
Ultra turrax	566.85	321.24

In the first study, the use of hand blender and ultra turrax in the grinding step was analyzed. Using the ultra turrax, an increase on the cell lysis and consequently on the release of lycopene and β-carotene into the extracellular environment enhancing the extraction process is foreseen. As shown in Table 8, the application of ultra turrax was

shown instead to limit the extraction phenomenon, as the amounts of lycopene and β-carotene able to be extracted were decreasing from 1270.81 μg g^{-1} to 566.85 μg g^{-1} and from 711.94 μg g^{-1} to 321.24 μg g^{-1}. Indeed, when the ultra-turrax is used it was necessary to conduct a pre-trituration step with the hand blender, enlarging the exposure time to oxygen leading to the degradation of the compounds *(123)*. In the second study, by fixing the trituration with hand blender as the pre-treatment as well as the extraction conditions aforementioned, solid-liquid experiments were conducted using either fresh or lyophilized biomass. Using fresh biomass, the concentrations of lycopene and β-carotene extracted were 1270.81 μg g^{-1} and 711.94 μg g^{-1}, respectively, while using the lyophilized one, the amounts reached were 885.88 μg g^{-1} and 494.88 μg g^{-1}, respectively. The lower values attained with the lyophilized biomass may be due to degradation of the compounds when exposed to light and oxygen during the lyophilization process *(123)*. At this point, it was not possible to overcome the limitation of using fresh biomass by its replacement by the lyophilized one; hence, a control (using acetone) using fresh biomass acquired at the same time will be systematically adopted, being thus all the results quantitatively presented in comparison with the acetone case.
However, the use of fresh biomass, thus triturated with magic wand was shown to be more effective, allowing the presence of higher concentrations of the compounds in acetone.

4.1.2. Searching for alternative solvents

As discussed before, acetone consists on the optimal conventional solvent for extracting lycopene and β-carotene from tomato biomass as it seems to possess a boosted capacity of penetrating the cells and chromoplasts. In this context, the release of both carotenoids into the extracellular medium was improved. However, acetone is not selective extracting both compounds and since the main idea of this work is to fractionate both compounds (meaning the separation of both compounds) other solvents were screened. Given the importance of finding new alternatives more selective for the extraction of lycopene or β-carotene, a vast screening comprising 25 distinct solvents was performed, including organic solvents and aqueous solutions of salts, ILs, polymers and surfactants. The solid-liquid experiments were performed according to the protocol already described, under specific conditions: S/L = 1/16, temperature of 4 ºC, constant stirring at 5000 rpm for 30 min and in the absence of light. All the results are presented

comparatively to those of acetone, using the same batch of tomatoes, as well as accompanied by the visual aspect of the experiments.

4.1.2.1. Organic solvents

Based on the results obtained above for acetone and considering that both lycopene and β-carotene are hydrophobic molecules, other organic solvents with different polarities were tested, in particular, *n*-hexane, petroleum ether, tetrahydrofuran, acetone and acetonitrile, ordered from the least to the most polar *(124)*. The results obtained are shown in Figure 6. In general, *n*-hexane and petroleum ether, the less polar solvents *(124)*, are those exhibiting lower efficiencies extracting β-carotene. In the specific case of *n*-hexane, this demonstrates a significantly higher capacity for dissolving lycopene, about 40 %, than for β-carotene, *circa* 5 %, being these results in agreement with literature *(125)*. As one of the most polar solvents, this work was studying the effect of acetonitrile, which presented poor affinity to dissolve both compounds. Included in the set of the most polar solvents is THF, which was able to extract the same concentrations of lycopene and β-carotene as acetone (*circa* 100 % of relative extraction efficiency). When analyzing the visual appearance of the extracts obtained from the extraction experiments (available in Figure 6), a high accordance with what was previously described is verified. Again, this fact is indicative of the low penetration capacity in biomass of the less polar solvents, consequently exhibiting lower relative extraction efficiencies.

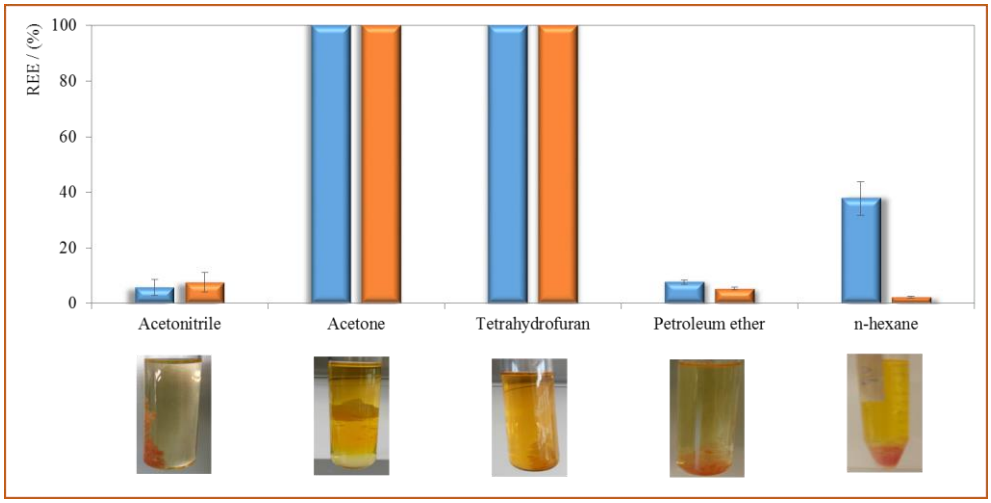


Figure 6: Relative extraction efficiencies (in percentage) of lycopene (blue bars) and β-carotene (orange bars) using distinct organic solvents. The visual aspect of the crude extracts obtained after this extraction is also depicted.

Within the solvents previously studied, it was found that acetone and THF showed higher extraction efficiencies. In this context, THF was selected to study the influence of the extraction time on the concentration of each compound aiming at maximizing the efficiencies attained and understanding if this parameter is relevant for the process here developed. This choice was also based on the criterion of THF being an useful solvent for possible subsequent stages aimed at purifying the target carotenoids as well as of being similar to acetone in terms of extractive capacity *(126,127)*. From here, it will be possible to determine if the extraction time adopted up to this point is the most suitable. As shown in Figure 7, between 0.5h and 2h30min, the concentrations of β-carotene and lycopene extracted were similar, suggesting that this parameter is not responsible for the discrepancy in the extraction aptitude of the solvents tested.

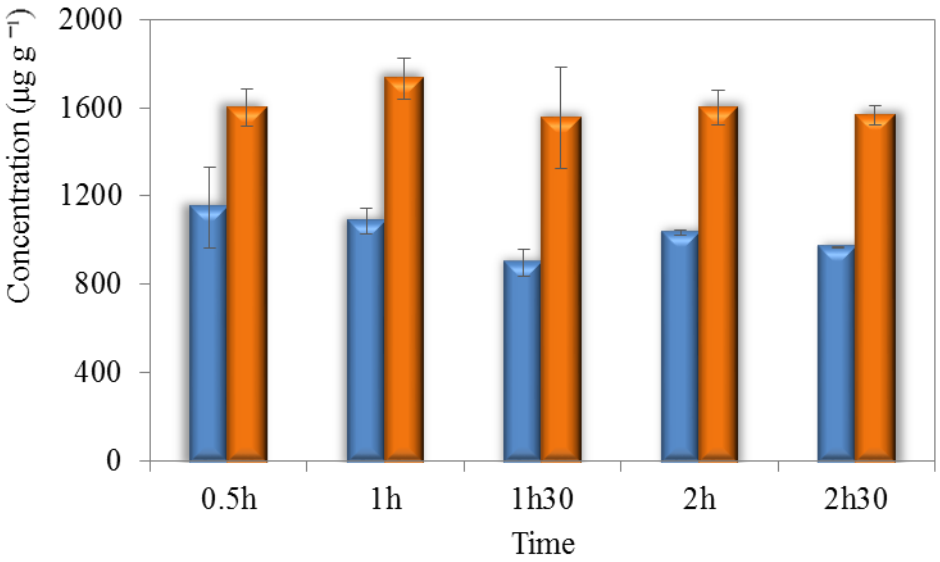


Figure 7: Concentration (µg g^{-1}) of lycopene (blue bars) and β-carotene (orange bars) with different times of extraction using THF.

Four distinct alcohols with crescent polarities (by the decrease in the number of carbons), namely 1-octanol, 1-butanol, 1-propanol and ethanol, respectively, were also tested (Figure 8). Ethanol and 1-propanol, the most polar alcohols, showed higher relative extraction efficiencies within the alcohols studied, with values closer to 20 % for both lycopene and β-carotene. This tendency is again justified by the higher capability of the most polar compounds to disrupt the cell membrane of the tomatoes biomass. As in previous trials, the relative extraction efficiency values can be confirmed by the biomass color displayed, when it is in contact with each one of the solvents at the

end of extraction (Figure 8). Despite the tendency verified, acetone is again the most effective solvent performing the solid-liquid extraction, as easily observed by the crude extracts color.

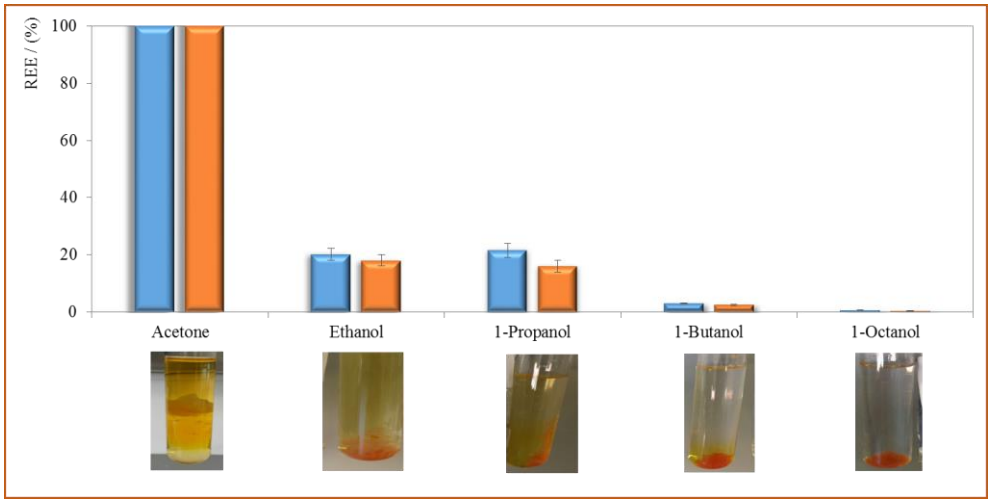


Figure 8: Relative extraction efficiencies (in percentage) of lycopene (blue bars) and β-carotene (orange bars) using four alcohols. The visual aspect of the crude extracts obtained is also depicted.

4.1.2.2. Solvents for the aqueous system

In an attempt to see if any of the classes of salts, ILs, polymers or copolymers/surfactants was efficient in the extraction of the target compounds, a screening with these solvents was made. However, the main purpose of this point of the study was to identify if any of these solvents would develop an extraction process characteristically in aqueous medium to replace the organic solvents and with selectivity in solubilize β-carotene or lycopene.

4.1.2.2.1. Salts

Sodium carbonate and aluminium sulphate, both inorganic salts, and sodium citrate, an organic salt, were also tested. The use of salts in aqueous solution intended to realize to what extent it could replace the organic solvent by aqueous solution. As organic salts tend to be biodegradable and non-toxic (4), sodium citrate was also included in this study. It must be noticed that these salts were all used as aqueous solutions at 5 wt% of salt concentration, with the exception of sodium carbonate, which was studied in an additional concentration of 15 wt%. As a general conclusion of the results obtained and shown in Figure 9, a poor effectiveness is shown by all aqueous solutions of salts investigated. Even so, sodium carbonate was the one presenting the highest efficiency of approximately 10 % and 15 % for lycopene and β-carotene, respectively. It was in an

optimization context that an additional higher concentration of salt was tested, however, without success, as proved by the image of the crude aqueous extracts obtained. This can be due to a salting-out effect of the salt, which is limiting the solubilization of both lycopene and β-carotene in the solution *(128)*.

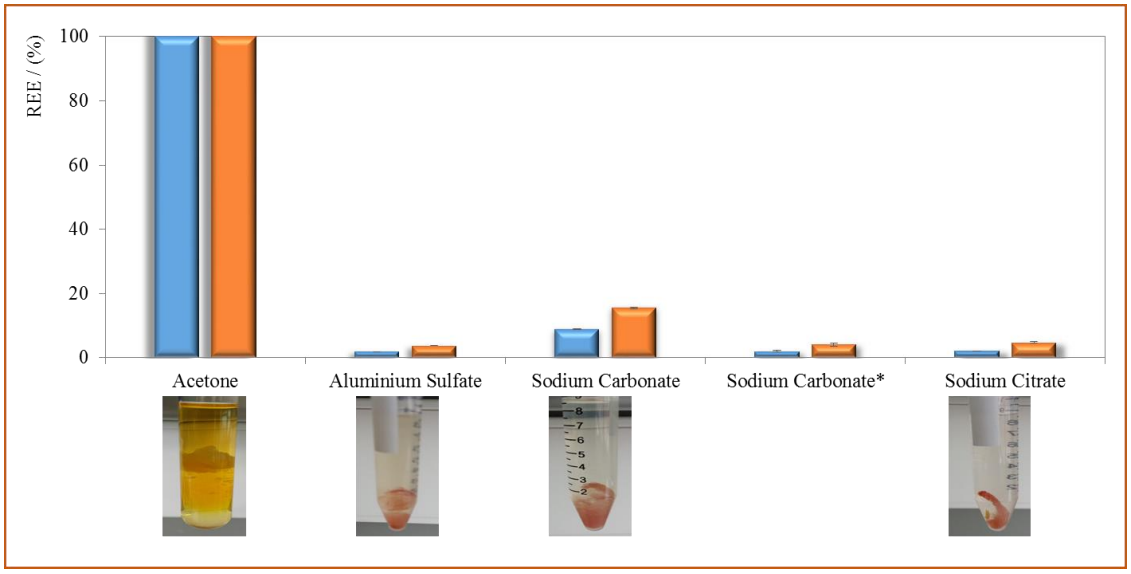


Figure 9: Relative extraction efficiencies (in percentage) of lycopene (blue bars) and β-carotene (orange bars) using organic salts at 5 wt% (and *at 15 wt%). The visual aspect of the crude extracts obtained is also depicted.

4.1.2.2.2. Ionic liquids

Ionic liquids are highly used in the design of new greener and more efficient extraction technologies in the field of cleaner manufacturing processes *(116)*. In this sense, seven structurally different ionic liquids were tested as extractive solvents in the solid-liquid extraction of lycopene and β-carotene from tomatoes. As for the salts, aqueous solutions at 5 wt% of each ionic liquid were employed and an additional concentration of 50 wt% was tested for $[P_{44414}]Cl$. From the analysis of Figure 10, it is clearly noticed that lycopene and β-carotene extraction is extremely limited. $[P_{44414}]Cl$, in any of the concentrations tested, is the most efficient extracting both carotenoids, as additionally indicated by the slight red/orange coloration of the solvent after extraction (Figure 10). Contrarily to what was observed for the conventional organic solvents, where the most polar compounds created more efficient processes, here, the less polar ionic liquid seems to be the best one, despite the lower extraction capacity, suggesting that besides the polarity of the solvent other specific interactions are occurring. A primordial role of the cell penetration capability is still notorious.

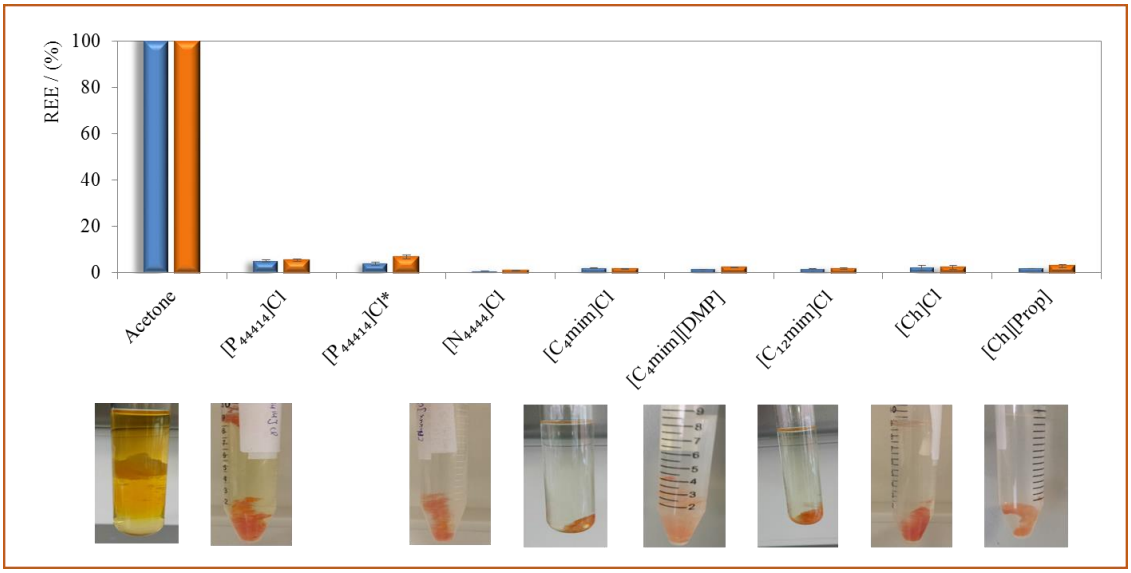


Figure 10: Relative extraction efficiencies (in percentage) of lycopene (blue bars) and β-carotene (orange bars) using ionic liquids' aqueous solutions (and * at 50 wt%). The visual aspect of the crude aqueous extracts obtained is also depicted.

4.1.2.2.3. Polymers

PEGs and PPGs are widely used in biochemistry processes due to their high biodegradability, low toxicity and low cost *(129)*. Taking into account all these benefits, the development of more environmentally friendly solutions for the extraction of the two carotenoids from tomatoes may pass by the utilization of these polymers. For the screening tests, aqueous solutions at 5 wt% of PEG 1000, PEG 2000 and 0.01 wt% of PPG 1200 were prepared. Figure 11 reveals that the relative extraction efficiencies obtained with these polymers were below 5 %. Despite these polymers' ability to increase the osmotic pressure of the medium *(130)*, no lysis of the cell membrane was observed, preventing the pigments extraction to the extracellular environment (also demonstrated in Figure 11), independently of the molecular weight. Moreover, Pluronic-L35, which is a block copolymer formed by the combination of PEG and PPG units, was introduced in this study [for an intermediate degree of polarity, since its structure can be manipulated by the proportion of PEG and PPG units *(131)*]. This is also reported to solubilize hydrophobic compounds afforded by the existence of a micellar environment, being thus a promising alternative *(132)*. However, just like the common polymers PEG and PPG, this copolymer was limited at extracting lycopene and β-carotene, independently of its concentration (see image of the extracts formed and depicted in Figure 11.

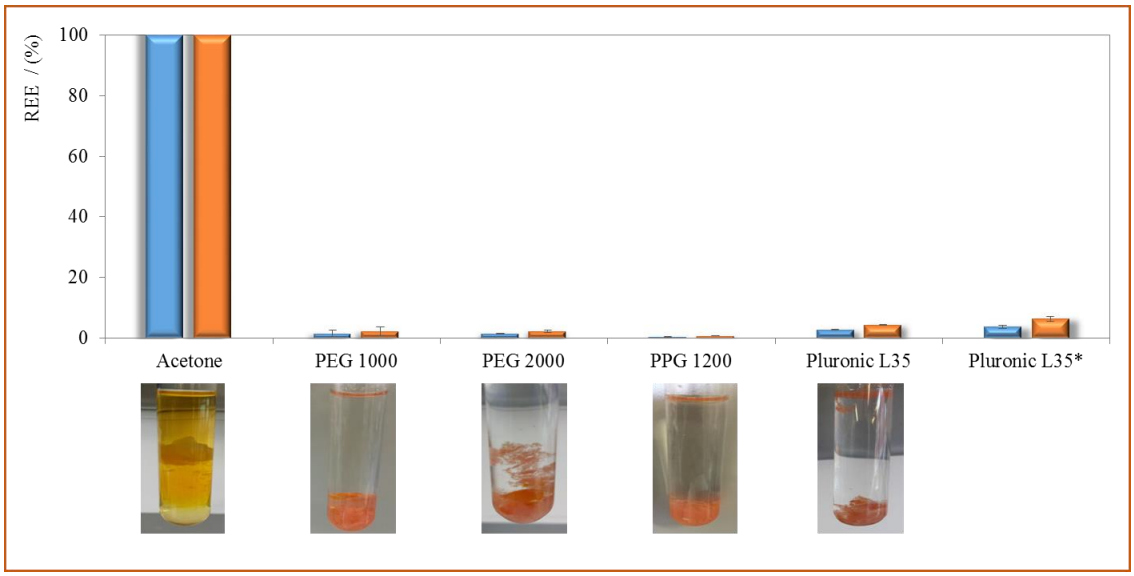


Figure 11: Relative extraction efficiencies (in percentage) of lycopene (blue bars) and β-carotene (orange) using polymers' aqueous solutions at 5 wt%. (* at 50 wt%). The visual aspect of the crude extracts obtained is also depicted.

4.1.2.2.4. Surfactants

Surfactants are amphiphilic molecules, *i.e.* present a polar, hydrophilic and sometimes charged 'head' and a non-polar, hydrophobic 'tail'. When its concentration is above a certain value, *i.e.* the critical micelle concentration (CMC), self-assembling aggregates are created. In this work, three distinct classes of surfactants were investigated: a nonionic, C_{11}-C_{13} 9 EO's, a cationic, CTAB, and an anionic surfactant, SDS. Initially, a concentration optimization study was performed by using C_{11}-C_{13} 9 EO's at 0.1 wt%; 1.0 wt%; 2.5 wt%; 5.0 wt% and 10 wt%. After, calculating the respective relative extraction efficiencies (Figure 12), it was observed that for the three lower concentrations, *i.e.* 0.1wt%; 1.0wt%; 2.5wt%, negligible amounts of lycopene and β-carotene were extracted from tomatoes (lower than 10 %). However, and still, low relative extraction efficiencies were obtained, when the surfactant concentration was increased up to 5 wt%, a slight increase, in particular for β-carotene relative extraction efficiency, was observed. For the highest surfactant concentration used (10 wt%), a reversed behavior can be seen as a slightly higher extraction efficiency for lycopene is obtained.

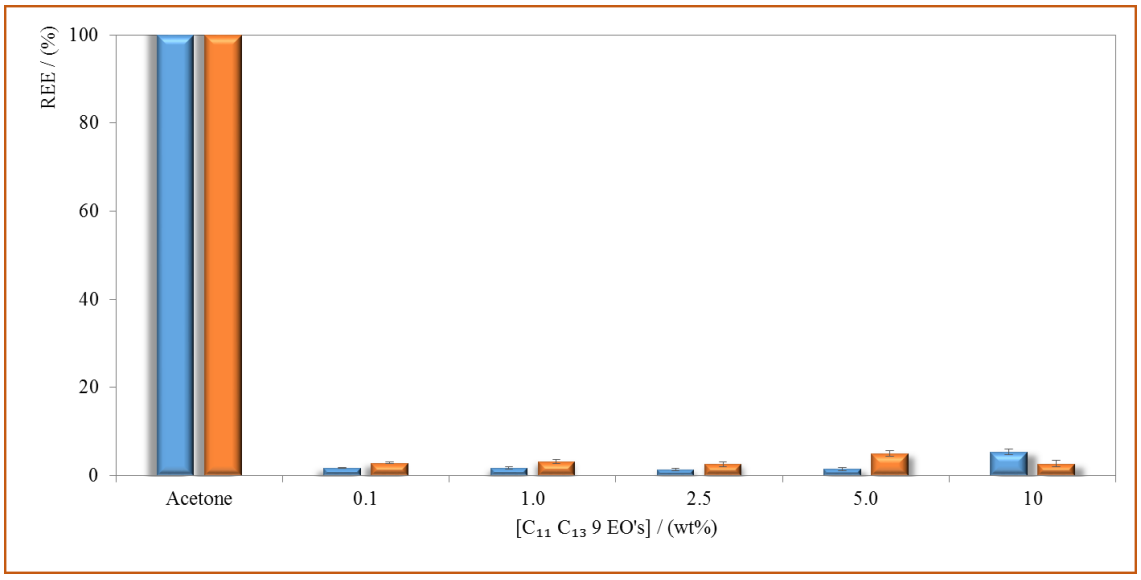


Figure 12: Relative extraction efficiencies (in percentage) of lycopene (blue bars) and β-carotene (orange bars) using C_{11} C_{13} 9 EO's at distinct concentrations.

Taking into account the results obtained for the nonionic surfactant and that the increase in concentration is not accompanied by a significant increase in the relative extraction efficiency, the surfactant concentrations used to perform the solid-liquid experiments with CTAB were 0.1 wt% and 1.0 wt% (in this case, no higher concentrations were tested due to solubility limitations) and with SDS being 0.1 wt%, 1.0 wt% and 2.5 wt%. The results obtained for the solid-liquid assays performed with CTAB are shown in Figure 13 and those obtained with SDS are provided in Figure 14.

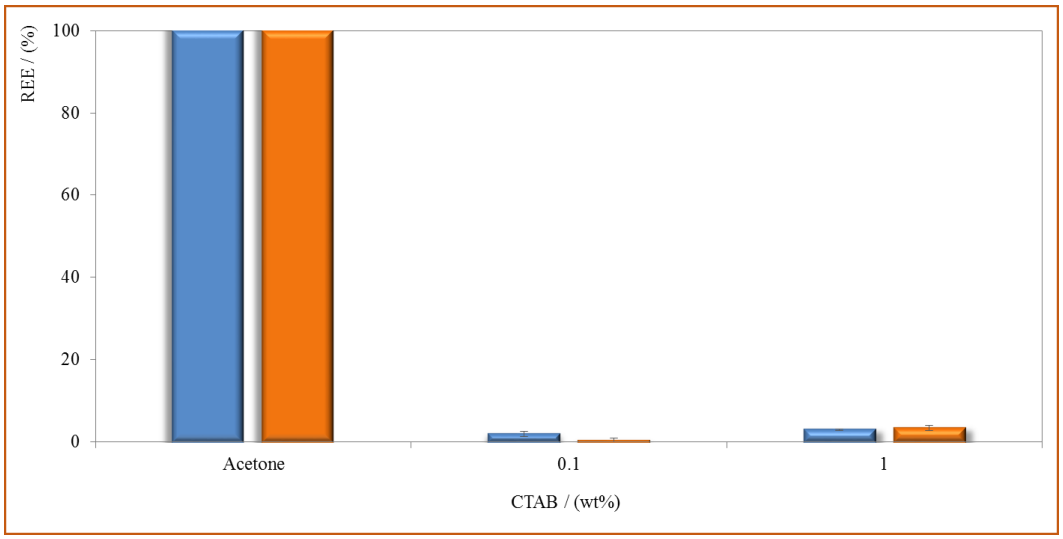


Figure 13: Relative extraction efficiencies (in percentage) of lycopene (blue bars) and β-carotene (orange bars) using CTAB at distinct concentrations.

Similarly to what was observed for C11-C13 9 EO's, the relative extraction efficiencies were always below 10%. More particularly, for the experiments conducted with either CTAB or SDS, it is possible to observe a slight increase in the relative extraction efficiency with the increase of the surfactant concentration, which is more significant than that occurring with C11-C13 9 EO's. Despite their lower capacity of extracting the target compounds, it is thus suggested that nonionic surfactants are less suitable to be used in this specific extraction process. Pappaioannou and Karabelas *(22)* have recently shown that the extraction of lycopene from tomato peel using surfactants can be significantly enhanced by adding an extra enzymatic pre-treatment step, supporting the idea that surfactants alone are not efficient at disrupting the chromoplasts where the two target carotenoids are located. The visual evidences also demonstrated in Figures 12, 13 and 14 are corroborating the poor success attained.

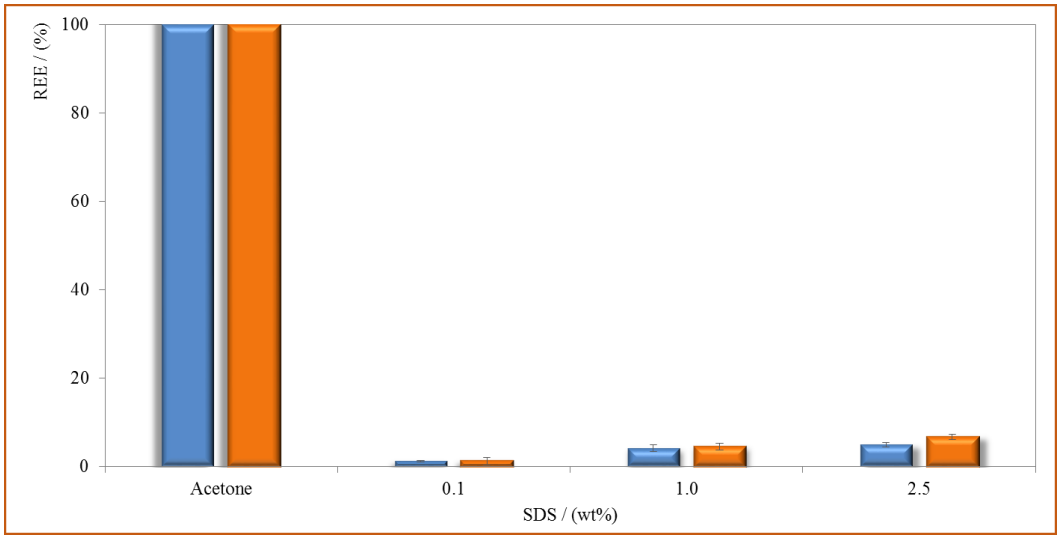


Figure 14: Relative extraction efficiencies (in percentage) of lycopene (blue bars) and β-carotene (orange bars) using SDS at distinct concentrations

4.1.3. Conclusions

Aiming at finding efficient and selective alternative solid-liquid extraction solvents for the fractionation of lycopene and β-carotene in just one-step process, several organic and alternative solvents were investigated. From the main results found for the organic solvents tested, it was concluded that especially the most polar, were the most efficient. Acetone and tetrahydrofuran exhibited the best performances at extracting lycopene and β-carotene from tomato. The group of results here collected suggests that the solvents

ability to penetrate the biomass cells, consequently promoting the chromoplasts disruption is of utmost importance for the success of the extraction process, whereas the extraction time was shown not to play an important role. Of course, the solubility of these two carotenoids in the solvents of interest needs to be significant. Ionic liquids, polymers and surfactants, known as some of the most selective classes of chemicals tested, have a poor ability to disrupt the chromoplasts releasing the target compounds. Still, the improved performance of both acetone and tetrahydrofuran is highlighted, since these are highly applied solvents, facilitating the following steps of purification and recycling of the compounds *(127,133)*.

4.2. Development of a fractionation process for lycopene and β-carotene

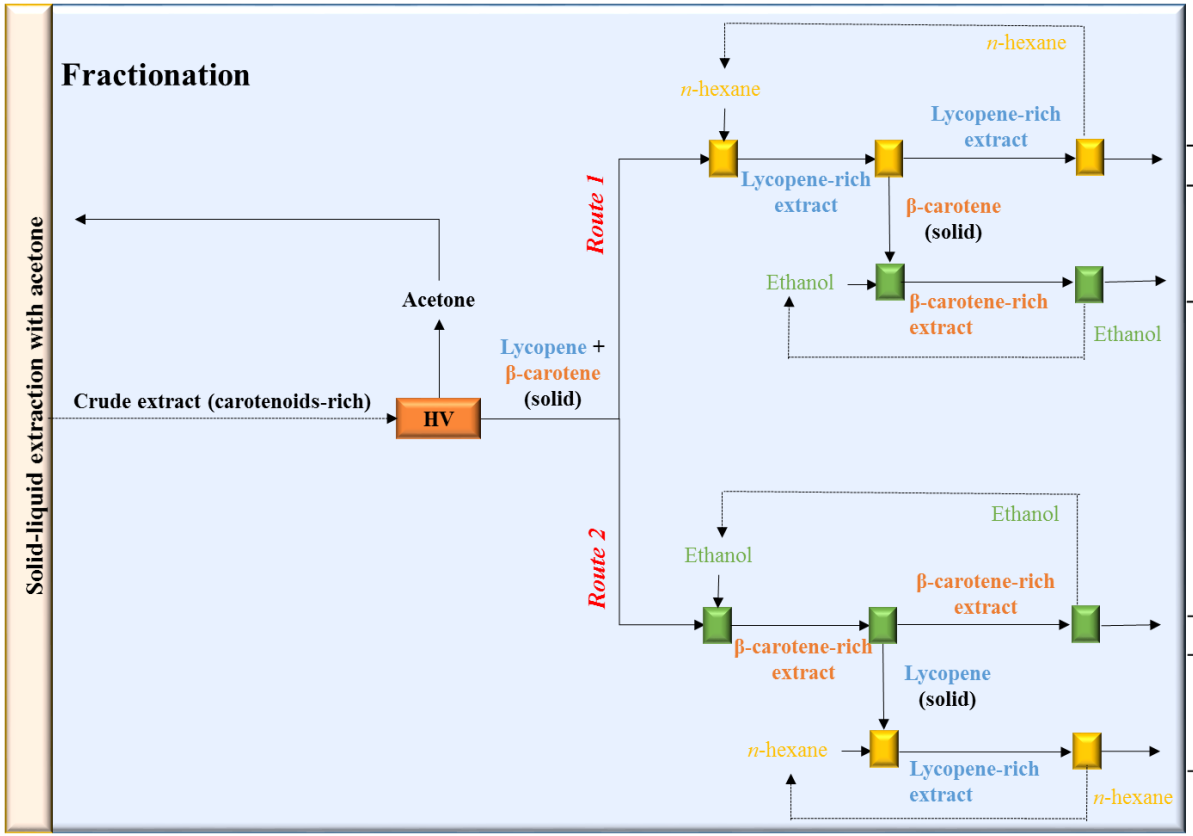
In this chapter, the main objective is to develop a selective process for the fractionation of lycopene and β-carotene previously extracted from tomato. In this sense, an integrated process diagram was idealized considering the main results collected in Chapter 3, followed by the study and proper development of an efficient fractionation route. The novel strategy herein implemented aiming at the fractionation of both compounds is based on high vacuum and on the distinct solubility of both target compounds in different solvents (as cautiously defined in the first chapter of this thesis). From the main results discussed in Chapter 3, acetone was shown to be the best solvent extracting both flavonoids simultaneously from the solid biomass of tomato. Moreover, and with the purpose of to isolate and purify both flavonoids, their separation was attempted through the alternate use of ethanol followed by *n*-hexane or *vice-versa*. As evidenced in Chapter 3, *n*-hexane exhibits an enhanced capacity to disrupt the cells of tomato when compared with ethanol, being therefore a more efficient solvent agent. In this chapter, these solvents were used as fractionation agents and their efficiency in the isolation of both flavonoids was carefully assessed. At the end, an integrated process was idealized taking into consideration the best solvents elected in Chapter 3, acetone, and the best fractionation route studied.

4.2.1. Fractionation studies

Two routes were adopted to carry out the fractionation studies, as aforementioned (Figure 15). Both routes start with the acetone extract rich in both flavonoids. The acetone is then completely evaporated being the solid sediment obtained from the acetone distillation used in both routes. Briefly, in Route 1, *n*-hexane is used to dissolve the solid sediment, then it is removed, and ethanol is immediately introduced in the flask containing the solid sediment remaining from the *n*-hexane dissolution step. Both crude extracts rich in ethanol and *n*-hexane were then analyzed and the respective

concentrations of both flavonoids in both extracts quantified. Route 2 is similar to Route 1, however the order of introduction of both solvents is the opposite, firstly ethanol is added and then *n*-hexane.

Considering the analyses to both routes (Figure 15), and since β-carotene has low solubility in *n*-hexane (solvent 1), it is possible to observe, as expected, a higher concentration of lycopene, as evidenced by the appearance of its two characteristic peaks at 470 nm and 502 nm in the visible spectrum, as described in literature *(134,135)* and confirmed by the standard spectrum (see Appendix B, Figure B.1.1.). However, the presence of β-carotene is also noticed (characteristic peak at approximately 450 nm). When adding the second solvent, *i.e.* ethanol, the spectrum profile remains similar, indicating the poor selectivity of this route of fractionation. It should be remarked that, the intensity of the peaks was lower than that obtained with solvent 1, an indication that the major part of the carotenoids has been recovered during its (*n*-hexane) addition. This is evidenced by the lycopene and β-carotene extraction efficiencies attaining values of 79.86 % to 56.61 %, respectively, whereas for the solvent 2 the values obtained were 2.98 % and 3.66 %, respectively. A loss of around 20-40 % of carotenoids is noticed, which can be associated to the method used, namely considering the potential significant impurity levels.



Route 1	EE (%)	
Solvent	**β-carotene**	**Lycopene**
n-hexane	56.61	79.86
Ethanol	3.66	2.98

Route 2	EE (%)	
Solvent	**β-carotene**	**Lycopene**
Ethanol	39.23	22.86
n-hexane	35.84	64.21

Figure 15: Schematic representation of the fractionation methodology involving Routes 1 and 2, along with the extraction efficiency data obtained.

Concerning Route 2, which allowed the understanding of the solvents' addition order, the spectrum obtained for ethanol (solvent 1) is depicted, indicating the presence of β-carotene (425 nm and 450 nm) in higher amounts than those of lycopene. This result proves the higher ability of ethanol to dissolve β-carotene. Contrarily to what was observed in Route 1, the spectrum profile obtained for the second solvent is adjusted by checking two characteristic peaks of lycopene with greater intensities at 470 nm and 502 nm. This analysis shows that considerable amounts of β-carotene are recovered with the first solvent. After the calculation of the respective extraction efficiencies it is concluded that 39.23 % of β-carotene and only 22.86% of lycopene were extracted with ethanol. Moreover, the second solvent extracts 35.84% of β-carotene and 64.21% of lycopene, indicating the accomplishment of a selective process. Here, it was observed a loss of around 13-25 % of the carotenoids, suggesting fewer losses, thus higher success of this route is attained. These data show an improved capacity of this method to be implemented in the fractionation stage.

4.2.2. Integrated process design

After the optimization studies carried out and discussed in Chapters 2 and 3, involving the solid-liquid extraction and the fractionation steps, respectively, an integrated process has been idealized. This comprises three main stages, namely ❶ solid-liquid extraction of the two carotenoids from tomatoes using acetone as the ideal solvent, ❷ the fractionation, *i.e.* separation of β-carotene from lycopene using ethanol and *n*-hexane in this specific order as selective solvents (Route 2) and, finally, ❸ the isolation of β-carotene and lycopene and reuse of the solvents. The schematic representation of the process developed is depicted in Figure 16. It should be noticed that Route 2 requires further optimization or additional stages to enhance the selectivity obtained. Although the stage ❸ was not focus of study, this was included in this process aiming at supporting its industrial relevance. This said, the solvents could be recycled and reused by HV at the same time that β-carotene and lycopene are precipitated.

This method yielded equivalent or superior extraction efficiencies at much lower times compared to processes currently most used. In the case of supercritical fluid extraction with CO_2, currently the technique most used *(136)*, extraction efficiencies at around 54% after 8-10 h of extraction were obtained *(137)* while in this study an extraction efficiency of about 61% for lycopene was attained after 1-2h.

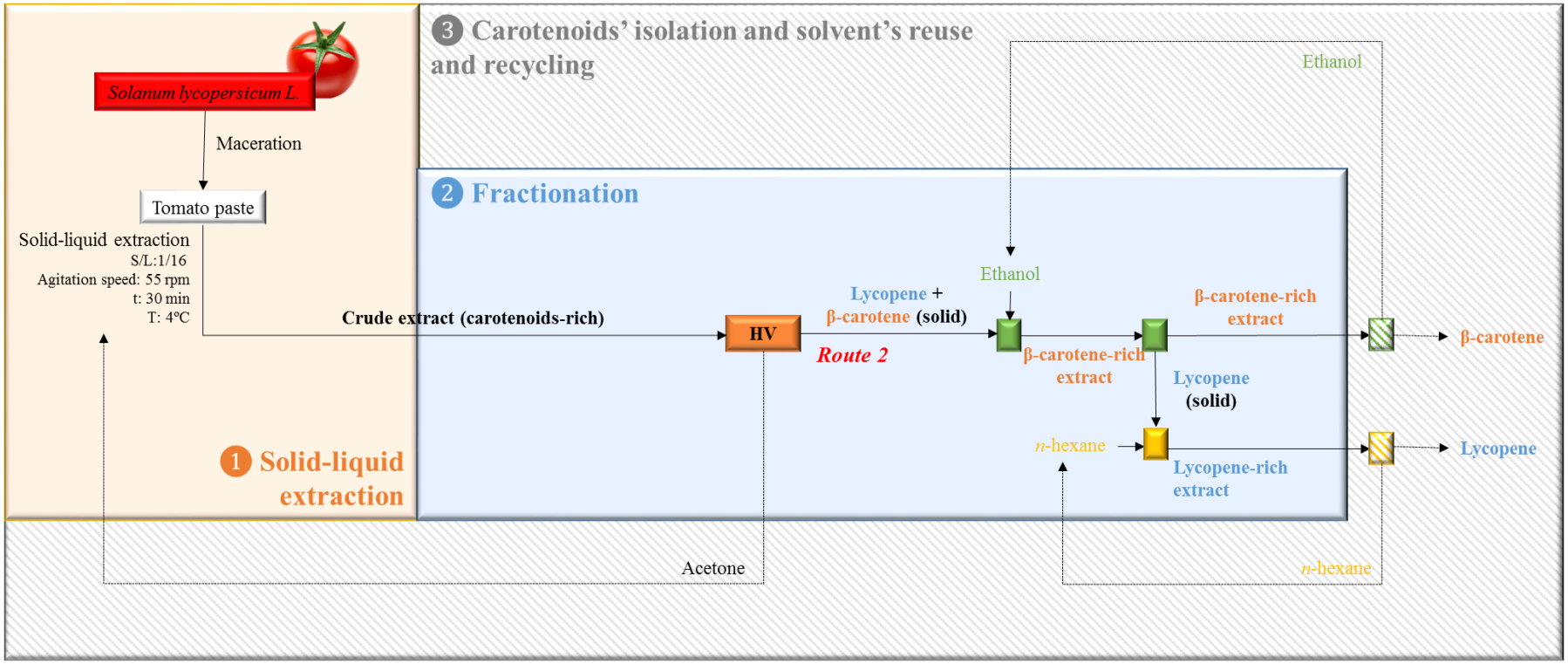


Figure 16: Process diagram for the selective extraction and purification of lycopene and β-carotene from tomato biomass.

4.2.3. Conclusions

In this chapter, it was possible to successfully accomplish the fractionation of lycopene and β-carotene by using ethanol and *n*-hexane, due their distinct solubilization abilities. In order to explore their potential application, the solvents' addition order was the condition varied, being shown to be of utmost importance. The extraction efficiencies for β-carotene and lycopene were approximately 35.23% and 24.68%, respectively, by the addition of ethanol followed by 35.84 % and 64.21 %, respectively (Route 2). These results indicate that certain selectivity is possible to be achieved, although deeper studies are required in the future. It should be remarked that the order of the solvents' addition (Route 1 *versus* Route 2) is relevant to achieve a successful fractionation step. At the end, it was possible to anticipate an integrated process with considerable performance, sustainability and industrial relevance.

5. FINAL REMARKS

5.1. General Conclusions

After the preliminary tests, it was found that the solvents with the highest potential to extract lycopene and β-carotene from fresh tomato were acetone and tetrahydrofuran rather than the remaining organic solvents and aqueous solutions of ILs, conventional salts, surfactants/copolymers and polymers. These results are justified by their higher aptitude to act as disrupting agents of the cytoplasmic membrane of tomato. This factor, combined with the polarity index of the solvents, was given evidences for the successful development of the solid-liquid extraction process. An attempt to fractionate the two target carotenoids was also made. It was shown that a continuous process adding ethanol followed by the addition of *n*-hexane (by this specific order) is considerably efficient at selectively isolating β-carotene and lycopene. In the end, these two steps (solid-liquid extraction and fractionation) were introduced in an integrated process of industrial relevance. Furthermore, the possibility of recycling and reuse the solvents diminishes the environmental footprint as well as costs of the process designed. This process requires fewer steps and it is a simpler and less expensive option compared with the conventional methods already reported in literature *(23)*.

5.2. Future work

Based on the results of this study, it will be interesting to further explore the development of the fractionation step. Route 2 here developed needs further optimization, for instance in the volume of solvents added, or even in the introduction of additional steps to increase the selectivity attained.

Finally, and in order to further support the sustainable character of the selective process developed, it will be important to actually carry out the entire process idealized in Figure 16. Hereafter, high Pressure Liquid Chromatography (HPLC) is needed for reliable analysis of samples, since this is a more versatile, sensitive and selective method. Additionally, true wastes should be used at this level, since in this study only model biomass was adopted. The evaluation of the purity and stability of the carotenoids purified as well as the analysis of the solvents aptitude to be reused, *i.e.* if they keep the efficiencies and how many times they can be recycled should also be addressed.

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Appendix A

Experimental data for the determination of the calibration curves

A.1. Determination of calibration curve for lycopene and β-carotene using acetone

The method that was used in this work for lycopene and β-carotene quantification uses UV-Vis spectrophotometry. The absorption spectra within a wavelength range between 330 nm and 600 nm were always acquired, since this is the range described in literature as the displaying the maximum absorption peaks of both carotenes *(138)*. All samples were analyzed in triplicate. The standard solutions were prepared from an initial solution at *circa* 20 μg mL^{-1} of lycopene and 18 μg mL^{-1} of β-carotene in acetone. Samples with lycopene concentration at *circa* 3.7 μg mL^{-1}; 2.5 μg mL^{-1}; 1.2 μg mL^{-1}; 0.75 μg mL^{-1}; 0.5 μg mL^{-1} and 0.25 μg mL^{-1} and β-carotene concentration at *circa* 3.3 μg mL^{-1}; 2.3 μg mL^{-1}; 1.1 μg mL^{-1}; 0.6 μg mL^{-1}; 0.45 μg mL^{-1}; 0.22 μg mL^{-1} were obtained and analyzed using a Shimadzu UV-1700, Pharma-Spec Spectrometer. At the end, it was possible to accurately determine each calibration curve, as depicted in Figures A.1.1 and A.1.2, with $r^2 > 0.99$. The absorbance output at the maximum absorbance peak, at an approximate wavelength of 470 nm for lycopene and 450 nm for β-carotene were those considered in the construction of each specific calibration curve. As lycopene and β-carotene pure standards were not available, a mixture of both (Licopeno Complex from NaturMil) was utilized. The preparation of the initial solution was thus done by considering the information taken from the capsules flyers. It should also be stressed that the volumetric flasks were always covered with aluminum foil in order to avoid any degradation of the target compounds. Furthermore, whenever possible (*i.e.* it was possible to guarantee complete solubilization of lycopene and β-carotene in the solvent) the calibration curves were also performed using other organic solvents, hampering influences coming from the extracting solvent investigated within the quantification. In preliminary studies calibration curves were prepared with four solvents, namely petroleum ether, n-hexane, acetone and THF. However, the differences between the curves were not significant and therefore the choice of acetone had to do with its greater ability to penetrate the biomass in comparison with the other two solvents, and able to dissolve carotene and lycopene.

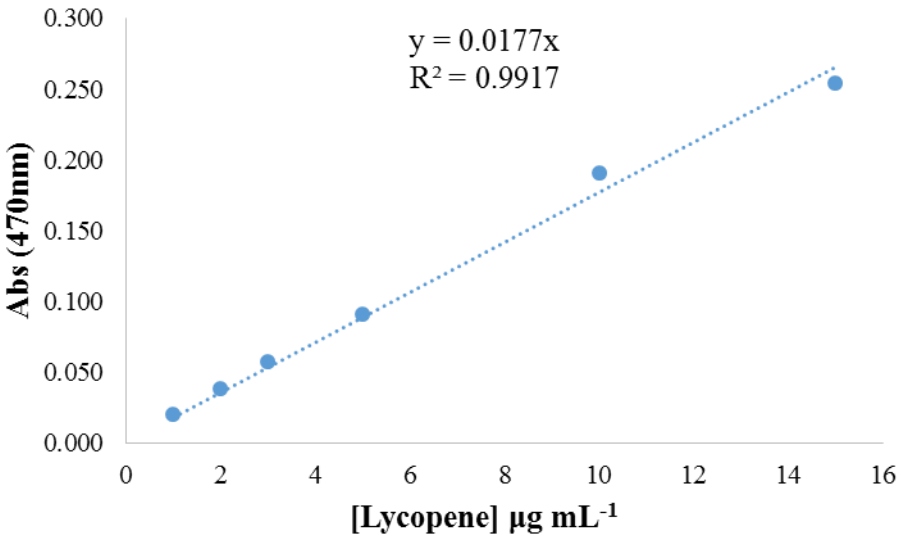

Figure A.1.1- Calibration curve obtained for lycopene in acetone by UV-Vis spectroscopy.

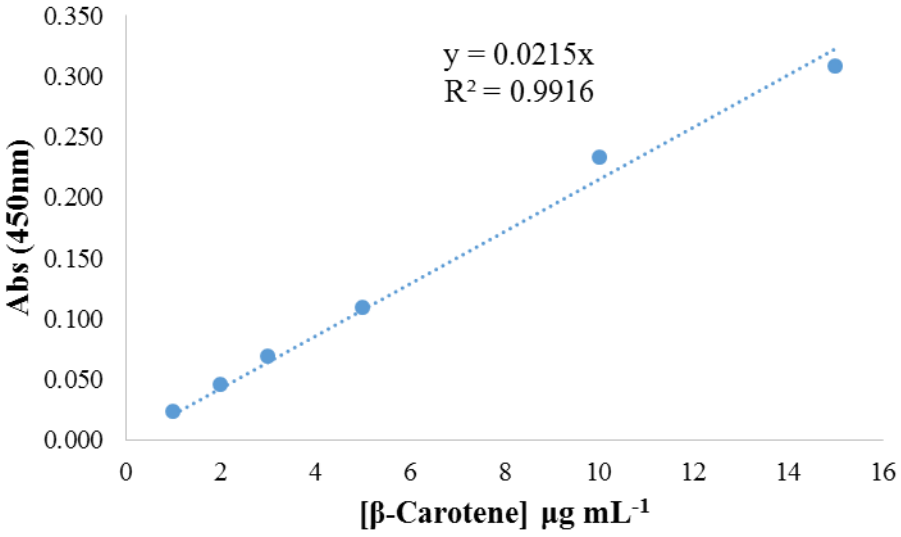

Figure A.1.2- Calibration curve obtained for β-carotene in acetone by UV-Vis spectroscopy.

Appendix B

Preliminary fractionation assays of lycopene and β-carotene from capsules using *n*-hexane and ethanol

B.1. Experimental procedure to determine the characteristic spectra of lycopene and β-carotene in *n*-hexane and ethanol useful for fractionation studies

The capsules used in this study were Licopeno Complex from NaturMil (coated and containing 5.0 mg of lycopene and 4.5 mg of β-carotene as aforementioned). The powder was directly weighed into a volumetric flask of 250 mL to reduce losses and *n*-hexane or ethanol. Each flask was subjected to stirring for about 30 minutes at 4 ° C. Due to the other compounds present in the capsule (including insoluble excipients) the obtained solution was filtrated using syringe filters of 45 μm of pore size. The filtered solution was analyzed in a spectrophotometer (Shimadzu UV-1700, Pharma-Spec Spectrometer). The spectra obtained are displayed in Figure B.1.1. Using the *n*-hexane, the higher absorbance value occurs at 470 nm, characteristic wavelength of lycopene, whereas in ethanol at 450 nm the maximum absorbance peak is indicative of β-carotene presence. This phenomenon runs counter to that described in the literature *(134,135)*, showing the low solubility of β-carotene in n-hexane and the inability to solubilize lycopene using ethanol *(139)*.

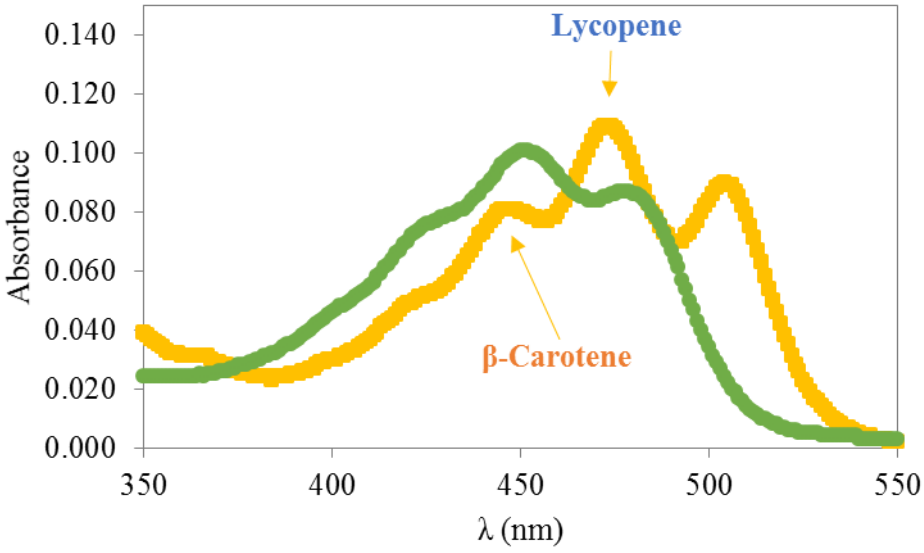

Figure B.1.1-Standard spectra of lycopene and β-carotene in *n*-hexane (yellow) and ethanol (green) obtained by UV-Vis spectroscopy.