University of Reading

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EXTRACTION AND PURIFICATION OF CAROTENOIDS FROM VEGETABLE WASTES: AN INTEGRATED APPROACH

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ABSTRACT

Vegetable by-products are abundant in interesting phytochemicals, such as phenolics and carotenoids, which exhibit high antioxidant activity and are valuable in the production of foods, chemicals and cosmetics. Different techniques have been used for the extraction of these molecules, with Supercritical Fluid Extraction (SFE) being one of the most sustainable, efficient and reliable approaches to this end. A variety of fruit and vegetable matrices have already been successfully extracted by SFE, including the targeting of such bioactives. However, there is a lack of studies dealing with further purification of the extracts for obtaining more valuable fractions that could potentially find finer applications as natural additives in the food and nutraceutical sectors. The aim of this work was to assess and optimise an integrated protocol for carotenoid extraction and purification from carrot peels and study the application of the optimised conditions to other carotenoid-rich vegetable matrices. The extraction process was evaluated through a Central Composite Design of Experiments at different temperatures, pressures and co-solvent concentrations, as well as by kinetics experiments and modelling, scalability potential and extract characterisation. The statistical and kinetic extraction models were validated successfully and the optimised conditions were: temperature 59.0 °C, pressure 349 bar, 15.5% ethanol, 15 g/min of CO_2 flow rate, and total extraction time of 30 min. These resulted in a carotenoid recovery of 86.1% and the process was shown to be potentially scalable, since recoveries as high as 96.7% were observed in runs performed with 10-fold the initial sample mass. Furthermore, the supercritical-fluid carotenoid-rich extracts were purified by Hydrophobic Interaction Chromatography, through the optimisation of batch and in-column adsorption parameters such as resin capacity, kinetics and elution. The adsorbent employed in

the purification stage showed an adsorption capacity of 10.4 μg of carotenoid per mg of resin and the global yield of the process was of 88.4% for total carotenoids and 92.1% for carotenes. Analyses of the final eluate confirmed the evolution of the purification, leading to a 2.1 and 4.7-fold increase in antioxidant activity and carotenoid concentration, respectively. Finally, the SFE-optimised extraction conditions were extrapolated to other vegetables samples, inherently rich in carotenoids. The model was deemed applicable to other vegetables, such as sweet potato, apricot, pumpkin, green and yellow pepper. It can be concluded that the protocol set up in this work can be employed with confidence to efficiently extract and purify carotenoids from vegetable matrices and represents a tangible alternative for waste valorisation.

DECLARATION OF AUTHORSHIP

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Mičael de Andrade Lima

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TABLE OF CONTENTS

LIST OF FIGURES

Appendices

LIST OF TABLES

Appendices

LIST OF ABBREVIATIONS

A_{1E}	Maximum attainable mass (g)	C_{eq}	Concentration in liquid phase
AA	Antioxidant Activity (%)	*CER*	Constant Extraction Rate
AC	Affinity Chromatography	*COM*	Cost of Manufacture
ABPR	Automated Back Pressure Regulate	*ChlB*	Chlorophyll B
ACar	α-carotene	*CoSol*	Co-solvent
Acet	Acetone	*C-REC*	Carotenoid recovery (%)
ADS	Adsorption stage	*CV*	Coefficience of Variance (%)
ADS %	Adsorbed fraction	D_{az}	Axial dispersion coefficient
APF	Apricot flesh	*DC*	Diffusion controlled stage
APP	Apricot peel	*DES*	Desorption stage
A_T	Column total area	*DoE*	Design of Experiments
b	Indicator of adsorption capacity	*DPPH*	2,2-diphenyl-1-picrylhydrazyl
B_{1E}	Mass transfer constant	*EtOH*	Ethanol
BCar	β-carotene	*EtOH%*	Ethanol concentration (%)
BHT	Butylated hydroxytoluene	*Ext*	Extract
BTC	Breakthrough curve	*FER*	Falling Extraction Rate
C	Final concentration (μg/g)	*GPF*	Green pepper flesh
C_0	Initial concentration (μg/mg)	*GRAS*	Generally Regarded As Safe
C_1	Esquivel's model constant	*HIC*	Hydrophobic Interaction Chromatography
C_2	Esquivel's model constant	*IEC, IEX*	Ion Exchange Chromatography
CAP	Capsanthin	*IsoProp*	Isopropanol
CCD	Central Composite Design	*J*	Interfacial mass flux

k, K_d	Langmuir dissociation constant	*SEC*	Size Exclusion Chromatography
K_y	Overall mass transfer coefficient	*SFE*	Supercritical Fluid Extraction
LUT	Lutein	*SPF*	Sweet potato flesh
LYC	Lycopene	*SPP*	Sweet potato peel
MeOH	Methanol	*STD*	Standard solution of β-carotene
n	Adsorption energy	*T*	Temperature (°C)
OEC	Overall Extraction Curve	T_c	Critical temperature
P	Pressure (bar)	*TCC*	Total carotenoid content
P_c	Critical pressure	t_{CER}	Time of CER phase (min)
PCF	Peach flesh	*TCR*	Total carotenoid recovery (%)
PCP	Peach peel	t_d	Dead time (min)
PF	Purification factor	t_E	Total extraction time (min)
PKF	Pumpkin flesh	t_{FER}	Time of FER phase (min)
PKP	Pumpkin peel	t_M	Time of max. extraction rate (min)
q	Adsorbed amount (μg/mg)	*TMF*	Tomato flesh
Q	Flow rate (mL/min)	*TMP*	Tomato peel
*q**	Resin adsorption capacity μg/mg	*V*	Volume of the column (m^3)
Q_{CO2}	CO_2 flow rate (g/min)	*v*	Solvent velocity (m/s)
q_m	Number of adsorption sites	*VLX*	Violaxanthin
q_{max}	Maximum resin capacity (μg/mg)	*XPW*	Pepper waste
Re	Reynolds number (-)	*Y*	Global mass yield (%)
RPF	Red pepper flesh	*Y**	Solute solubility in S-CO_2
RSM	Response Surface Methodology	*YPF*	Yellow pepper flesh
S-CO_2	Supercritical CO_2	*ZEA*	Zeaxanthin

Greek letters

ε	Bed porosity (-)
ρ	Density (kg/m^3)
ρ_s	Solvent density (kg/m^3)

THESIS PRESENTATION

This thesis is organised in six chapters, with the experimental results being compiled and presented as a collection of individual manuscripts, each of which dealing with a different stage of the integrated protocol proposed. There is also an Appendix section, where complementary data can be found. Thus being, the breakdown of the entire work is as follows:

In **Chapter 1**, the scientific context in which this work is inserted is presented as a short discourse on the current situation of the extraction and purification of bioactive molecules along with its challenges and weaknesses. Also, the main aim of the work is presented, as well as the individual objectives that need to be addressed in order to successfully build and validate the proposed process.

In **Chapter 2**, a comprehensive Literature Review is put together to introduce the extraction and purification techniques to be employed: Supercritical Fluid Extraction (SFE) and Hydrophobic Interaction Chromatography (HIC), respectively. The principles, factors of influence, state of the art, limitations, current applications and the relevance/potential to this specific study are all discussed.

Chapter 3 shows the first set of experimental results, which deals with SFE as the extraction technique for carotenoids from carrot peels. This encompassed an extensive study of parameter optimisation via Design of Experiments and statistical validation, experiments of extraction kinetics, mathematical modelling, scalability potential and extract characterisation.

Following on the aforementioned results, **Chapter 4** presents a novel purification protocol for the supercritical-fluid carotenoid extract employing preparative adsorption by HIC. Batch experiments identify important resin and kinetic

parameters and subsequent in-column assays optimise and validate the semi-continuous purification process by quantifying recoveries, activities and purity degree of the final fractions.

In **Chapter 5**, the optimum extraction conditions from Chapter 3 are applied to other carotenoid-rich fruit and vegetable matrices to assess the feasibility of using these conditions as a predetermined set of values for extracting carotenoids by SFE and discusses how this is limited by compound polarity and vegetable composition.

The thesis is finalised in **Chapter 6**, where the implications on and applications of this work in the food, chemical, nutraceutical and pharmaceutical industries are discussed, along with its challenges, limitations and necessary future improvements.

Additional data related to and mentioned in the experimental chapters can be found in the form of **Appendices**, placed at the very end of the thesis. These provide detailed information that can aid referencing for future replications.

CHAPTER 1

INTRODUCTION

The utilisation of vegetable processing residues or wastes as starting materials for the extraction of natural compounds represents a promising route towards reducing the environmental impact of current waste management activities such as landfill disposal and incineration. Conventional extraction methods primarily involve the use of relatively toxic solvents, such as hexane, dimethylformamide (DMF), and ethyl ether, which can potentially lead to considerable environmental issues and constitute time-consuming operations. The significant commercial interest for the extraction of bioactive compounds from natural resources and by-products combined with the need for sustainable approaches has led to the exploration of Supercritical Fluid Extraction (SFE) technology as a very effective means of extraction.

SFE is acknowledged as an efficient, fast and environmental-friendly method that has currently been actively used, among other applications, for extracting bioactive compounds from various matrices. In order to improve the efficiency of the extraction of such molecules from complex structures, as is the case with vegetable wastes, there is a need to understand not only the mass transfer phenomena taking place during SFE but also the factors of influence to the specific process and how this correlates to the macromolecular composition of such wastes. Among the various types of vegetable waste, carrot waste is a pertinent example where SFE can be applied for the extraction of added-value compounds, such as carotenoids, which can be used in the food and nutraceutical and cosmetic industries as natural pigments, aroma and flavour precursors and imparters of antioxidant properties.

Also, due to the scarcity of studies dedicated to the downstream processing of SFE extracts, the recovery and purification of the molecules extracted constitute highly relevant knowledge to the area. For the targeting of carotenoids, a potential

technique to this end is Hydrophobic Interaction Chromatography, which is relatively cheap and easy to set up. The development of an integrated process of extraction and purification is scientifically and economically interesting, since this can undoubtedly potentialise the process as a whole through the yielding of final extracts with a high degree of purity, which are valuable commodities on an industrial level.

The overall aim of this PhD is to set up an efficient valorisation route for carrot waste, with potential application to other vegetable residues, involving the integrated extraction of carotenoids by Supercritical CO_2 and the purification of the obtained extracts by Hydrophobic Interaction Chromatography.

In order to meet this aim, the following objectives are established:

- Analysis of the macro and micro composition of carrot peels to generate descriptive and comparative data;
- Optimisation study via Design of Experiments and Response Surface Methodology to identify the best SFE conditions (temperature, pressure and co-solvent concentration) for mass yield and total carotenoid recovery;
- Analysis of the extraction kinetics through mathematical modelling to evaluate the solubility and the mass transfer rates pertaining the extraction of carotenoids from the solid matrix;
- Assessment of scalability potential to confirm the model reliability and extraction behaviour when working with larger amounts of sample;
- Compositional analysis of the extracts as to protein, lipid, carbohydrate and micronutrient content for characterisation purposes, envisaging the future purification protocol;

- Establishment of a thorough purification protocol for carotenoids using Hydrophobic Interaction Chromatography, including studies in batch and in column, by assessing and optimising all the parameters inherent in this process;
- Analysis of the final extracts as to chemical composition, carotenoid recovery, purification degree, antioxidant activity and degradation rates.
- Extrapolation of the conditions optimised for carrot peels to other carotenoid-rich vegetable matrices to assess the possibility of their generalisation and their correlation to different vegetable structures.

In the next Chapter, a thorough literature review on the theoretical and practical aspects of the techniques of extraction and purification employed in this work is presented. The underlying principles and parameters of influence, applications and the state of the art are summarised and discussed in order to identify the challenges and build the scientific background necessary to execute and complete the work proposed.

CHAPTER 2

LITERATURE REVIEW

2.1 WASTE MANAGEMENT AND VALORISATION APPROACHES

One of the main challenges in the food industry is undoubtedly waste management. Pre-consumer waste, generated from post-harvest to food processing, are produced at a large scale annually worldwide. Post-harvesting and processing waste is an ongoing issue and, in some countries, such as the USA, it can account for over 50% of the total waste produced, with 60% of this figure consisting of organic matter. This renders their disposal energy and chemical intensive [1]. The Food and Agriculture Organization of the United Nations (FAO) reported that roughly one third of all the food produced in the world for human consumption every year, which accounts for approximately 1.3 billion tonnes, is lost or wasted. In Europe alone, over 90 million tonnes of food-related waste are disposed of annually [2].

There are different types of food waste and their classification is based on the stage of the production chain where they are generated [3]. Vegetable waste can originate in agricultural production, where losses occur due to mechanical damage and/or spillage, during harvest or post-harvest operations. Additional losses can also take place during transportation between the farm and the distribution centres or during industrial processing (when crops are sorted out, during peeling, slicing and boiling, due to process interruptions and accidental spillage, etc.). Distribution (e.g. at wholesale markets, supermarkets, retailers and wet markets) and consumption (including losses on a household level) also contribute to waste generation [4]. The waste produced in the vegetable sector can encompass the whole product, as well as damaged, rotten or non-edible parts, in the form of peels, tubers, roots, seeds, bagasse and pomace.

It is a fact that numerous industry sectors have already started taking actions to reduce food waste across all the stages on their production chain and making sure the waste that is generated receives the adequate treatment before being discarded on the environment or given another proper end. However, the bodies responsible for a major pressure on industrials and individuals for that mindset to be adopted are still national and local governments, with their environmental policies and legislations [5]. For instance, regarding Europe, the European Commission adopted in 2014 a communication and a legislative proposal to review the current recycling and other waste-related goals [6]. Australia has become the first country to set a target to cut the food waste it generates by 50% by the year 2030 [7]. In South Korea, a new policy was adopted in 2013 in Seoul that made households pay for recycling according to the amount of food they discarded. Since then, the volume of waste has decreased by 10% (more than 300 tonnes a day), in comparison to five years ago [7].

The UK is ranked seventh in the world production of carrots, and as such, carrots are a pertinent contribution for waste generation in the country. During the processing stages, around 11% of the vegetable is lost in the form of peels, tubers, and attached flesh, which results in 70,000 tons of by-products [8]. Carrot waste possess a variety of valuable components, such as carotenoids and phenolic compounds, that exert a range of functional properties and can be potentially extracted and exploited as added-value ingredients.

Due to the high volumes of fruit and vegetable wasted, developing and establishing waste management practices in order to minimise the impact on the environment and add value to by-product streams is of high importance. As previously

mentioned, fruits, vegetables and the by-products thereof are rich in a variety of compounds including carbohydrates (e.g. dietary fibre, oligosaccharides), aromatic compounds and phytochemicals (e.g., polyphenols, glucosinolates, carotenoids). Among these, polyphenols, and more specifically phenolic acids are highlighted, due to their inherent antioxidant properties and potential benefits to human health, as they have been associated with the prevention of asthma, diabetes or cancer [9–11]. Another very important group of phytochemicals that have attracted considerable interest is carotenoids. These compounds are natural pigments that constitute essential nutrients in the human diet, and exert antioxidant and potentially cancer-preventive properties [12–14]. Carotenoids have found applications in the food sector, as food ingredients and natural additives, as well as in the cosmetic, personal care and nutraceutical sectors.

The extraction of phytochemicals from vegetable matrices is commonly carried out with the aid of conventional chemical solvents, due to their ease of use, efficiency, relatively low price and wide applicability [15]. In the case of carotenoids, different solvents, such as acetone, methanol, ethanol, hexane and tetrahydrofuran are commonly used due to the non-polar character of most of these molecules [16].

With the growing environmental concern and the advent of green technologies, new methods for extracting these classes of phytochemicals have been suggested and are currently being investigated, including microwave and ultrasound assisted extraction (MAE, UAE), subcritical water extraction (SWE), enzymatic extraction and supercritical fluid extraction (SFE) [17–20]. SFE employs mainly supercritical CO_2 and is a method for which considerable knowledge and skills already exist in terms of process engineering and design, as it is used commercially for the

decaffeination of coffee and tea [21,22], the extraction of flavours, lipids and alcohol [19] and speciality bioactives for cosmetic applications [23]. In the next section, a detailed description of the technique is provided along with its principles. Also, its potential within the area of fruit and vegetable waste valorisation is discussed.

2.2 SUPERCRITICAL FLUID EXTRACTION: FUNDAMENTALS

2.2.1 Introduction

The traditional methods used for the extraction of phytochemicals from fruit and vegetable matrices have been intensively studied, and for some technologies, such as liquid-liquid or solid-phase extraction, the processes are already well developed and documented. However, many of these methodologies involve extractions with conventional organic solvents which, despite their efficiency, represent a major environmental concern due to the toxicity of some of these solvents. For instance, formic acid, ethyl acetate, acetonitrile and dimethylformamide (DMF) have been deemed very harmful to the environment because they have a low net calorific value, which implies low environmental credits in their incineration and residue treatment, combined with the imposition of a relatively high environmental impact during their production [24]. Hexane and toluene have been reported as being strong emitters of greenhouse gases during their manufacturing and especially when they are oxidised in the environment or in an incinerator [25]. Other downsides of employing such solvents include the fact that extractions require several hours to achieve satisfactory recoveries and that the final solutions are often dilute and, therefore, require additional concentration steps, which could result in degradation and loss of the compounds of interest. Thus, the rising concerns with regards to environmental issues has led researchers to focus on developing 'green' technologies that, while having a minimum impact on the environment, can also be highly efficient, safe, scalable and economically viable.

Supercritical Fluid Extraction (SFE) is a fast, effective and 'clean' method for the extraction of natural products from plant matrices, such as fruits and vegetables. The ease of tuning the operating conditions in order to increase the solvation power

of the extracting fluid renders SFE a promising choice for the recovery of several types of substances. The technology is an important process in a number of different industries due to the possibility of delivering products without toxic residues, with no degradation of the active components and in some cases, with considerable purity. Studies on the extraction of essential oils, phenolic compounds, carotenoids, tocopherols, tocotrienols, alkaloids and others classes of chemical compounds have already been published [19]. Also, several matrices have been used, such as seeds, fruits, leaves, flowers, rhizomes, roots, peels of fruits, and even branches of trees [26].

The application of supercritical fluids dates back to the second half of the 19th century, and has been first documented by Hannay and Hogarth [27], who studied the solvation power of ethanol under supercritical conditions for dissolving chloride, iron, potassium bromide and potassium iodide. However, no earlier than almost one century later, when it was used for the first time to decaffeinate coffee beans [28], did the technique receive considerable attention by the scientific community. A number of industrial applications have been developed and are now widely used, including the removal of fat from milk, extraction and recovery of flavours, aromas and volatiles from natural products (including de-alcoholisation of beverages), production of herb extracts and removal of pesticides from vegetables [19].

2.2.2 SFE Principles

2.2.2.1 Overview

The supercritical state of a fluid (either a liquid or a gas) refers to the distinctive state attained when it is subjected to temperatures and pressures beyond its critical point. A critical point is defined as the characteristic temperature (T_c) and pressure (P_c) above which the distinction between the gas and liquid phases no longer exists [29]. In supercritical state, the specific properties that distinguish gases from liquids seem to merge in such a way that the supercritical fluid can no longer be liquefied by modifying its temperature or pressure. A supercritical fluid possesses gas-like properties of diffusion, viscosity and surface tension, and liquid-like density and solvation power (the capability of having an ionic or polar compound dissolving in a nonpolar substance). The combination of these properties renders SFE highly suitable for extracting compounds within a short time and with better yields, when compared to conventional liquid-state solvents [29].

Figure 2.1 shows a simplified overview of a typical SFE system. The system works as follows: First, the solvent pump starts driving the solvent through the line into the heat exchanger, so that the solvent can reach its critical conditions. It then flows into the pressurised extraction vessel, where it is uniformly distributed throughout the fixed bed (the dried and milled matrix). The extraction is then initiated, the soluble compounds start dissolving in the solvent throughout the process and the phases are separated later in the collection vessels, or separators, due to a sudden pressure drop to atmospheric pressure (or, alternatively, a fast temperature rise). The solvent is then cooled, recompressed and driven back into the storage tank.

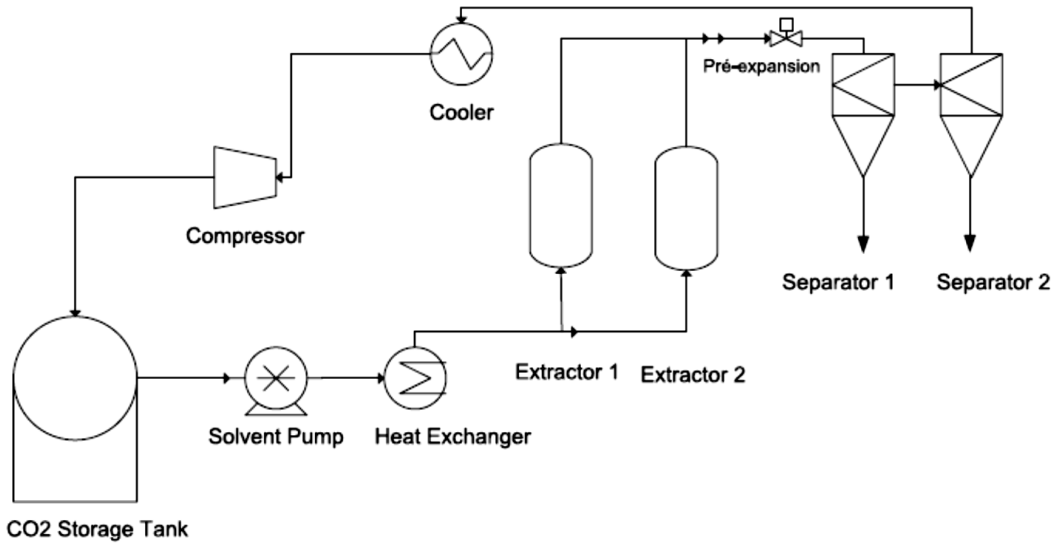


Fig. 2.1 Simplified diagram of an SFE unit (Source: [26])

The extraction efficiency may vary, depending on factors such as the composition, moisture and particle size of the matrix, the nature of the target compounds, the co-solvents used, the operational temperature and pressure and the system design. Most of these factors are discussed in detail in the following sections.

2.2.2.2 Solvents

most frequently used solvent in SFE is carbon dioxide and this is due to its inherent characteristics. Its critical temperature of 31 °C, very close to room temperature, along with a low critical pressure of around 74 bar [26], grants the possibility of performing extractions under moderate conditions, which is advantageous for both minimising the impact of extraction on thermolabile compounds and reducing operational costs. Other beneficial properties of CO_2 include its low cost, capability of solvent recovery, recognition as safe (GRAS status), its nontoxic and non-flammable nature, non-reacting with the food matrix, and easy separation from the

target compounds. Several other solvents that can be used under supercritical conditions include water, ethanol, methanol, propane and ethane [26].

2.2.2.3 Co-solvent

The main drawback of carbon dioxide is its very low polarity which, in spite of making it theoretically ideal for the extraction of neutral lipids, oils, carotenoids, and many other non-polar substances, renders it unsuitable for the extraction of more polar compounds, such as polyphenols and carbohydrates or for high-molecular-weight compounds. Fortunately, this is a limitation that can be easily overcome by employing a modifier (also known as 'co-solvent' or 'entrainer'), such as methanol, ethanol and water. Usually, just a small amount (around 2 – 10%) of co-solvent is enough to significantly enhance the polarity of carbon dioxide and allow the interaction of more polar or heavier compounds with the solvent [23].

The application of co-solvents in SFE has been strongly dominated by ethanol. It is an innocuous solvent for human health and the environment, and as such, presents great advantage compared to hexane or even methanol, particularly when SFE is intended for applications in the food, cosmetic or pharmaceutical industries. Being substantially polar, the addition of small amounts of ethanol increases considerably the polarity of the supercritical solvent, usually resulting in higher extraction yields [19]. Methanol can also be used to this end, as it is even more polar than ethanol, but its use raises hazard concerns for the human health and for that reason, it is not as broadly employed.

The addition of the co-solvent to the system is more commonly implemented by an independent pump, connected to the main solvent pump (so that both can enter the

extractor at the same time), with its concentration controlled by the workstation. Alternatively, the co-solvent can be mixed along with the samples in the extraction vessel. The amount of co-solvent will vary from case to case and can be quantified through process optimisation studies.

2.2.2.4 Temperature and pressure

Pressure (*P*) and temperature (*T*) are undoubtedly two major parameters in SFE processes. The main effect imposed by the *P vs. T* binomial is the change of solvent density, which is directly correlated to its capacity of dissolving the solutes throughout the extraction.

De Melo et al. (2014) [19] compiled information from a large number of articles in the literature and it was shown that the vast majority of the works employ pressure values from 100 to 400 bar, with the absence of higher pressure values being attributed to equipment limitations. In terms of temperature, most works report values between 40 – 60 °C, a relatively narrower region compared to pressure. Relevant research in literature has been mainly focused on exploring the effect of pressure at lower temperatures, probably due to the higher degree of density variation under these conditions, but also due to fact that high temperatures lead to degradation of some thermolabile compounds.

2.2.2.5 Solvent flow rate

Flow rate (*Q*) is another noteworthy factor since a number of parameters, such as the axial dispersion, the convective mass transfer coefficient, and the concentration

in the fluid phase, are strongly influenced by this variable. In practical terms, if a low interstitial velocity (flow rate) is employed, the film resistance and/or the accumulation in the fluid phase may prevail over intraparticular diffusion and solubility, and eventually compromise extraction yields. On the other hand, if the extractor is run under very high interstitial velocities, there will also be a decrease on the yields, due to an insufficient contact time between the solute and the solvent [26]. Also, high velocities may also lead to an overuse of SC-CO_2 that will then be spent in excess and result in higher utility and energy costs. All the above parameters are deemed important in order to increase the economic viability of a SFE process [19]. According to De Melo et al. [19], common flow rates can usually vary from less than 1.0 up to 140.0 g_{CO2}/g_{sample}.

2.2.2.6 Sample properties

Some studies have reported the effect of other variables inherent in the samples being extracted. In most cases, decreasing the particle size favours the extraction, as observed in the extraction of oil from fennel [30], and of volatile oils from coriander seeds [31]. Lower particle sizes result in a greater area of contact between the S-CO_2 and the substrate and consequently, in less substrate being trapped in the non-accessible regions of the solid insoluble matter.

The moisture content of samples has also been shown to have an effect [32]. Theoretically, the water present in vegetable matrix competes with the solute and interacts with the solvent, decreasing the yield of the process. For this reason, drying the raw material in sample preparation steps is a common practice. However, in some cases, the presence of low concentrations of water is necessary to allow

interactions of the solvent with the solute, as in the extraction of caffeine from green coffee beans, or due to its role in the swelling of the solid matrix cells, which facilitates the flow of the solvent into the cell [26]. Freeze-drying is a very common technique to this end due to the high-quality dry materials obtained with this practice. However, the costs associated with the process can easily compromise the economical viability of the protocol and therefore, other drying techniques could also be assessed.

2.2.3 Optimisation of conditions

An important step in developing an SFE protocol is the optimisation of key operating conditions in order to enhance the extraction of targeted compounds. The selection of these conditions will depend on the specific targeted compound or compound family and on the raw material used. The solvating power of a supercritical fluid is known to be directly linked with pressure and temperature; therefore, these two factors are considered critical. Besides the solvation power of the solvent, the extraction time is another key parameter to increase yields and productivities [26]. Fluid flow rate, moisture content, co-solvent choice and concentration are also parameters to be considered for process optimisation, which also influence the process economics.

Optimal conditions for extraction can be achieved using statistical, kinetic and/or thermodynamic models, which aid in understanding and describing the extraction process. The use of such tools is one of the most common strategies in order to set up robust extraction methods [33].

Statistical modelling is used to identify the critical values of variables that impose a real influence on the process, and as such, is commonly employed for optimisation purposes. Central Composite Designs [34–40], Box-Behnken Designs [41–45] and Orthogonal Arrays [46,47] are commonly employed tools in SFE. Among these, Response Surface Methodology (RSM) seems to be widely accepted as the method of result presentation, since the obtained 3D graphs offer a good visual representation of the optimised regions. Such models can be obtained with a Design of Experiments (DoE) approach, where the parameters to be tested are decided along with their levels and a batch of experiments is carried out under the different pre-set combinations of those conditions. The results are then submitted to statistical software for data analysis and model fitting.

Studies on mass transfer (kinetics) and phase equilibria (thermodynamics) are also necessary, and these phenomena should be taken into account to enable a more detailed understanding of the underlying extraction mechanisms, inherent limitations, predict process efficiencies and estimate associated costs.

2.2.4 Mass transfer kinetics and balance equations

The understanding of fundamental mechanisms by which the compounds of interest migrate from the solid phase of the matrix to the fluid phase of the solvent is of paramount importance in SFE. This comprehension can only be achieved by a thorough study on the transport phenomena that govern the technique, both at molecular and macroscopic levels. All the models employed to describe these type of processes derive from fundamental mass transfer and balance equations that are

adapted to specific applications, by taking into account their inherent characteristics and particularities.

The extraction process during SFE from a kinetic point of view can be described briefly as follows: the solid substrate absorbs the supercritical solvent, which promotes the dilatation of the cellular structures. This facilitates the solvent flow by decreasing the mass transfer resistance; concurrently, the soluble compounds are dissolved by the solvent and then transferred by diffusion to the surface of the solid. Finally, the compounds are transported by the solvent, separated and then removed from the extractor [26]. The following concepts explain the main fundamentals of mass transfer kinetics in the fixed bed of a standard SFE system, as summarised by Meireles [29]. For more details on the topic, this literature is highly recommended.

In Figure 2.2, a "zoomed-in" schematic diagram of a very thin slice of the extraction bed (called *control surface*) is shown. The blank region represents the void fraction of the column, where CO_2, or any other solvent, circulates. In turn, the hatched area represents the solid phase, i.e., the set of particles of food matrix.

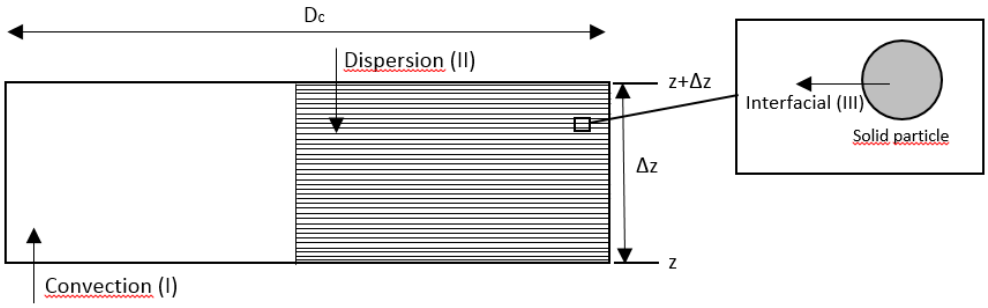


Fig. 2.2. Representation of the mass transport mechanisms in the SFE fixed bed. D_c is the column diameter, z is the axial position of the control surface and Δ_z is the infinitesimal axial height of the control surface.

The mass transfer in SFE occurs by three main mechanisms: (I) by convective transport in the void fraction of the bed, (II) by axial dispersion of the solid phase into the void space, and (III) by an interfacial mass transfer occurring on the interphase between the solid particles and the fluid phase.

The convective mass transfer (I) happens as a result of solvent flow. Due to this movement, the compounds that are more easily accessible and/or weakly attached to the solid particles are transferred to the fluid phase and quickly led out of the column. Assuming that the particle bed is homogeneous, the solvent velocity through the void fraction in the column is given by:

$$v = \frac{Q}{A_T \varepsilon} \tag{2.1}$$

where v is the solvent velocity, Q is the volumetric flow rate of the solvent, A_T is the column area and ε is the bed porosity (i.e., void fraction).

Using Eq. 2.1, the convective mass flux can be calculated. The solute concentration (Y) in the fluid phase is given by the ratio between the mass of solute and the mass of solvent. Y is a function of the solvent flow rate (since it should increase with

increasing the flow due to the interfacial mass transfer), of the axial position (the distance from the bottom of the bed, as the concentration should increase as the *z*-value increases) and also of time (since interfacial mass flux decreases with extraction time). Once again assuming that the bed is homogeneous, *Y* should not be a function of the radial position. Thus, we have:

$$Convective\ flux\ =\ \rho v Y(z,t) \tag{2.2}$$

where ρ is the solvent density.

The axial mass transfer coefficient corresponds to the mass transported by axial dispersion (II), due to the gradient of *Y* concentration along the bed. Thus, mass transfer will happen in the opposite direction of the flow, to minimise the differences in the *Y*-value along *z*. The term is expressed according to Fick's first law, as follows:

$$Axial\ dispersion\ flux\ =\ \rho D_{az}\frac{\partial Y(z,t)}{\partial z} \tag{2.3}$$

where D_{az} is the axial dispersion coefficient.

Lastly, the interfacial mass transfer (III) needs to be determined. This transfer modality may occur either by convection due to the solvent movement around the particles or by diffusion. In cases where the compound of interest is mostly found outside the solid particles, convection will be the main contributor to the flux. When the solute is mostly inside the particles, diffusion will govern the transfer. In cases where the solute is both inside and outside the solids, both mechanisms should be taken into account. The term for the interfacial mass flux is represented by *J* and is defined as the interphase mass transfer rate by the column volume. *J* then should have the solute concentration in the solid and in the fluid as independent variables,

i.e., $J = J(X,Y)$, with X being the mean solute concentration in the solid particles and given by the ratio between the mass of solute and solid particles free of solute.

If we consider the bed fraction in Figure 2.2 as the control surface, we can write down the mass balance equation in the *fluid phase*, with all the aforementioned terms:

$$A_T \varepsilon \rho v Y|_z + A_T \varepsilon \rho D_{az} \frac{\partial Y}{\partial z}|_{z+\Delta z} + J(Y,X) A_T \Delta z = A_T \varepsilon \rho v Y|_{z+\Delta z} + A_T \varepsilon \rho D_{az} \frac{\partial Y}{\partial z}|_z + \\ + \frac{\partial}{\partial t}(\rho A_T \Delta z Y) \quad (2.4)$$

The left-hand side of Eq. 2.4 represents the solute that enters the control surface with its convective, dispersive and interfacial terms, respectively. On the right-hand side, the first two terms represent the mass leaving the control and the last term makes up for the mass accumulation. Rearranging Eq. 2.4 gives:

$$\frac{D_{az}\frac{\partial Y}{\partial z}|_{z+\Delta z} - D_{az}\frac{\partial Y}{\partial z}|_z}{\Delta z} + \frac{J(Y,X)}{\rho \varepsilon} = \frac{vY|_{z+\Delta z} - vY|_z}{\Delta z} + \frac{\partial Y}{\partial t} \quad (2.5)$$

Recalling the fact that the region in Figure 2.2 is an infinitesimal slab, Δz tends to zero. Applying this limit in Eq. 2.5, we can define the final expression for the differential mass balance in the void space of the column (fluid phase), given by Eq. 2.6 below:

$$\frac{\partial}{\partial z}\left(D_{az}\frac{\partial Y}{\partial z}\right) + \frac{J(Y,X)}{\rho \varepsilon} = v\frac{\partial Y}{\partial z} + \frac{\partial Y}{\partial t} \quad (2.6)$$

For the mass balance in the *solid phase*, a similar process of calculation is followed, but this time, taking the set of solid particles in Figure 2.2 as the surface control. The expression obtained in this case is:

$$0 = J(X,Y) A \Delta z + \frac{\partial}{\partial t}[X \rho_s (1-\varepsilon) A \Delta z] \quad (2.7)$$

where ρ_s is the density of the solid free of solute. Since ρ_s is a constant, the final mass balance in the solid phase is:

$$\frac{\partial X}{\partial t} = -\frac{J\,(X,Y)}{\rho_s(1-\varepsilon)} \tag{2.8}$$

The vast majority of the models found in literature describing the Overall Extraction Curve (OEC, curve that shows the variation of solute concentration in the fluid phase at the exit of the extractor against time) derive from Eq. 2.6.

As discussed before, the interfacial term *J(Y,X)* can be driven both by convection and diffusion. If convection governs the mass transfer, the term is defined as:

$$J(Y,X) = \rho\varepsilon K_Y a(Y^* - Y) \tag{2.9}$$

where K_Y is the overall mass transfer coefficient, a is the interfacial area per column volume and Y^* is the solute solubility in the solvent; the term $\rho\varepsilon$ is used to correct the units for the mass flux. Replacing this term into Eq. 2.6 and 2.8, respectively, gives:

$$\frac{\partial}{\partial z}\left(D_{az}\frac{\partial Y}{\partial z}\right) + K_Y a\,(Y^* - Y) = v\frac{\partial Y}{\partial z} + \frac{\partial Y}{\partial t} \tag{2.10}$$

$$\frac{\partial X}{\partial t} = -\frac{\rho\varepsilon K_Y a(Y^*-Y)}{\rho_s(1-\varepsilon)} \tag{2.11}$$

The solution to Eq. 2.10 provides the OEC in the beginning of the extraction, where the main mechanism is convection in the fluid phase. However, solving this equation can be very complicated and some simplifications and assumptions are usually made to build the mathematical models found in the literature, some of which are described in the next section.

2.2.5 Mathematical modelling

The aim of modelling a particular SFE process is to obtain the Overall Extraction Curve (OEC), which is used to describe the whole extraction and deliver critical information that can be employed to optimise, predict and scale up the process.

2.2.5.1 Empirical models

The first category of models includes empirical correlations to obtain the curves. They rely on the nonlinear shape of the OEC and hence use nonlinear functions to fit it. Such examples represent the works of Naik [48] and Esquivel [49], who employed models very similar to the Langmuir model, broadly used in the modelling of adsorption processes. The general form of these equations is:

$$m_E = \frac{A_{1E}t}{B_{1E}+t} \tag{2.12}$$

where m_E is the cumulative mass of solute obtained, A_{1E} and B_{1E} are constants and t is the extraction time. Physically speaking, A_{1E} represents the maximum mass of solute that can be obtained during the extraction and B_{1E} is a mass-transfer coefficient.

Although in some cases these models are capable of delivering satisfactory fittings, they do not convey much mechanistic information about the process. This is due the fact that the adjustable parameters in the models – A_{1E} and B_{1E} – do not account for the different types of mass transfer mechanisms discussed earlier. An example is the work of Comim et al. [50], who attempted to fit the data obtained after SFE extraction of banana peels to Esquivel's model. According to the authors, the most probable causes for the poor model fit were the low number of adjustable

parameters and possibly the position of the solute inside the solid particles, which hindered solvent accessibility. This parameter is not taken into consideration by the model.

2.2.5.2 Diffusion Model

Other models use analogies from heat transfer principles to describe the mass diffusion in porous media. In these cases, the mass transfer is assumed to take place by diffusion and therefore an apparent diffusion coefficient needs to be determined. Such approach is used in the work of Crank [51], which was later adapted by Reverchon [52] and resulted in the following equation:

$$m_E = \left(1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{-n^2\pi^2 D_{ap}t}{R_p^2}\right)x_0 m_t \quad (2.13)$$

where D_{ap} is the apparent diffusion coefficient, R is the particle radius, x_0 is the global yield and m_t is the total particle mass packed into the column.

This model results in poor fit for most SFE systems due to the apparent diffusion coefficient not being able to describe adequately the convective mass transfer that occurs in the early stages of the extraction process [53,54].

2.2.5.3 Desorption model

The third group of models are those that derive from the differential mass balance equations discussed in the previous section and account for all the mass transfer mechanisms involved; these models are preferred over empirical and diffusive ones. For instance, the Tan and Liou model [55] (also referred to as Desorption Model),

considers the variation in the solid phase concentration with time as a first-order equation and neglects the axial dispersion in the system. With these restrictions, after solving the equations by making the adequate assumptions and applying the pertinent boundary conditions, the final expression for Tan and Liou's OEC model is given by:

$$m_E = \frac{Q(1-\varepsilon)x_0\,\rho_s}{\rho\varepsilon k_d}\left[1-\exp\left(\frac{k_d H}{v}\right)\right]\exp[(-k_d t)-1] \quad (2.14)$$

where k_d is the first-order (desorption) constant.

Despite some authors trying to use an Arrhenius dependence on k_d with temperature to make correlations, the model sometimes fails to attain good fits. However, it is still used to describe SFE in a variety of raw materials, such as eucalyptus bark [53], rosemary [56], shitake mushroom [57], turmeric [58] and pupunha [59].

2.2.5.4 Logistic model

This model was proposed by Martínez [60] and neglects axial dispersion, as well as accumulation in the bed, and assumes that the interfacial mass transfer only depends on the composition of the extract along the process. A logistic equation that is usually applied to model population growth was adopted to describe the variation of the extract composition with time [19]. Martínez's final equation is given by:

$$mE = \frac{x_0}{\exp(b*t_m)}\left(\frac{1+\exp(b*t_m)}{1+\exp[b*(t_m-t)]}\right) \quad (2.15)$$

where t_m is an adjustable parameter and its value represents the time where the process has reached its maximum extraction rate.

2.2.5.5 Broken-Intact Cells (BIC) model

Sovová [61] presented a very interesting approach for the development of a model, which is one of the most widely accepted to fit the OECs of SFEs due to its accuracy and ability to be used for scaling-up.

The author started by the principle that the cell walls of plant materials act as a barrier to solute extraction. The samples to be used in the extraction vessels had initially been dried and milled to reduce particle size, and as such, they would be prone to deliver higher extraction rates and yields. Since the compounds of interest are assumed to be exposed at the surface of the 'broken cells', solute dissolution in the solvent is facilitated, making convection the main mass transfer mechanism in the fluid phase. The 'intact cells' (cells not broken by the milling process), still had their compounds segregated within them, making the extraction of the compounds there located a difficult task. Hence, this model is generally referred to as the BIC model (Broken-Intact Cells).

The interfacial mass transfer term during the stage of convection in the fluid phase has a similar form to that of Eq. 8. This stage is characterised by a straight line observed from the beginning of the extraction process until t_{CER} (Constant Extraction Rate, the time when this stage is finished) is reached and the extraction enters a Falling Extraction Rate period (*FER*). In FER, there is a slow decrease in the rate of the extraction due to the exhaustion of free solute in the cell surface. Here, both convection and diffusion are important.

After t_{FER}, when all the easily extractable solute has been exhausted, the extraction curve profile will look like an almost-straight line, with a very low slope, and the mass transfer will occur only by effective diffusion of the solutes from the particles.

After neglecting the mass balance dispersion term and the variation in the fluid phase concentration with time (since the residence time of the solvent in the column is very low), and considering a transient behaviour for the solid phase, the final equation for describing the model is given in the form of a piecewise function:

$$For\ t < t_{CER}, \qquad m_E = QY^*[1 - exp(-Z_1)]t$$

$$For\ t_{CER} \leq t < t_{FER}, \qquad m_E = QY^*[t - t_{CER}\ exp(Z_w) - Z_1)]$$

$$For\ t \geq t_{FER}, \qquad m_E = m_{SI} * \left\{X_0 - \frac{Y^*}{W}\ln\left[1 + \left[exp\left(\frac{Wx_0}{Y^*}\right) - 1\right] exp\left[\frac{WQ(t_{CER} - t)}{m_{SI}}\right]\frac{X_k}{X_0}\right]\right\} \tag{2.16}$$

where

$$Z_1 = \frac{m_{IS}K_y a\rho}{Q(1-\varepsilon)\rho_s}$$

$$W = \frac{m_{IS}K_x a}{Q(1-\varepsilon)}$$

$$Z_W = \frac{Z_1 Y^*}{Wx_0}\ln\left\{\frac{x_0 \exp\left[\frac{WQ}{m_{SI}}(t_{CER} - t)\right] - X_k}{(X_0 - Xk)}\right\} \tag{2.17}$$

Later, Sovová [62] proposed an improved model to the above, which took into account the fluid phase variation with time and changed the interfacial mass transfer term. However, the complexity of the model increased considerably and hence its application is very limited.

2.2.5.6 Fitting of different models and concluding notes

In Figure 2.3, a typical OEC is reproduced from the work of Mezzomo et al. [63], along with some model fits for the extraction of peach almond oil. According to the authors, the Sovová's model fitted best the experimental conditions.

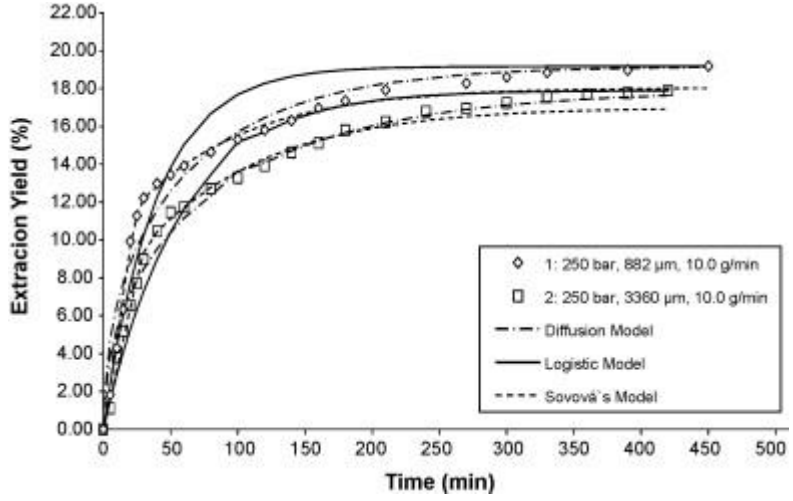


Fig. 2.3. OEC for peach almond oil extraction at two different conditions, fitted to the Logistic [60], Diffusion [52] and BIC [61] models.

Many other authors have successfully employed Sovová's model to fit their experimental data on the extraction of compounds from various food matrices [32,64–69]. In almost all cases, the Sovová's model presented the best fit to experimental data, especially when the samples were previously grinded and milled. A possible explanation for that lies on the fact that the parameters of the model have a clear physical meaning (the mass transfer coefficients, for both solid and fluid phase, and the solution concentration in the unbroken/intact cells) and therefore the model succeeds in depicting the whole extraction behaviour.

Models like these are essential for optimisation studies and scale-up. Although the study of scaling up methodologies is out of the scope of this review, these models

can be employed to aid the choice of the extractor volume and mass load, to predict extraction yields and other crucial parameters that enable the transition of the process from lab to pilot and industrial scales. Moreover, modelling provides knowledge on the dominant mass transfer mechanisms for each type of food matrix (whether convective, diffusive or a combination of both phenomena), which is considered very important for scaling up studies [70]. Del Valle [71] have suggested a few comprehensive approaches for scaling up SFE plants whose starting point for this were some of the models described in this section, a fact which reflects how vital they are for addressing SFE scalability strategies.

2.3 CAROTENOIDS AND CAROTENOID EXTRACTION BY SFE

As previously discussed, SFE is already widely used at industrial scale for common applications [71]. For more specific applications, the technique is mostly research-oriented and its full migration into industrial scale is still under development. For instance, one area in which SFE has been extensively studied in the last fifteen years and has showed high efficiency is the recovery of valuable compounds, such as antioxidants, from vegetable matrices.

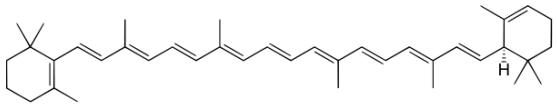
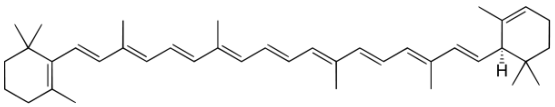
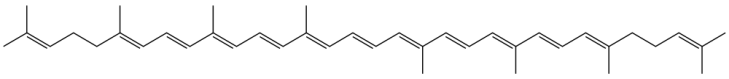
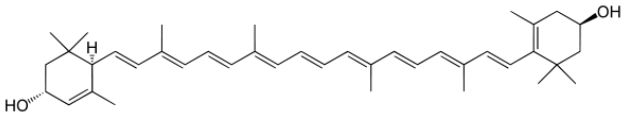
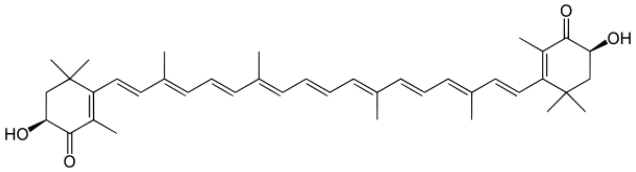
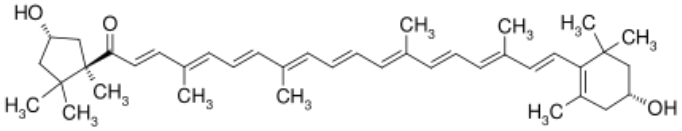
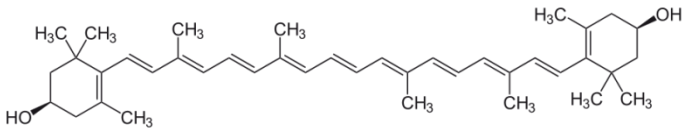
Antioxidants are a very effective group of chemicals that, among other roles, can extend the shelf life of food products [72]. Their main action is on the inhibition or delay of autoxidation. There is a rising interest in natural antioxidants, as currently-used synthetic antioxidants have been suspected to cause or promote undesirable effects on human health [73]. Therefore, the use of fruit and vegetable by-products and waste streams for the recovery of naturally occurring carotenoids (one of the main representative of natural antioxidants) stands both as a very promising path to valorise agricultural waste and as a safe route for enriching and/or conserving food products.

2.3.1 Overview

From a chemical perspective, carotenoids are C_{40} tetraterpenoids formed by eight C_5 isoprenoid units joined head-to-tail, except at the centre, where a tail-to-tail linkage reverses the order, resulting in a symmetrical molecule. An important feature is a centrally-located, extended conjugated double-bond system, which constitutes the light-absorbing chromophore that gives carotenoids their attractive colour and provides the visible absorption spectrum that serves as a basis for their

identification and quantification. Their basic skeleton may be modified in many ways, including cyclization, hydrogenation, dehydrogenation, introduction of oxygen functions, rearrangement, chain shortening, or combinations thereof, resulting in a variety of different of structures [16]. Carotenoid composition in both fruit and vegetables can be variable and complex. However, the most common pattern observed is that fruits have one or two main carotenoids and very low concentrations or even traces of other minor carotenoids. Table 2.1 presents the structure of the most common carotenoids found in fruit and vegetables.

Table 2.1. Chemical structures of the most common naturally-occurring carotenoids

Common Name	*Chemical structure*
α-Carotene	
β-Carotene	
Lycopene	
Lutein	
Astaxanthin	
Capsanthin	
Zeaxanthin	

The considerable interest of the food industry in these compounds stems from their potential use as natural pigments, as in most countries the use of food additives (food colorants included), is governed by strict regulations [16]. Replacing synthetic for natural pigments is very important and the extraction and recovery of these from fruits and vegetables is a plausible pathway for so accomplishing. The cosmetic industry also employs these molecules by adding them to a diverse range of

products, not only for colouring purposes, but also for the antioxidant activities they exert [16,74].

However, the importance of carotenoids in food goes far beyond their purpose as pigments or additives. A number of biological functions and actions have been increasingly attributed to these compounds. β- Carotene, for instance, plays a major role in the human body as the main precursor of vitamin A, which is involved in vision, cell differentiation, synthesis of glycoprotein, mucus secretions from epithelial tissues, reproduction, overall growth and development of bones, etc. [75,76]. Vitamin A deficiency is pointed to be the most common dietary deficiency in the world. Carotenoids have also been linked with the enhancement of the immune system and decreased risk of degenerative diseases such as cancer, cardiovascular disease, age related muscular degeneration and cataract formation [77]. Additionally, carotenoids have also been identified as a potential inhibitor of the Alzheimer's disease [74].

Carotenes are the major carotenoid representatives in a few root crops (e.g., carrot, sweet potato, pumpkin), whereas xanthophylls predominate in maize (seeds). Even within the same type or family of crops, compositional and quantitative differences exist due to factors such as cultivar/variety, stage of maturity, climate and geographic site of production, location in plant, conditions during agricultural production, post-harvest handling, processing, and storage [16]. Differences among cultivars are also reported; for instance, the mean β−carotene content of sweet potato cultivars, varies from 10 to 26,600 μg/100 g [78–80]. Among the aforementioned factors, the stage of maturity seems to be the key factor influencing the carotenoid composition of a specific plant [16]. For example, in ripened fruits,

carotenoids are located in the chromoplasts and hydroxycarotenoids are mostly esterified with fatty acids [16,38].

Carrots are one of the most carotenoid-rich vegetables found in nature, with β-carotene being its major component. The total carotenoid content in the edible part of carrot roots can range from 460 to 30,000 μg/100g, depending on the cultivar [81–84]. Other vegetable sources containing carotenoids include paprika, red and yellow pepper, apricot, pumpkin, peach, tomato, watermelon, grape, pear, guava, papaya and mango.

2.3.2. Carotenoid extraction by SFE

Table 2.2 presents data compiled from a number of studies investigating the extraction of carotenoids by SFE under different conditions. The most common co-solvent employed for carotenoids are ethanol and methanol. However, some authors used vegetable oils as entrainers (e.g. olive, hazelnut, sunflower), and observed an increased carotenoid solubility [63,85,86]. In terms of the operating conditions, temperatures ranged between 40 – 90 °C, and pressures, between 172 to 507 bar.

Table 1.2. SFE parameters, conditions and results of carotenoid extraction from different vegetable food matrices.

Food matrix	Target Comp.	Co-solvent	Mass load (g)	Extraction Parameters (range)				Recovery (%, w/w)	Carotenoid concentr. (µg/g)		Statistical models / Variables of influence	Kinetic Models	Refs
				T (°C)	P (Bar)	Q CO_2	T_E (min)		UV	HPLC			
Apricot bagasse	BCar	-	2.0	40 - 60	304 - 507	1.0 mL/min	90	3-6	-	-	ANOVA / T, P, PSize.	Crank-Nicholson	[87]
Apricot pomace	BCar	1-20% H_20 / EtOH	1.0	40 - 60	304 - 507	1.0 mL/min	90	1-8	67 - 101	-	Central Composite / T, P, CoSol	-	[88]
Citrus waste	TC	0-20% EtOH, IsoProp, Acet, EtAc.	1.0	70	172 - 448	1.0 – 2.5 mL / min	-	38-73	-	550	Central Composite / P, CoSol	-	[89]
Paprika	TC	-	720	60 – 80	300 - 500	415 g/min	-	81-85	1150	-	P, Moist	-	[90]
Pitanga	TC	-	5.6	40 / 60	100 - 400	4.1 g/min	120	0.7 – 48.0	-	138 - 5474	-	-	[91]
Pink Shrimp	TC	2-5% Hexane; Isoprop, sunflower oil	16.0	40 - 60	100 - 300	8.3 – 13.3 g/min	420		-	3.48 – 1,223	-	Martínez, Gaspar, Sovová	[32]
Pumpkin	TC	0-10% EtOH	0.4	40 - 70	250 - 350	1.5 mL/min	-	19-74	-	29 - 110	Central Composite / T, P, CoSol	-	[38]
Pumpkin	ACar, BCar, LUT	MeOH, EtOH, H_2O and Olive Oil	2.0	50 / 80	250	1.5 mL/min	-	40 - 76	-	81.8 – 472	One-way ANOVA / CoSol, T	-	[86]

Food matrix	Target Comp.	Co-solvent	Mass load (g)	Extraction Parameters (range)				Recovery (%, w/w)	Carotenoid concentr. (µg/g)		Statistical models / Variables of influence	Kinetic Models	Refs
				T (°C)	P (Bar)	Q CO_2	T_E (min)		UV	HPLC			
Red pepper waste	BCar	0 - 15% EtOH	30.0	45 / 60	200 - 300	33.3 mL/min	45 - 120	12.9 – 68.1	-	-	T, P, PS	-	[92]
Tomato	LYC	Enzyme-aided	24	86	500	4 mL/min	270	14-38	-	-	/ Enzyme action		[93]
Tomato	LYC	0-10% Hazelnut oil	-	65	425	230 g/min	480	31-38	-	4200	-	-	[85]
Tomato	BCar, LYC	EtOH, Canola Oil	10	40 / 70	400	500/1200 L/min	720	-	-	130-600 (Lyc)	-	-	[39]
Tomato juice	LYC	-	15	40 - 80	200 - 350	0.85 – 1.7 g/min	180 - 360	7.7 - 77	-	-	One-way ANOVA / T, P, CoSol	-	[94]
Tomato peel	LYC	5/15% EtOH	1.2	40 / 70	250 / 450	-	30	24 - 33	-	17 – 24	Central Composite Rotatable / T, P	-	[34]
Watermelon	LYC	0-15% EtOH	0.5	70 - 90	207 - 414	1.5 mL/min	35	-	-	38	Temp	-	[40]

*EtOH = Ethanol; MeOH = Methanol; Acet = Acetone; IsoProp = Isopropanol; EtAc = Ethyl acetate; Q CO_2 = Solvent flow rate; T_E = Extraction time; TC = Total carotenoids; ACar = α-carotene; BCar = β-carotene; LYC= lycopene; LUT = Lutein; Var. of inf. = variables of influence; CoSol = Co-solvent; T = Temperature; P = Pressure; Moist = Moisture; PSize = Particle Size.

Looking closer into the conditions used in the works cited, it is evident that mid-range pressures (250 – 350 bar) improve carotenoid recovery. This can be explained by the fact that, when combined with optimum temperatures, better recovery yields are obtained due to higher molecule solubility as a consequence of increased solvent density. However, some studies reported a decrease in lycopene, α- and β-carotene extraction when pressures were raised above 350 bar [91,95]. This may be due to the over-increased polarity of the supercritical fluid imparted by a further density increase, a phenomenon often related to what is known as the Crossover Effect. At very high pressures, the selectivity of the supercritical fluid is diminished, which leads to dilution of target compounds in the extracts [96]. Although temperature increase also has a clear effect on carotenoid yield in SFE processes, pressure is the factor that can affect carotenoid recovery the most, since these constitute large molecules with a low vapour pressure. Also, very high temperatures (above 70 °C) can lead to carotenoid degradation and isomerisation [96].

Extraction time and flow rate are also reported to influence the extraction process and outcome. Longer extraction times can lead to decreased carotenoid recovery, possibly due to degradation [97,98]. Therefore, an increased solvent flow rate should be used to decrease the extraction time [19].

One interesting fact to notice is that many authors have employed the whole fruit or vegetable as a raw material in the process. Not many of them have turned their attention to assess the wastes thereof (bagasse, pomace, seeds, leaves, peels, etc.). These parts can be rich

sources of bioactive compounds, and in the case of phenolics, for example, they are actually more abundant in these parts than in the flesh for nearly all the vegetables tested in the literature. In the case of carotenoids, they are to be found mostly in the flesh than in other parts, however, studying peels and crops of carotenoid-rich fruits and vegetables (such as carrots, peppers, pumpkins, tomatoes and sweet potatoes) can be an interesting approach, since these all still contain an appreciable amount of such compounds.

Some few studies have compared SFE and conventional solvents in terms of their ability to extract carotenoids. For pumpkin, lutein and lycopene recoveries were much higher in SC-CO_2 extracts (7.0 and 5.7 μg/g dry weight, respectively) than those in organic solvent extract (0.1 μg/g, for both compounds) [38]. *Cis-β*-carotene increased by more than two-fold in the SC-CO_2 extracts (13.7 μg/g), even at a relatively low temperature of 40 °C, over those in conventional solvent extracts (5.2 μg/g). Also, SFE has additional advantages over Soxhlet extraction, requiring 5-fold shorter extraction times and lower solvent consumption [99].

2.4 PREPARATIVE CHROMATOGRAPHY FOR THE PURIFICATION OF BIOACTIVE COMPOUNDS

2.4.1 Purification approaches

The separation and purification of molecules plays such a major role in chemical and biochemical processes that it encompasses an entire field of study dedicated to the development and advancement of dedicated techniques. The collective set of unit operations involved in obtaining a target product in its end form for a specific application is called *downstream processing*. This can range from very simple steps – filtration, centrifugation, precipitation – to high-resolution operations, such as chromatography or crystallisation. The choice of the technique depends on several factors: end-use of the target molecule, its physicochemical characteristics, degree and nature of impurities, economic viability, as well as volume of production [100]. For the purification of bioactive molecules, more refined technologies, such as liquid-liquid extraction, adsorption chromatography, membrane separation and electrophoresis are used due to the nature and the final applications given to these compounds.

One challenge in purification strategies is the fact that, depending on the target molecule and its end-use, expenses with such techniques can add up to 80% of the total production cost and, moreover, usually more than one unit operation is needed to meet the required standards. Therefore, the development of new approaches and the improvement of already-existing purification protocols is essential for reducing the number of stages and consequently, maximising yields and minimising costs.

2.4.2 Chromatographic techniques

Chromatography is perhaps one of the most efficient and extensively employed separation techniques available nowadays. Having found a plethora of applications in several fields, it represents a solute fractionation technique that relies on the dynamic distribution of different molecules between two phases: a stationary (or binding) solid phase and a mobile (or carrier) fluid phase [101]. Although mostly applied for analytical purposes, it is a valuable tool as a preparative technique, i.e., for the recovery and purification of molecules of interest. The different modalities of chromatography are depicted in Figure 2.4 and are discussed in the following paragraphs.

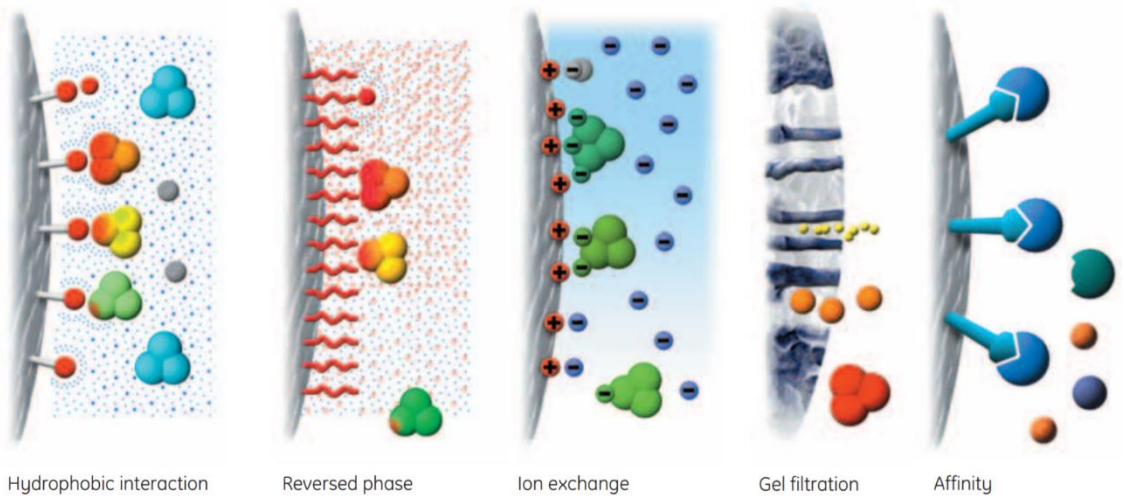


Fig. 2.4. Different chromatographic modalities. (Source: [102])

Different chromatographic modalities exist to allow separation to occur by different mechanisms. The most common is Ion Exchange Chromatography (IEX, IEC), a chemical process in which the stationary phase is constituted by an adsorbent support or matrix –

usually a polymeric resin – to where ionisable functional groups are added. Thus, there are anionic exchangers, which have positively-charged active sites and retain anions, or cationic exchangers, with negatively-charged sites that retain cations. The mobile phase used is usually a buffer of specific salts. Its use has been vastly studied in the separation of proteins and DNA/RNA, metals and minerals, phenolics and there are also numerous industrial applications, such as in sugar and sweetener production, water purification and decontamination and in pharmaceutical production. [103].

Another chemical modality in chromatography is Affinity Chromatography (AC), which utilises groups of very high biological specificity that are chemically attached to a support. These groups (antigens, substrates, lectin, etc.) remove only their respective complementary compounds (antibodies, enzymes and sugars, respectively) from the mobile phase [104]. Size Exclusion Chromatography (SEC, Gel Filtration), on the other hand, is a purely-mechanical process in which the stationary phase selects molecules according to their molecular size: the smaller penetrate easily into the pores, while the larger are excluded from all pores and flow in between the particles. The mid-sized molecules migrate with varying velocities due to their selective penetration, leaving the column in the order related to their size. This technique is largely employed for separating small molecules, such as nucleotides, primers, dyes, and contaminants [104].

However, for the purification of compounds such as carotenoids, a suitable modality would be Hydrophobic Interaction Chromatography (HIC), which, given the strong hydrophobicity

of both the molecule and the ligand, stands as a promising option that is relatively cheap and capable of delivering high yields with considerable purity resolution. The technique separates biomolecules under relatively mild conditions according to differences in their hydrophobicity [101] and is majorly used for protein purification as a complement to IEX or SEC [105]. Carotenoids, as highly hydrophobic molecules, are usually found dissolved in the polar solvents employed as co-solvents in supercritical fluid extracts. Carbohydrates and proteins, which are strong hydrophilic and amphiphilic molecules, are also coextracted. The latter compounds represent the main impurities found in these extracts, which leads to the hypothesis that HIC could be an excellent choice for a relatively-cheap yet efficient purification protocol.

2.4.3 Adsorption in fixed bed

2.4.3.1 Fundamentals

The process of adsorption of components present in a fluid mixture flowing through a column packed with a bed of porous adsorbent material is a commonly-employed technique, especially in food and chemical engineering processes. In addition to industrial applications, this process allows for lab- and pilot-scale chromatographic separations for both preparative and analytical purposes.

Adsorption in fixed bed usually involves a cycle started by an equilibration stage and followed by sample injection (load or adsorption step), washing, elution and regeneration.

In the first stage, the mobile phase free from solutes is allowed to flow through the column to equilibrate the solid phase accordingly. Then, injection of the sample follows, when it comes into contact with the adsorbent, responsible for the adsorption of the material of interest ('adsorbate') on its surface. During the washing step, the column is fed with the mobile phase – usually the same as that used in the injection step. This is necessary so that adsorbate fractions that were weakly bound or not adsorbed can be carried out of the system. Depending on the type of chromatographic modality, elution can be achieved by increasing or decreasing the salt concentration in the buffer, lowering or increasing the pH and/or temperature, using an isocratic elution or increasing the concentration of a competing agent [100]. The last step, regeneration, is aimed at the removal of solutes still bound to the adsorbent, so that a new adsorption cycle can be started. In liquid-phase adsorption, this step usually consists of using a strong solvent, usually of alkaline character, through the bed.

2.4.3.2 Factors of influence and protocol development

Four parameters have been reported to influence the efficiency of HIC: the type of buffer used and its ionic strength (i.e. salts concentration), temperature and pH [101]. The first two are based on the fact that some compounds can manifest different degrees of hydrophobicity under different salts and ion concentrations, favouring or disfavouring the binding to the adsorbent. As hydrophobic interactions are temperature-dependent, temperature can also

greatly affects the process. While higher temperatures usually show a positive influence, lower are known to compromise considerably the resin binding efficiency [102]. Flow rate can also affect the process: HIC is considered a slow process, if contrasted with ion exchange or affinity chromatography [101], therefore very fast flow rates result in low residence times and, consequently, adsorbate-adsorbent interactions become limited. On the other hand, very slow flow rates result in insufficient driving force to overcome the adsorbent external resistance film, impeding the adsorbate from getting to the adsorption sites – the particle pores. They also imply higher time consumption and, consequently, lower productivities.

Therefore, whenever new protocols for purifying molecules are developed, comprehensive studies assessing the influence of all these factors must be performed to identify the operational conditions that deliver the best resolution and recovery, under the lowest time frame. This can be achieved by obtaining and analysing breakthrough curves (BTCs) and equilibrium isotherms, explained in the next section.

The resin adsorption capacity (q^*, μg adsorbate / mg adsorbent) is fundamental information for evaluating the binding efficiency of the adsorbate to the adsorbent and for calculating the exact amount of resin needed for every run, given any solution with a known adsorbate concentration. To obtain q^* in a batch process, we use the following correlation:

$$q\ (mg/mg) = \frac{V_{sol}(C_0 - C_{eq})}{m_{ads}} \tag{2.18}$$

where q is the amount of adsorbate per milligram of resin (mg/mg), C_{eq} is the total adsorbate concentration in the liquid phase (μg/mL) in equilibrium with q, C_0 is the initial

concentration in the liquid phase, m_{ads} is the adsorbent dry mass (mg) and V_{sol} is the volume of solution in contact with the adsorbent.

Adsorption kinetics are also essential to evaluate the minimum time needed for the resin saturation to take place and therefore optimise process time, once q^* is known. The investigation of the adsorption and desorption behaviours under this scenario is crucial due to two main reasons: the feasibility of working in semi-continuous mode, which is economically interesting and allows for a more realistic representation of an industrial process, and also of performing the fine-tuning of other parameters that will enable the use of mathematical models to describe the process and aid in future assessments of technique scaling-up.

2.4.3.3 Breakthrough curves and adsorption isotherms

The adsorption of a substance present in a fluid phase on the surface of a solid phase, in a specific system, generates a thermodynamically-defined distribution between these phases once the equilibrium is reached. A common way of describing such distribution is to express the amount of adsorbed substance by an amount of adsorbent (q^*) as a function of the adsorbate concentration (C_0) in solution. Such expression is defined as an *adsorption isotherm* and is extremely useful for calculating q_{max}, the maximum amount of adsorbent that the resin is able to adsorb. The isotherm needs to be calculated at a constant temperature,

since this parameter directly affects the adsorptive phenomenon and to this end, Breakthrough Curves (BTC) are built. In Figure 2.5 a general BTC is depicted.

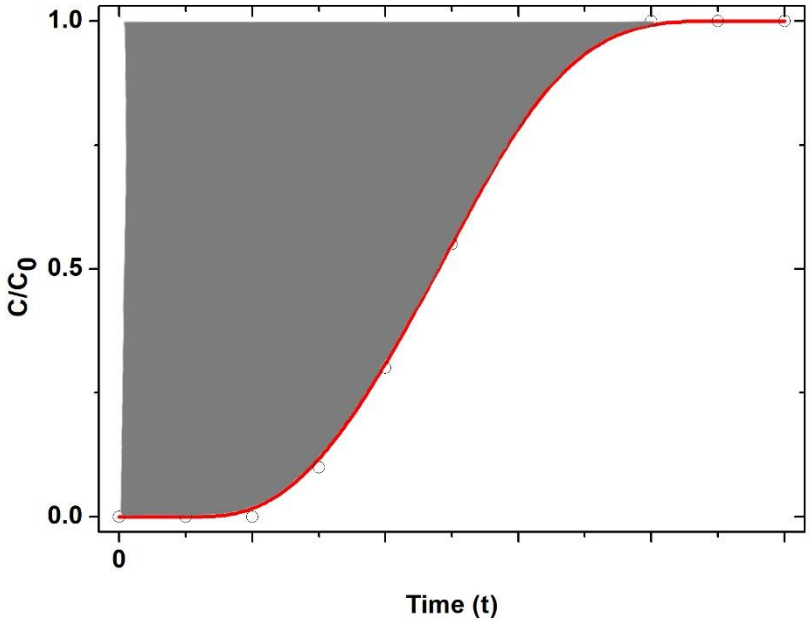


Figure 2.5. Example of a common Breakthrough Curve (BTC).

As seen in Figure 2.5, a BTC is a graph that shows the progress of the adsorption step, built by plotting the ratio between the concentration in the column outlet (C) and that at the column inlet (C_0) up until saturation is reached, versus time. Using this ratio of input and output concentrations (C/C_0), the BTCs are obtained and the amount of carotenoids adsorbed (q^*) on the resin (in mg/mL), for each concentration and in equilibrium with C_0 in the column, is calculated using equation 1.19, which is the expression for the final mass balance in the column:

$$q^* = C_0 . \left[Q . \int_{t_d}^{\infty} \left(1 - \frac{C}{C_0}\right) dt \, . V . \varepsilon \right] \qquad (2.19)$$

where C_o is the initial concentration of the adsorbate (μg/mL), m_{ads} is the mass of the adsorbent and V is the volume of the packed bed.

The expression $\int_{t_d}^{\infty}\left(1-\frac{C}{C_0}\right)dt$ in the equation is equivalent to the area lateral to the BTC (hatched region in Figure 2.5) and correspond to the total carotenoid amount adsorbed. Solving and rearranging its terms, we get to the final expression:

$$q^* = \frac{C_0(A_h Q - \varepsilon V_c)}{(1-\varepsilon)V_c} \quad (2.20)$$

where C_0 is the concentration the column inlet (μg/mL) and A_{h} is the lateral area limited by the curve.

Thus being, the equilibrium isotherms can be plotted, with each q^* providing one point for the isotherm.

2.4.3.4 Mathematical modelling

Several models have been used to describe the behaviour of adsorption isotherms, with the model of Langmuir [106] still being the most widely used. It has allowed a correct description of the experimental data in several studies involving dilute solutions of a strongly adsorbed component in a pure adsorbent [107]. The Langmuir isotherm can adequately explain the experimental data of solutions at low or moderate concentrations and assumes the existence of a monolayer where adsorbates accumulate on the surface of the adsorbent. At high concentrations, however, the activity coefficients of the species in solution become

concentration-dependent, therefore deviations from the Langmuir model are observed and consequently, modifications of this model are proposed, such as the Langmuir-Freundlich model. However, the derivation of the Langmuir isotherm is still considered the starting basis for most theoretical models of the adsorption phenomenon.

For adsorption in liquid phase, Equation 2.21 represents the Langmuir model:

$$q^* = \frac{q_m k C_{eq}}{1+kC_{eq}} \tag{2.21}$$

where q_m is the number of adsorption sites and k is the Langmuir dissociation constant (mL/mg), related to the adsorption energy.

The Freundlich model [108], that assumes a logarithmic distribution of active sites, reads:

$$q^* = b * {C_{eq}}^{1/n} \tag{2.22}$$

where b and n are, respectively, indicators of adsorption capacity and of adsorption energy.

The Langmuir-Freundlich modification is proved efficient when dealing with high concentrations of adsorbate:

$$q^* = \frac{q_m * {C_{eq}}^b}{k+{C_{eq}}^b} \tag{2.23}$$

The Henry's law is a reduction of Langmuir's when C_{eq} tends to 0, i.e., when the solutions present very low adsorbate concentration:

$$q^* = H * C_{eq} \quad (2.24)$$

where H is the Henry constant.

2.4.3.5 Carotenoid purification

Carotenoid purification by hydrophobic chromatography for preparative purposes has apparently never been attempted before. As commented, the technique is largely used for the purification of proteins and these are molecules very different from the first, in structure, charge and polarity. Although constituting a challenge, given the inherent properties in carotenoids, this possibility is envisaged provided that an efficient linking between the adsorption process engineering and the underlying carotenoid science and chemistry is successfully made and that the limitations of each area are observed.

On a more specific note, for example, to assess the protocol efficiency, apart from simply calculating carotenoid recoveries and extract concentrations, a crucial factor that would need to be monitored throughout the process is the antioxidant activity of the fractions. In this specific scenario, an efficient process should be able to guarantee that the maximum compound recovery is attained and that its bioactivity is maintained or, at least, that eventual losses are kept to a minimum. Also, temperature and pH are parameters that reportedly affect the adsorption in HIC. However, since carotenoids can be degraded when submitted

to high temperatures, light, oxygen and very drastic pH values, a trade-off must be observed in the search for a middle-ground solution. Every effort must be made to prevent degradation and, when it occurs, it must be accounted for. This knowledge is important to describe the process as a whole and also to help understand recovery calculations, by assessing, for instance, how much TCC was lost due to oxidation and not to inefficient binding.

2.5 CONCLUDING REMARKS

In this literature review, all the underlying scientific principles along with the past, current and potential future applications regarding the techniques to be used for achieving the aim and objectives of this work (Chapter 1) have been presented and discussed. Now, the following chapters will lay out the methodologies, results and discussion related to the SFE extraction of carrot peels (Chapter 3), HIC purification of carotenoid-rich SFE extracts (Chapter 4) and the feasibility of the application of the optimum conditions to other vegetable matrices (Chapter 5).

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CHAPTER 3

Optimisation and modelling of Supercritical CO_2 Extraction process of carotenoids from carrot peels

Authors: Micael de Andrade Lima, Dimitris Charalmpopoulos, Afroditi Chatzifragkou.

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Contributions: Micael de Andrade Lima performed all the analysis mentioned in the text, treated, presented and discussed the outcome data and wrote the first draft of this manuscript. Afroditi Chatzifragkou and Dimitris Charalampopoulos were responsible for all the corrections, input on and proofreading of the main text from its draft status until its final version, now available in press.

PREFACE

After general preliminary experiments involving methodology assessment and validation, plotting of standard calibration curves (Appendix A) and screening of initial analysis parameters (Appendix B), the first in-depth study necessary for the implementation of the proposed protocol was understood to be a thorough optimisation of the operational conditions via an Experimental Design and a kinetic modelling of the extraction.

Carrots were chosen as the model vegetable due to their very high carotenoid content and to the fact it is one of the most consumed – and consequently, waste-generating – vegetable crops in the UK and in the world. Therefore, finding the best conditions for this particular matrix would facilitate their future extrapolation to other vegetables with less abundant carotenoid concentrations. Also, in this first part of the work, both flesh and peels were characterised to generate comparative data between these two parts of the matrix, but only the peels were eventually submitted to supercritical extraction. Pressure, temperature and co-solvent concentration were the parameters chosen for this assessment, along with time, which was optimised by extraction kinetics.

Already with a view to enabling the following stage in the protocol – the implementation of a dedicated purification process – the final extracts deriving from the best conditions found were submitted to a chemical characterisation, which, when compared to those obtained from other less ideal conditions, would also allow for the knowledge of how influential the aforementioned parameters are to the final composition of the extracts.

Optimisation and modelling of Supercritical CO_2 Extraction process of carotenoids from carrot peels

ABSTRACT

This work aimed to assess and optimise the extraction of carotenoids from carrot peels by supercritical CO_2 (S-CO_2), utilising ethanol as co-solvent. The evaluated variables were temperature, pressure and co-solvent concentration, with 5.0 g of sample being used in each run. According to the validated model, the optimal conditions for maximum mass yield (5.31%, d.b.) were found at 58.5 °C, 306 bar and 14.3% of ethanol, and at 59.0 °C, 349 bar and 15.5% ethanol for carotenoid recovery (86.1% w/w). Kinetic experiments showed that 97% of the total extractable carotenoid content was recovered after only 30 min, whereas model fitting confirmed the fast extraction trend and desorbing nature of carotenoids from the sample matrix. The process is potentially scalable, as demonstrated by runs performed with a 10-fold initial sample size (50.0 g), which led to even higher recoveries (96.7% w/w) and indicated that S-CO_2 can be as efficient as a conventional solvent extraction for recovering high value compounds from vegetable by-products.

Keywords: carotenoids; supercritical CO2; carrot peels; modelling; recovery

3.1 INTRODUCTION

Due to the increasingly high volumes of waste generated by the food processing industry, developing and establishing waste management practices is paramount. Fruit, vegetables and their by-products are known to contain a variety of valuable compounds including carbohydrates (e.g. dietary fibre, oligosaccharides), aromatic compounds and phytochemicals (e.g., polyphenols, glucosinolates, carotenoids) [1].

Carrots are one of the most consumed vegetables with over 37 million tonnes produced every year worldwide [2]. Such a vast production results in proportionally large amounts of waste, as during carrot processing around 11% of the initial mass is lost, mainly in the form of peels, tubers and attached flesh. Carrots are enriched with phytochemicals of high importance, such as carotenoids and phenolic compounds [3] that could be extracted and utilised as natural additives (e.g. colourants) for food and pharmaceutical applications. In particular, carotenoids are ubiquitous compounds in vegetables and constitute essential nutrients in the human diet, exerting antioxidant and potentially cancer-preventive properties [4–6].

The extraction of phytochemicals from vegetable matrices is commonly carried out with the aid of conventional chemical solvents, due to their ease of use, efficiency, relatively low cost and wide applicability [7]. The mechanism of extraction rests on the differences in the solubility of the matrix (insoluble residue) and the compounds of interest which, by having high affinity with the liquid solvent phase, promptly diffuse into it. However, conventional

solvents require several hours to achieve satisfactory recoveries. Also, the end solutions are often dilute and therefore, an additional concentration step is needed, which could result in degradation or bioactivity losses of the components of interest. In the case of carotenoids, different solvents such as acetone, methanol, hexane and tetrahydrofuran (THF) are commonly used due to the non-polar nature of such phytochemicals [8]. However, the toxicity of many of these solvents can raise both health and environmental concerns.

With the development of a more environment-friendly mindset and the advent of green technologies, new methods for extracting these classes of phytochemicals have been suggested and are currently being studied, including microwave and ultrasound assisted extraction, subcritical water extraction, enzyme-aided extraction and supercritical fluid extraction (SFE) [9–12]. SFE technology employs mainly CO_2 and is considered a fast, efficient and "clean" method for the extraction of natural products from biomass matrices, such as fruit and vegetables. The extraction of compounds happens similarly to conventional solvents, but fluids in supercritical state possess gas-like properties of diffusion, viscosity and surface tension, as well as liquid-like densities and solvation powers. These properties combined render S-CO_2 ideal for extracting compounds in a shorter time with higher yields, when compared to conventional liquid-state solvents [13]. Supercritical CO_2 results in the recovery of compounds without toxic residues and cause no degradation to active components, which can be then recovered in high purity. It is currently used commercially for the decaffeination of coffee and tea [14,15], the extraction of flavours from plant leaves,

lipids from milk and fish oils, alcohol from beverages [16] and specialty bioactives for cosmetic applications, such as antiaging creams [13].

The aim of this work was to evaluate the use carrot peels as the starting material for the extraction of carotenoids by supercritical CO_2. Although previous studies dealing with the extraction of carrot flesh or other vegetable matrices by SFE have been performed [17,18], these were more focused on statistical approach rather than on the variable effects of process kinetics and extract characterisation.

The efficiency of the process was evaluated with respect to global mass yield (%, ratio between total extracted mass and the amount of initial sample) and carotenoid recovery. A 2^3 Central Composite Design of Experiments (DoE) was carried out to optimise the process conditions and the extraction kinetics, whereas the fit of different models to the data was assessed. Also, experiments using a 10-fold initial sample size were performed to attest the scalability potential of the process. Finally, the obtained extracts were compositionally characterised and insights on their potential applications were given.

3.2 MATERIALS AND METHODS

3.2.1 Sample preparation

Samples of Nantes carrots (*Daucus carota*) grown in the UK and harvested in February 2015, were purchased from a local supermarket chain in Reading (UK). The carrots were washed and manually peeled. In our experiments, the peels represented 9.5% of the total vegetable mass loss, a figure quite close to those reported to occur during the industrial processing of carrots (11%). However, it is important to highlight that the peeling techniques used by the food industry (abrasion, caustic and steam peeling) differ from that used in this work (manual peeling) and despite their similar composition, the samples employed here cannot be considered a perfect representation of the actual vegetable residue.

The samples of peels and flesh were frozen at -20 °C for 36-48h, freeze dried (VirTis SP Scientific, UK) for 72h, milled with a grinder for 2 min and sieved to exclude particles with diameter greater than 750 μm. The final samples presented a mean particle diameter of 205 μm (70 mesh) for peels and 245 μm (60 mesh) for flesh. The samples were then stored in containers away from light and kept at -20 °C until further analysis.

3.2.2 Total carotenoid content (TCC) determination and identification

Carotenoids were analysed according to a protocol optimised for carrot matrices [19]. Briefly, 1.0 - 2.0 g of freeze-dried samples, both of flesh and peel, were weighed and added to 6 mL of methanol. After vigorous mixing, samples were centrifuged for 5 min at 2500 x *g*

and the supernatant was separated; a new extraction was performed twice with 8 mL of a mixture of hexane and acetone (1:1). Subsequently, the organic solvent fractions were combined, 25 mL of saturated NaCl were added, and the mixture was shaken in a separator funnel. After phase separation, the lower, water-phase was re-extracted with 8 mL of hexane and the resulting supernatant was combined with the first. The combined fractions were evaporated under nitrogen stream and re-dissolved in methanol prior to High Pressure Liquid Chromatography (HPLC) analysis. An Agilent Infinity 1260 series HPLC system was used, coupled with a 1260 DAD detector (Agilent Technologies, UK). An YMC-C30 silica-based reversed-phase column (250 x 4.6 mm) was used in the separation of carotenoids with a gradient method consisting of (A) methanol/MTBE/water (82:16:2) and (B) methanol/MTBE/water (23:75:2) as mobile phase. The gradient started at 100% of A. Solvent B was then increased to 50% (0 - 45 min) and further increased to 100% (46 – 55 min), with this condition being held for 5 minutes, totalling 60 min per run. The injection volume was 100 μL and the flow rate was kept constant at 1.0 mL/min. For carotenoid identification and quantification, previously-built calibration curves of external commercial standards (α-carotene, β-carotene, lutein and lycopene; Sigma-Aldrich) were used. All detected peaks were analysed at 450 nm (Appendix A, Figure A.1 – A.4).

3.2.3 S-CO_2 extraction parameters and optimisation of experimental conditions

Freeze-dried samples of carrot peels deriving from the same batch (to avoid variability and minimise errors) were subjected to S-CO_2 extraction in a S-CO_2 rig (SciMed, UK). The apparatus consisted of a recirculating chiller, CO_2 line, solvent and co-solvent pumps, heat exchanger, 200-mL extraction vessel, automated backpressure regulator (ABPR), collection vessel and a controlling computer. For every run, 5.0 g of dried peel samples (mean particle diameter of 0.205 mm) were thoroughly mixed with 95 g of inert glass beads (Sigma-Aldrich, UK, 1.0 mm diameter) to ensure bed homogenisation, placed in the extraction vessel and submitted to a CO_2 flow rate of 15 g/min (interstitial velocity 1.33×10^{-5} m/s), chosen due to better results found in preliminary studies when under this condition (Appendix B, Tables B.1 and B.2). Ethanol was used as co-solvent and extraction time was fixed at 80 minutes.

In order to optimise the process, a non-factorial 2^3 Central Composite Design of Experiments (DoE) with three factors at three levels was employed. The three independent variables assessed in the study were temperature (*T*, at 50, 60 and 70 °C), pressure (*P*, at 150, 250 and 350 bar) and co-solvent concentration (*EtOH*, at 5, 10 and 15% v/v). The dependent variables (or responses) assessed were the global mass yield *Y*, defined as the % (g/g) of mass recovered in the extracts with relation to the initial mass load (5.0 g), and total carotenoid content (TCC) recovery *C-REC*, defined as a percentage (%, mg/mg) of the initial TCC. Fourteen different experiments including the low, high and axial points of all the parameters were conducted along with a central point replicated three times to calculate experimental errors, totalling 17 runs. At the end of every run, the extracts obtained in

ethanol, were evaporated to dryness in a rotavapor (RE 120, Büchi, UK), weighed and re-dissolved in methanol for TCC analysis, as described above.

Response Surface Methodology (RSM) was used to represent the model obtained in the form of a 3D graph. All terms in the model equation were tested statistically by F-test at a 95% interval of confidence. The values of the determination coefficient (R^2) and the coefficient of variance (*CV*, %) were also used to confirm the quality of the fitted polynomial model. Lastly, after identifying the critical points by localising the graph global maximum (point where the derivative of the curve is zero), additional triplicate experiments were performed at these critical conditions in order to determine the validity of the optimised conditions. The average values of the experiments were compared to the predicted values given by the model to confirm its accuracy.

3.2.4 Extraction kinetics, data modelling and assessment of scalability potential

Kinetic experiments were also carried out to optimise the extraction time, as a function of both global mass yield and carotenoid recovery. The runs were conducted in triplicate at the critical conditions obtained from the DoE study, with all other fixed parameters being kept constant. Extracts were withdrawn every 5 minutes during the first 30 minutes of extraction and every 10 minutes after this point until the end of the run (80 minutes). At every time point, the extraction would be paused, the accumulated extract removed from the extraction vessel for analysis and the extraction would resume. The results regarding each specific time

point would be calculated by summing the TCC or the yield in the current extract to the TCCs or yields of the previous extracts. The Overall Extraction Curves (OECs) for both responses obtained with these studies were fitted to empirical [20,21], desorption [22] and logistic [23] models. Also, in order to assess the influence of higher mass loads on the behaviour of the extraction and how the attained model would respond in larger scales, runs were performed in the SFE using the full capacity of the extraction vessel (50.0 g of sample) while keeping all other parameters fixed. The extraction time was set at 210 min for these runs.

3.2.5 Analytical methods

The extracts of four selected conditions were characterised as to their macronutrient profile (total protein, lipid and carbohydrate content) in order to understand how these are affected by changes in the variable parameters *P*, *T* and *CoSol%*. The extracts deriving from each condition were evaluated in triplicate.

The total protein content was determined by the Kjeldahl method [24]. The amount of nitrogen and protein present (%, w/w) was calculated as follows:

$$\%\, Nitrogen = \frac{V_{H2SO4}(\mathrm{mL}) * \mathrm{N} * 14{,}007 * 100}{W_{sample}(mg)} \quad (3.1)$$

$$\%\, Protein = \%\, Nitrogen * 6.25 \quad (3.2)$$

where V_{H2SO4} is the volume (mL) of sulfuric acid 0.1N consumed during titration, N is the normality of H_2SO_4, W_{sample} is the exact weight of the sample submitted to digestion and 6.25 is the nitrogen-to-protein general conversion factor for vegetable foods [24].

The lipid content was determined gravimetrically, using the standard Soxhlet method [25]. Calculation of the lipid content was done by weight difference.

The total carbohydrate content was determined according to the NREL protocol [26]. Approximately 300 mg of sample was submitted to acid hydrolysis with 3 mL of H_2SO_4 (72%, v/v) followed by incubation at 30 ^oC for 1 h. The liquid phase was then diluted to 3% H_2SO_4 (v/v) and autoclaved at 121 ^oC for 30 min. After cooling down, the pH value of the supernatants was adjusted to 5.0 using $CaCO_3$ and the supernatants were filtered and subject to analysis in a HPLC system coupled with DAD/RI detectors (Agilent Infinity, 1260 series). The column used was an Aminex HPX-87H (300 x 7.8 mm), the isocratic mobile phase employed was 0.005 M H_2SO_4 and the flow rate was 0.6 mL/min. The injection volume was 20 μL and the quantification of sugars was performed with the aid of previously-plotted calibration curves of external individual standards of glucose, fructose, xylose, arabinose and galacturonic acid (Sigma-Aldrich).

Finally, the moisture content of the dried extracts was measured by a halogen moisture analyser (Mettler Toledo, UK). The apparatus was equipped with an oven operating at 105 °C and a precision scale, which determined the water content by gravimetry from the sorption isotherms plotted by the equipment.

3.3 RESULTS AND DISCUSSION

3.3.1 Carotenoid content of carrot samples

The individual carotenoids identified and their concentrations, along with the total carotenoid content (TCC) in the samples of carrot flesh and peels are presented in Table 3.1. The respective chromatograms can be found in Appendix C, Figures C.1 and C.2. The TCC in carrots can vary from 4.6 to 548 μg/g, depending on the different cultivars [27–30].

Table 3.1. Carotenoid content in carrot flesh and peel samples

Carotenoid	Carrot flesh		Carrot peels	
	Concentration (μg/g)	**% Total**	**Concentration (μg/g)**	**% Total**
Lutein	7.1 ± 0.8	2.0	1.9 ± 0.3	1.2
Lycopene	30.2 ± 2.6	8.5	8.4 ± 1.1	3.9
α-carotene	106.6 ± 7.3	30.1	67.6 ± 5.6	32.9
β-carotene	210.0 ± 12.1	59.4	127.8 ± 9.4	62.0
Total	**353.9 ± 22.8**	100	**205.6 ± 16.4**	100

The results obtained in the current study are within the range of values reported in the literature. *β*-Carotene was identified as the main carotenoid in the samples, representing

around 60% of the TCC in both flesh and peel, followed by α-carotene, making up for around 30% of the TCC in both samples. In the peels, these two carotenoids accounted for approximately 95% of TCC. Other carotenoids identified were lycopene and lutein, but were present in much lower concentrations (1.9 - 30.2 μg/g).

It is noted that a considerably high TCC was present in the peels, which amounted to around 60% of that in the flesh. It is likely that a considerable part of the high TCC found in peels is due to the flesh still attached to them after the peeling process. This highlights the potential of using this particular by-product for the recovery of carotenoids.

3.3.2 Optimisation of S-CO_2 extraction of carotenoids

Dried carrot peels were subjected to S-CO_2, targeting the recovery of carotenoids. Table 3.2 shows the values obtained for both mass yield and carotenoid recovery, under the 17 conditions assessed, according to the non-factorial Central Composite Design of Experiments (DoE). The presence of values outside the fixed limits (runs 9-14), is a characteristic inherent in the CCD and is useful for observing the behaviour of regions out of the chosen range, as this can indicate a trend in case the critical points are spotted outside these boundaries. A few studies in the literature using different raw materials have employed conditions other than those proposed in this study, with pressures as low as 100 bar and as high as 500 bar and temperatures varying from 40 to 90 °C [31–36]. Although in theory, the nonpolar nature of carotenoids should allow their extraction in S-CO_2 without the addition of a modifier,

preliminary studies (Appendix B, Table B.1 and B.2) revealed that the exclusion of a co-solvent from the extraction process resulted in carotenoid recoveries of no higher than 30% (w/w), mainly due to the high molecular weight of the targeted compounds. Besides, the same preliminary studies showed that the use of ethanol resulted in better carotenoid recoveries compared to methanol (Appendix B, Tables B.1 and B.2). By also considering its low toxicity, it was decided to employ ethanol as a co-solvent for all experiments.

Table 3.2. Experimental conditions and obtained values for mass yield and total carotenoid content recovery

Run	T (°C)	P (Bar)	EtOH (% v/v)	Y (% w/w)	C-REC (%)
01	50.0	150	5.0	1.15	34.9
02	50.0	150	15.0	2.59	51.5
03	50.0	350	5.0	0.90	67.7
04	50.0	350	15.0	4.00	78.8
05	70.0	150	5.0	0.31	64.9
06	70.0	150	15.0	2.31	57.1
07	70.0	350	5.0	1.34	66.8
08	70.0	350	15.0	3.26	81.2
09	43.2	250	10.0	3.77	43.0
10	76.8	250	10.0	2.96	68.2
11	60.0	82	10.0	3.09	46.6
12	60.0	408	10.0	5.37	68.7
13	60.0	250	1.6	0.98	41.5
14	60.0	250	18.4	5.70	82.5
15	60.0	250	10.0	4.44	77.1
16	60.0	250	10.0	4.51	79.1
17	60.0	250	10.0	4.43	77.8

There was a considerable variation in the mass yield and total carotenoid recovery values for the different conditions assessed, which highlights the high relevance of this statistical process optimisation study. In terms of the response tested, the global mass yield ranged from 0.31% (run 5) to 5.70% (run 14) and the C-REC from 34.9% (run 1) to 82.5% (run 14).

Figure 3.1 depicts the Pareto Charts of Effects, which demonstrate the influence of the variables and their interactions on global mass yield and carotenoid recovery (at a 95% level of significance). All variables that surpassed the line at p = 5% were deemed to have affected significantly the mass yield or the recovery of carotenoids.

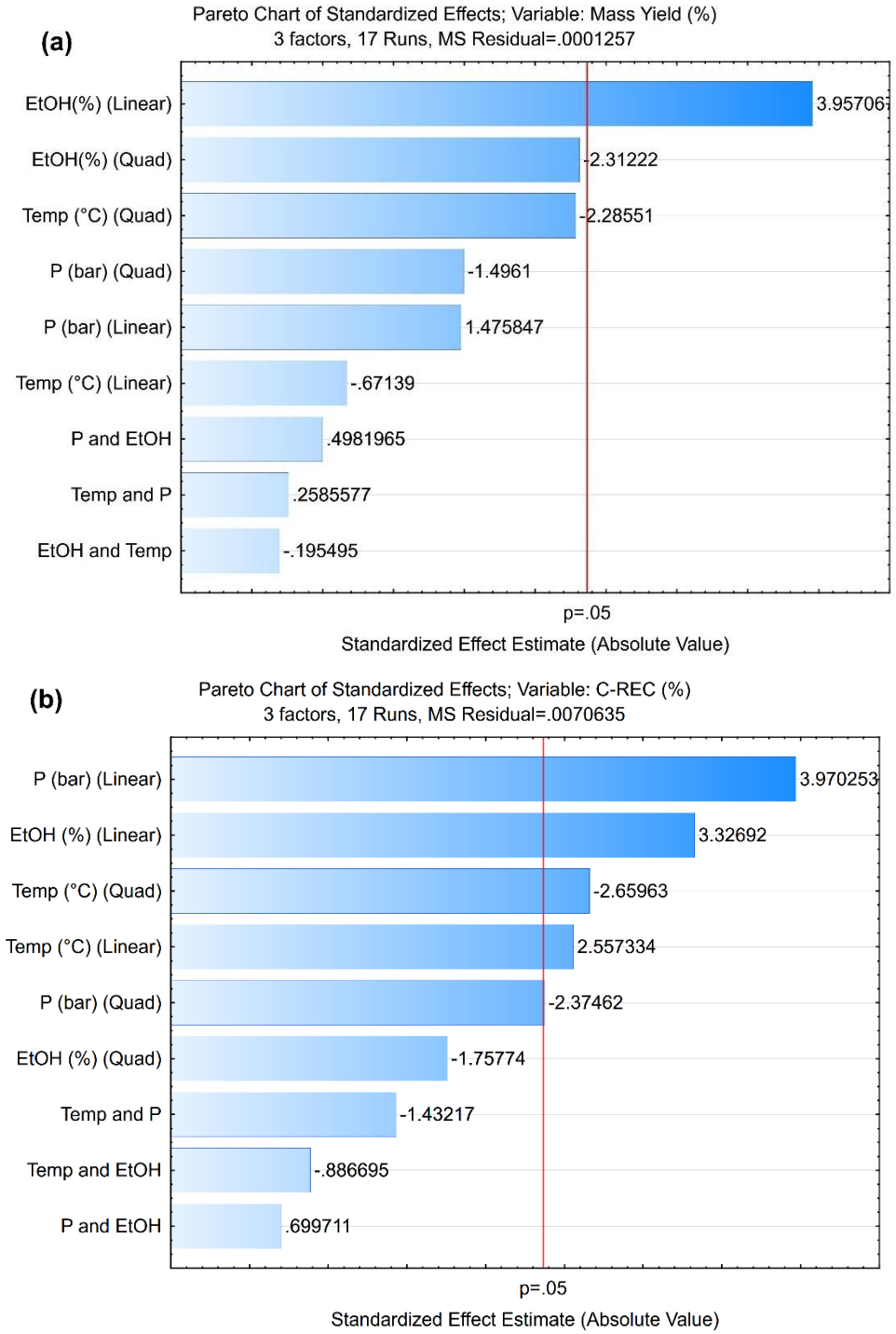

Fig. 3.1. Pareto Chart demonstrating the effects of the variables and their interactions on (a) mass yield and (b) carotenoid recovery, at a 95% significance level.

With regards to mass yield, it can be observed that only the linear term of co-solvent concentration affected significantly the extraction, while none of the quadratic terms or the interactions of the linear terms showed a significant effect ($p > 0.05$). Ethanol is highly efficient in increasing the polarity of CO_2, enabling the dissolution of polar macronutrients, e.g. carbohydrates and amphipathic lipids or proteins. This can explain the higher mass yields that were obtained as ethanol levels increased. It was noticed, however, that both the quadratic term of the co-solvent concentration and the temperature did influence the extraction at 94.6 and 94.4% of significance, respectively. Although not crossing the 95%-threshold set for this experiment, these parameters are certainly important, as they can impose an effect on the process to a certain extent.

With regards to carotenoid recovery, all linear variables presented a statistically significant influence, with pressure imposing the greatest effect on the process. Pressure, in conjunction with temperature, plays a major role in increasing the solvation power of CO_2, favouring the extraction of micronutrients, and effectively phytochemicals. Although a temperature increase showed a clear effect on carotenoid yield in S-CO_2, pressure is the factor that mostly influenced carotenoid recovery, since carotenoids are large molecules and their vapour pressure is low [37]. Additionally, higher pressures are believed to disrupt the vegetable cell walls and other stronger chemical interactions between different compounds (lipids, carbohydrates) and the vegetable cell wall structures, which can cause carotenoids to dissociate from these structures and consequently, to be more easily expelled from the extraction bed [38]. Higher co-solvent concentrations also resulted in positive influences to

the process. Although carotenoids usually present very low polarity, they are high-molecular-weight molecules and the presence of an entrainer (e.g. ethanol, methanol, etc.) facilitates their extraction, since it can aid the dissolution of heavier molecules in CO_2. Also, by observing both the linear and quadratic temperature effects on the graph, it can be noted that temperature showed a dual behaviour: increasing the temperature had a positive influence on the process (linear term) but very high temperatures affected the extraction negatively (quadratic term). This is advantageous not only from an economical point of view, but it is also a qualitative benefit, since high temperatures can lead to carotenoid degradation and isomerisation [37], compromising their stability and bioactivity. Additionally, quadratic pressure was also found to be statistically significant to the model.

Besides identifying the optimal conditions, another important function of these models is the fact that once statistically validated, they enable the prediction of the response variables (mass yield or carotenoid recovery), given any conditions within the studied range.

For mass yield (%) the equation is:

$$[Y]\% = 0.87887 * [T] - 0.0075781 * [T]^2 + 0.84782 * [CS] - 0.030666 * [CS]^2 + 0.0001025 * [T] * [P] - 0.00155 * [T] * [CS] + 0.000395 * [P] * [CS] - 28.2153 \quad (3.3)$$

where [Y] = yield; [T] = temperature (°C); [CS] = co-solvent concentration (%); [P] = pressure (bar).

For carotenoid recovery (%) the equation is:

$$[C\_REC]\% = 10.17256 * [T] - 0.06667 * [T]^2 + 7.16074 * [CS] - 0.17624 * [CS]^2 - 0.004255 * [T] * [P] - 0.052695 * [T] * [CS] + 0.004158 * [P] * [CS] - 237.1932 \quad (3.4)$$

where [Y] = yield; [T] = temperature (°C); [CS] = co-solvent concentration (%); [P] = pressure (bar).

In order to confirm the adequacy of the model, an analysis of variance (ANOVA) (Appendix D) was performed along with an F-test (lack of fit), for validation. For mass yield, the R^2-value was 0.808 and that the F-value (3.06) was higher than the tabulated F value ($F_{9,7}$ = 2.72) (Appendix D, Table D.1). For carotenoid recovery, the R^2-value was of 0.870 and the F-experimental value (5.26) was almost twice the F-tabulated value ($F_{9,7}$ = 2.72) (Appendix D, Table D.2). Hence, for both cases, it can be deduced that the models can satisfactorily describe the extraction process.

The conditions for maximising the responses were identified by determining the absolute maxima of the response surface graphs generated (Figures 3.2a and b). For global mass yield, these conditions were: temperature of 58.5°C, pressure of 306 bar and at 14.3% (v/v) of co-solvent. This set of values predicted an extract mass of 5.31% (w/w). The maximum carotenoid recovery (86.1%), in turn, was found at 59.0°C, 349 bar and with the aid of 15.5% of co-solvent, conditions very similar to those for mass yield. The critical points were then tested by performing three experiments under the critical conditions and comparing these to the predicted results. A low variance of the experimental values (5.38% of mass and 87.0%

of carotenoid recovery) with the predicted responses was found. The models are very consistent and therefore valid to describe the whole process.

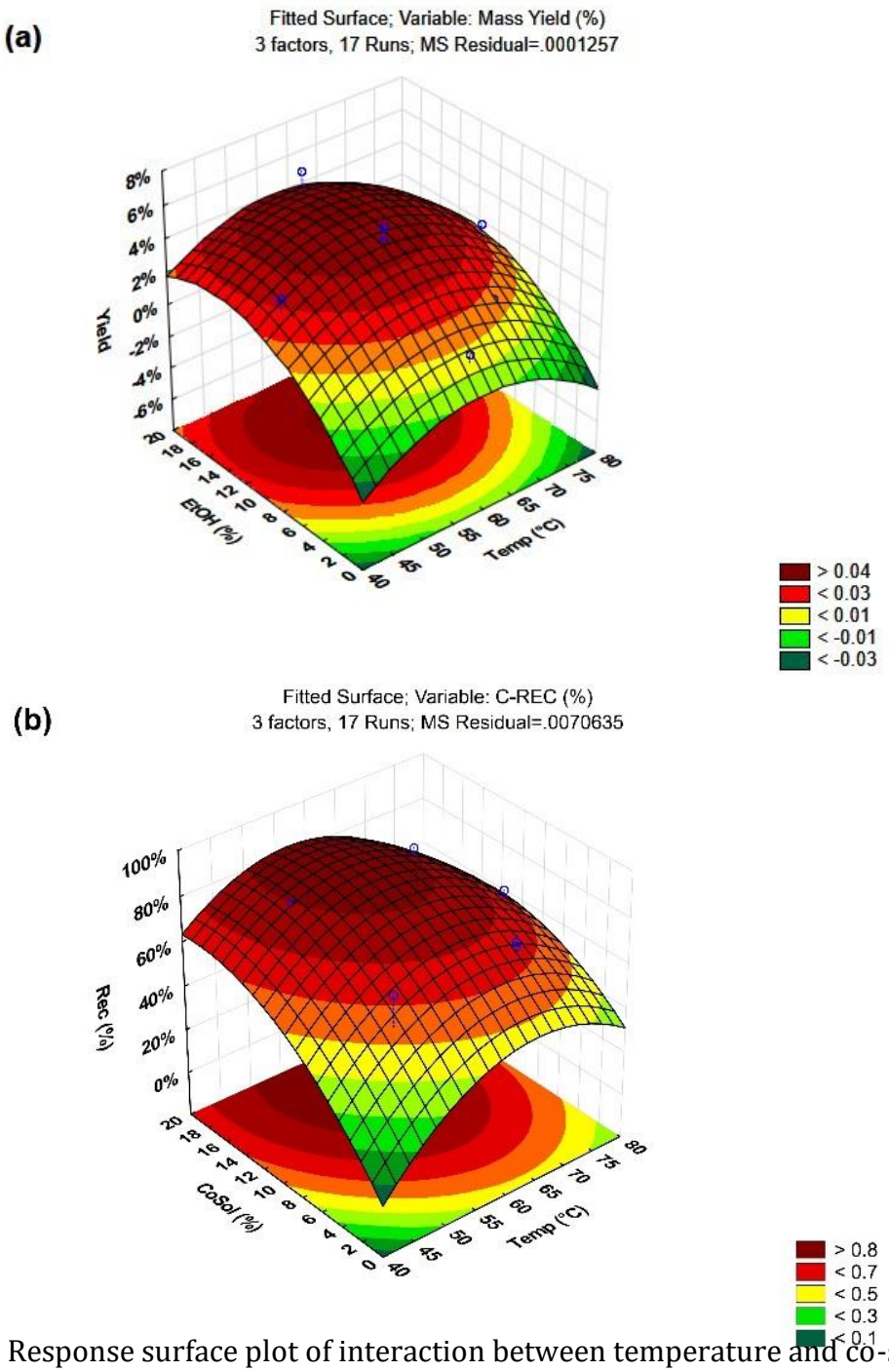

Fig. 3.2. Response surface plot of interaction between temperature and co-solvent concentration effects on total mass yield at 300 bar (**a**) and carotenoid recovery at 350 bar (**b**). The blue dots in both graphs represents each condition with their variations.

Table 3.3 presents a list of studies involving the optimisation of the extraction via S-CO_2 of different food materials (for global mass and carotenoid recovery), with the key variables influencing the process and the recovery values for each material.

Table 3.3. Variables influencing the mass yield and carotenoid recovery of different fruit and vegetables at 95% of confidence.

Food matrix	Compounds of interest	Maximum recovery (%)	Variables of influence (at p=0.05)				Reference
			Temperature	Pressure	CoSol Conc.	Other significant variables	
Mass Yield							
Banana peel	Mass	6.9	**X**	√	-	-	[39]
Grape peel	Mass	13.2	√	√	**X**	-	[40]
Grape seed	Mass	12.3	√	√	-	-	[41]
Guava seeds	Mass	19.0	√	√	-	-	[42]
Passiflora seed	Oil	25.7	√	√	-	Extraction time	[43]
Peach almond	Oil	24.0	√	√	-	-	[44]
Pomegranate seed	Mass	13.9	**X**	√	-	CO_2 Flow rate	[45]
Pumpkin seed	Oil	31.5	√	√	-	Extraction time	[46]
Tomato peel	Mass	-	√	√	**X**	-	[47]
Carrot peel	Mass	5.4	**x**	**x**	√	-	*This work*
Carotenoid recovery							
Apricot bagasse	BCar	6.5	√	√	-	CO_2 flow rate, particle size	[34]
Apricot pomace	BCar	8.0	√	√	√	-	[48]
Carrot	TCC	-	√	√	√	-	[49]
Citrus press cake	TCC	73.0	**x**	√	√	-	[50]

Food matrix	Compounds of interest	Maximum recovery (%)	Variables of influence (at p=0.05)				Reference
			Temperature	Pressure	CoSol Conc.	Other significant variables	
Paprika	TCC	86.0	-	√	-	Moisture content	[51]
Pumpkin	TCC	74.0	√	√	√	-	[32]
Pumpkin	ACar, BCar, LUT	76.0	√	-	√	-	[52]
Red pepper waste	BCar	68.1	√	√	√	Extraction time	[53]
Tomato juice	LYC	77.0	√	√	√	-	[36]
Tomato skin	LYC	33.0	√	√	x	-	[47]
Watermelon	LYC	37.0	√	x	x	-	[33]
Carrot peels	TCC	87.0	√	√	√	-	*This work*

√ = significant influence; x = no significant influence; - = not tested. ACar = α-carotene; BCar = β-carotene; LYC = Lycopene; LUT = Lutein; TCC = Total Carotenoid Content.

Based on literature findings, pressure is the most reported parameter to influence the mass yield of solid biomass residues extracted under SFE process. The ranges tested in these studies are usually between 200 – 450 bar. Temperature has also been shown to affect the extraction in most of the processes, particularly in the range of 50 – 70 °C. Exceptions to this are banana peels [39] and pomegranate seeds [45], where temperature does not impart a statistically significant effect to the process. Co-solvent concentration has only been investigated in grape peels [40] and tomato peels [47] and, in both cases, there was no significant effect on extraction. Other factors that have been reported to play significant roles are extraction time and CO_2 flow rate. In terms of the raw materials used, not many studies have focused on the use of by-products/solid residues for SFE. In the case of peels specifically, only banana, grape, mango and tomato have been investigated, and therefore a more comprehensive comparison with literature data is hindered.

With regards to the results of the current study, carrot peels generated a lower amount of extracted mass compared to banana (6.9%) and grape (13.2%). One explanation for that might lie in the different composition of these matrices, the nature and amount of their components (carbohydrates, proteins, lipids, etc.) and the strength of the interactions between these and the vegetable structures. Dietary fibre is abundant in carrots, with around 88% of the vegetable being composed of cellulose and hemicellulose, and both of them constitute materials with rigid structures [54]. The latter is further reinforced by the fact that in the case of mango peels, which are abundant in lignin, mass yields even lower (3.15%) [55] than those for carrots were reported. The fact that only the co-solvent concentration

was shown to significantly affect the extraction of mass from carrot peels (while for banana and grape, pressure and temperature were the most influential parameters) indicates that for food matrices that have high cellulose content, the amount of entrainer is potentially more important than the temperature *vs.* pressure binomial. A noteworthy point, however, is the fact that the global mass yield is only one of the indicators for assessing the effectiveness of the extraction. When the raw material is a medicinal plant or food, the most important factor for the selection of an extraction technique is the profile of the extract obtained and the amount of the bioactive compounds present [56]. In many cases, depending on the application, lower mass extracts may be advantageous as this can minimise the need for further downstream unit operations.

On the other hand, for carotenoid recovery (Table 3.3), almost all studies confirm the influence of temperature, pressure and co-solvent on the extraction, with the exception of tomato skin and watermelon. The highest carotenoid recovery (86%) was observed for paprika samples, a value virtually identical to that obtained in this work. High recoveries were also achieved for tomato juice, pumpkin and citrus press cake (77, 76 and 73%, respectively). This indicates that the conditions obtained in this work can ensure very high recoveries of carotenoid fractions with a low level of extracted mass (0.269 g from 5.0 g of raw sample) and could potentially be applied to other vegetable waste matrices as well.

3.3.3 Kinetic experiments and data modelling

Figures 3.3a and 3.3b show the kinetic profiles (Overall Extraction Curves, OEC) of the experiments carried out under the optimised conditions fitted with the Naik, Esquivel, Martínez and Tan and Liou models. From the models discussed in the literature review (Chapter 2, section 2.2.5), these presented the best fits.

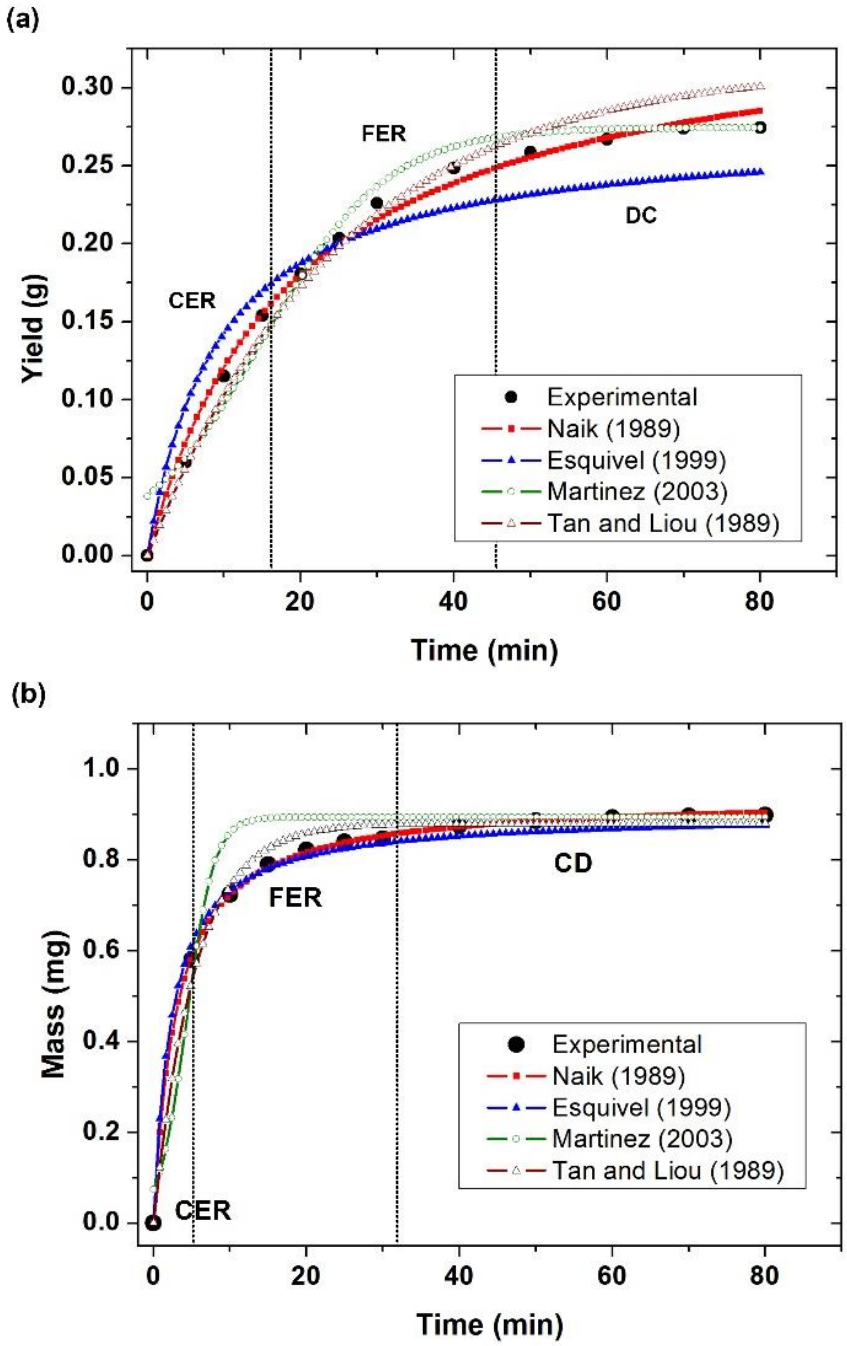

Fig. 3.3. Kinetics of SFE extraction under optimal conditions, modelled by the Naik, Esquivel, Martínez and Tan and Liou models. (**a**) Mass yield. T = 58.5°C; P = 306 Bar; EtOH = 14.3%. (**b**) Total Carotenoid Recovery. T = 59.0 °C; P = 349 Bar; EtOH = 15.5%. CER = Constant Extraction Rate, FER = Falling Extraction Rate; CD = Diffusion-controlled stage.

As extensively explained in section 2.2.5.5., Sovová [57] presented an interesting approach for classifying the stages of the kinetics of the OECs when working with supercritical fluid extractions. The samples used in the process were initially dried and milled to reduce particle size, increasing contact surface and promoting higher extraction rates in the first few minutes due to fact that the compounds of interest had been exposed in the surface of the "broken cells". In this CER (Constant Extraction Rate) stage, solute dissolution in the solvent happens fast since convection is the main mass transfer mechanism. The process then enters the FER (Falling Extraction Rate) stage due to the exhaustion of the free solute in the cell surface, with the "intact cells still having their compounds segregated within their structure. This slows down the extraction rate (indicated by a curved line) and makes the extraction of the compounds more difficult. In FER, mass transfer happens by both convection and diffusion. When all the easily extractable solute is exhausted, the extraction rate can be seen as an almost-straight line, with a very low slope, and mass transfer can occur only by effective diffusion of the solute from the solid particles to the CO_2. Hence, this stage is known as DC (Diffusion Controlled).

Based on Figure 3.3a, it can be stated that the global mass extraction rate was high in the first minutes (50% of the yield was reached by 11 min and 75% by 25 min) and started to decrease due to the exhaustion of free solute as the process progressed, causing the extraction to enter the FER phase after 17 minutes. The optimum time for this extraction was found to be between 40 and 50 min (just after the beginning of DC stage, when respectively 90% and 95% of the extractable mass had already been recovered). A 5% increase in the

yield is not enough to justify extending the extraction, as this would require additional solvent, energy and processing time.

For carotenoids (Figure 3.3b), the extraction rate in the first moments of the extraction is visibly higher than that for yield, given the very steep slope observed in the CER phase, which lasted for just 6 minutes. After only 5 min of process, 65% of all the extractable carotenoids had already been recovered. This value increased to 88% after 15 min and to about 97% by 30 min (when the total recovery had reached 87.0%). The process can be interrupted at this point, since extending it for a further 50 min does not justify the recovery of the remaining 3%. Moreover, longer extraction times can lead to decreased carotenoid recovery, possibly due to heat degradation within the extraction vessel [58,59]. It can be assumed that with the milling process, the carotenoids are exposed in the surface of the solid particles and therefore their dissolution in CO_2 occurs in fact mostly by convection, justifying the very fast extraction rate observed in the early stages of the kinetic experiments.

Table 3.4 presents the adjustable parameters used to fit the four models.

Table 3.4. Nonlinear adjustable parameters of the models for total yield and carotenoid recovery.

Response	Model	Parameter	Physical meaning	Value	Error (±)
YIELD (mg)	Naik [19]	A_{1E} (g)	Maximum attainable mass	0.354	0.0098
		B_{1E}	Mass transfer constant	19.24	1.55
		R^2	-	0.993	-
	Esquível [18]	C_1	None	9.264	1.386
		R^2	-	0.926	-
	Martínez [21]	C_2	None	0.122	0.0145
		t_M (min)	Time of max. extract. rate	14.95	0.951
		R^2	-	0.968	-
	Tan and Liou [20]	K_d (min^{-1})	Desorption constant	0.039	0.0018
		R^2	-	0.977	-
CAROTENOID RECOVERY (mg)	Naik [19]	A_{1E} (mg)	Maximum attainable mass	0.938	0.00271
		B_{1E}	Mass transfer constant	2.988	0.06639
		R^2	-	0.999	-
	Esquível [18]	C_1	None	2.351	0.15653
		R^2	-	0.993	-
	Martínez [21]	C_2	None	0.561	0.17195
		t_M (min)	Time of max. extract. rate	4.345	0.58074
		R^2	-	0.922	-
	Tan and Liou [20]	K_d (min^{-1})	Desorption constant	0.185	0.00101
		R^2	-	0.989	-

As commented in section 2.2.5., the models of Naik [20] and Esquível [21] are classified as empirical models. They rely on the non-linear shape of the OEC and hence use appropriate functions to fit the data. As observed in Fig. 3.3 and in Table 3.4, Naik's and Esquível's models describe the extraction kinetics very well, given the high R^2-values and the low errors for both yield and carotenoids. Once again, the disadvantage of these models, is that some of the adjustable parameters (B_{1E} and C_1, respectively) have no practical physical meaning and, despite the very good fit, the models deliver very little information that can be of use for further studies.

The model of Martínez [23] (logistic model) assumes that the extract is a mixture of compounds or group of compounds with similar chemical structures and introduces the parameter t_m, defined as the time when the extraction reaches its maximum rate, which can be a useful indicator for controlling the process. Having a t_m of 4.3 min in the Martinez's model confirms the very fast extraction trends for TCC, compared with that in the mass yield experiments, which had a t_m of 14.9 min. The high R^2 (0.92 in both graphs) demonstrated a good fit, although not the best among the models.

The Tan and Liou model (desorption model) [22] uses analogies to the adsorption phenomena (section 2.4.3) and takes into account that the solutes dissolve in the solvent following a desorption process in the solid particles. The desorption (or distribution constant) coefficient K_d indicates how strong the compounds are adsorbed to the solid phase: the lower the value, the stronger the interactions between the solute and the particles

surface and consequently, the more difficult their extraction. K_d values in the order of 10^{-2} and 10^{-3}, such as those found for global yield, indicate a slow desorbing fraction [60], thus requiring more contact time to recover all the extractable material. This reinforces the theory that macro-extractants, such as carbohydrates or lipids, are more strongly-bound to the carrot peel structures. The K_d constant is almost 5-fold higher for carotenoids than it is for mass yield, confirming that the carotenoids are easily disposed in the surface of the sample matrix. This is a strong indication that with grinding, carotenoids are exposed at the outermost layers of the particles and mass transfer is therefore favoured, since an extraction with a K_d value of 0.185 can be considered fast [60]. However, to confirm these claims, high-end microscopy analyses such as SEMs (Scanning Electron Microscopy) should be carried out.

The models of Naik, Esquível and Tan and Liou can be employed to describe the process with confidence, given the high R^2-values and low errors presented. In general, it can be observed that the experimental kinetics for *C-REC* fits the models proposed slightly better than *Y* does.

3.3.4 Assessment of scalability potential

Various scale-up routes for SFE (and other extraction processes) have been reported and suggested in the scientific literature. However, a general consensus on the key criteria for scaling-up procedures has not yet been reached [61]. The most commonly used procedures involve maintaining constant: (1) the solvent mass to sample mass ratio, (2) the flow rate to

sample mass ratio, (3) both aforementioned conditions along with a dimensionless number, e.g., *Re* [11]. Due to equipment and process limitations, none of these approaches could be applied in the present study, as this would involve changing extractor dimensions and/or sample particle size. Therefore, it was decided to keep all the optimised conditions constant and increase solely the sample mass. It is envisaged that, despite the limitations, this approach would still provide useful information of the extraction behaviour under the optimised conditions on a more real-scale application. To this end, a 210-minute triplicate run under the best conditions was performed, in which the mass load was increased by 10-fold (from 5.0 g to 50.0 g) in the absence of glass beads. As emphasised, all other parameters were kept constant. The averaged results (in %) were plotted against time, along with data obtained in the previous experiments, and are depicted in Figure 3.4.

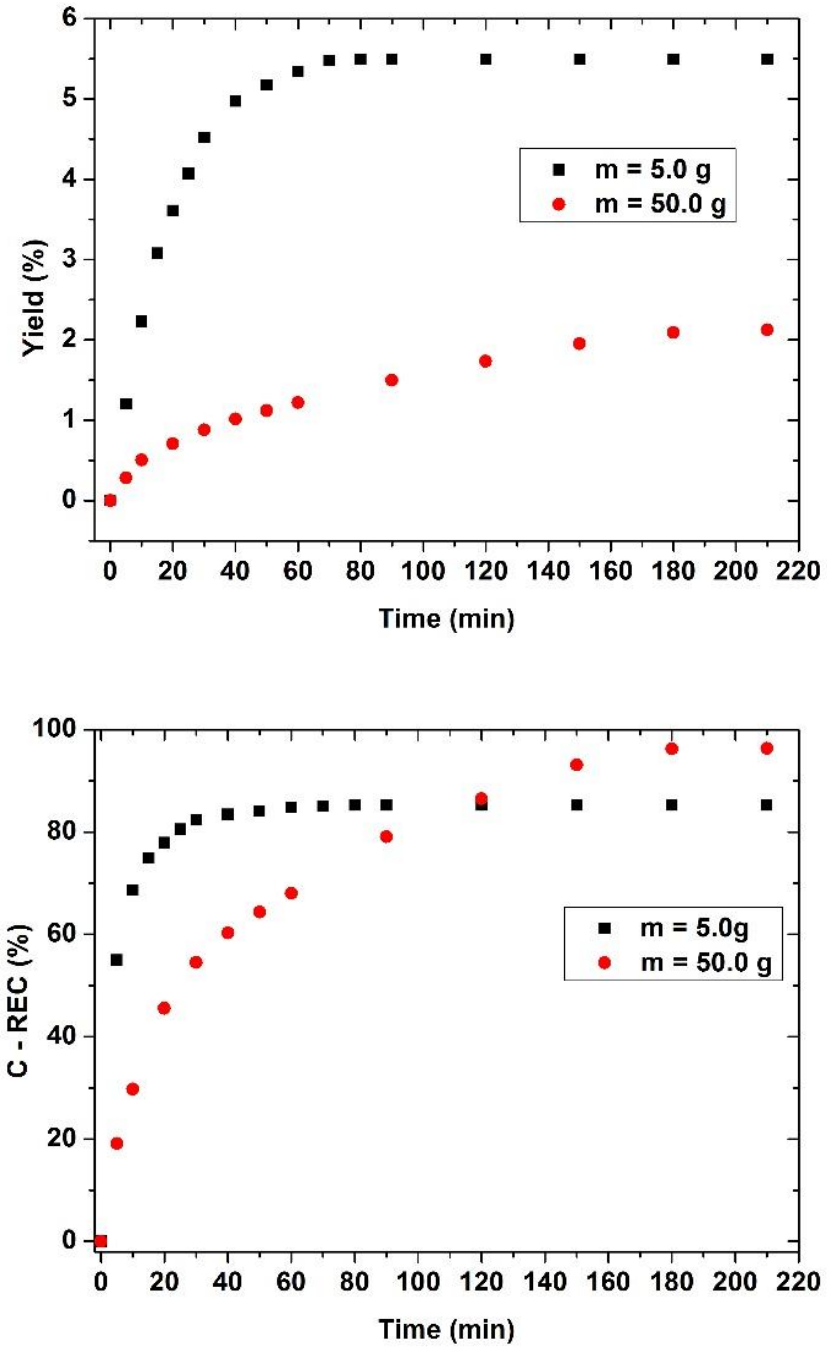

Fig. 3.4. Effect of increasing mass load on (**a**) global mass yield and (**b**) total carotenoid recovery.

When the mass load was increased from 5.0 g to 50.0 g, the global yield decreased from 5.38% to 2.09%. Also, increasing the sample mass affected positively the carotenoid recovery, which reached 96.2% in the full-vessel run, with a total of 9.89 mg of carotenoids being extracted. As expected, the CER phase lasted longer in this experiment (~27.0 min) than in the previous (~6 min). This was due to the fact that the CO_2 flow rate was kept constant while the mass was increased. The scaled-up experiment reached the same 87%-recovery mark attained in the small-scale experiment (Fig. 3.3b) at around 115 min and then proceeded to reach its plateau (96.2%) at the 180-min mark, which was the optimum time for this process.

A considerable decrease of the extracted mass was noted, whereas at the same time, carotenoid recovery was found to increase. This observation is most likely to occur due to the longer residence time of the CO_2 in the extraction bed and to a higher selectivity exhibited by the solvent. In the first experiment, the extraction bed had a 6-cm height (sample plus glass beads), while in the second, the full vessel (15 cm) was loaded with sample. In this scenario, the bed presented a much lower porosity with almost no head-space so the fluid had more pathways to flow through. This ensured a higher contact time between the phases, less pronounced dispersion effects, faster saturation of the CO_2 with the compounds of interest and consequently, more effective mass transfer rates. Additionally, an increase in selectivity could be taking place due to the presence of higher sample mass, since by applying the same flow rate, the fluid saturated preferentially with the carotenoids that were present

in abundance within the full capacity vessel and much less with more polar macro-compounds, such as carbohydrates.

3.3.5 Compositional analysis of extracts

Different combinations of high and low *P*, *T* and co-solvent concentrations during S-CO_2 extraction can result in distinct extract profiles. Table 3.5 shows the mass of the final extracts, their composition breakdown in percentage of individual macronutrients (*% EXT*), as well as the amount recovered for each component from the original carrot peel samples in d.b. *(% REC*) under each of the four tested conditions, using 5.0 g of sample and 95.0 g of glass beads for each run.

Table 3.5. Extract characterisation under four different extraction conditions.

Sample	Conditions			Ext. mass (mg)	Lipids		Protein (%)		Carbohydrates (%)		Moisture (%)
	T (°C)	P (bar)	EtOH %		%EXT	%REC	%EXT	%REC	%EXT	%REC	-
RAW	-	-	-	-	1.40±0.25	-	2.85±0.35	-	76.2±4.1	-	5.9
EXT 1	60	305	15.5	216.5±10.7[a]	12.2±1.6	30.3±2.2	20.8±1.6	30.6±2.1	57.6±3.2	25.8±1.3	8.1
EXT 2	60	300	0	58.1±4.2[b]	62.0±3.5	41.1±4.6	15.1±0.9	6.0±0.85	17.0±2.6	2.5±0.40	1.9
EXT 3	50	150	5.0	62.1±3.6[b]	24.2±2.1	17.1±0.8	12.5±0.7	9.5±1.1	57.0±4.1	8.9±1.1	4.1
EXT 4	70	350	2.0	64.3±3.9[b]	35.3±2.8	22.3±1.4	17.1±1.9	7.5±0.65	33.9±4.0	5.5±0.75	5.8

%EXT = compound percentage in the extract; %REC = % of compound recovered from the raw sample.

Extract 1 represented the optimal conditions, as attained by the DoE studies, and exhibited the highest amount of carbohydrates among all runs (57.6%), as well as the highest recovery of carbohydrates (25%). This is due to the relatively high percentage of ethanol used as modifier (15.5%), which added polarity to the CO_2 and resulted in the extraction of very polar compounds, such as carbohydrates. This reflects on the total extracted mass (216.5 mg), which was around 4-fold higher than those obtained in all the other conditions. As a consequence of the increased polarity, lipids – highly nonpolar compounds – were present at the lowest amounts among the runs, making up only 12% of this particular extract. The opposite was observed in extract 2, where no co-solvent was used. The amount of extracted mass (58.1 mg) was the lowest observed, with 62% of it consisting of lipids and only 17% of carbohydrates.

Extracts 3 and 4 represented intermediate conditions of *T*, *P* and *EtOH%*. Specifically, extract 3 (low pressure, low temperature) yielded the highest protein amount (22.5%), even though the protein values did not change dramatically among runs. Another noteworthy observation is that this extract, despite the much lower values of *P*, *T* and *EtOH%* compared to the optimal extraction conditions (extract 1), contained very similar amounts of total carbohydrates to extract 1. Also, when comparing extracts 3 and 4 (high pressure, high temperature and slightly less EtOH%), it can be noticed that the carbohydrate content decreased considerably, which seems to indicate that carbohydrates are much more sensitive to changes in co-solvent concentrations than in pressure or temperature, i.e., in the solvation power of CO_2.

Although the carbohydrate content varied among extracts, HPLC analysis showed that their sugar profile did not change considerably (Appendix E, Table E.1). All extracts were

composed of around 45-50% glucose (deriving mainly from beta-glucans and hemicelluloses and to a lesser extent, from cellulose), 20-27% of galacturonic acid (from pectins), 18-22% of xylose and 6-7% of arabinose, both from arabinoxylans.

Based on their composition and depending on the desired final application, these extracts could be potentially utilised directly as ingredients for food (as natural food colourants or processing ingredients) or animal feed applications (animal feed ingredients). Moreover, additional downstream operations could be performed, in cases where a considerably higher purity of carotenoids is required (e.g. employment as fine supplements or as additives in cosmetic and pharmaceutical formulations, etc.). Potential methodologies that could be applied to the SC-CO_2 extracts to obtain carotenoids in a purer form include conventional solvent extraction, adsorption with hydrophobic interaction resins and ultrafiltration. Such processes would constitute promising alternatives to the chemical synthesis route that is currently used for the production of commercial carotenoids [62]. In addition, a number of applications could be further investigated for the solid residues left after the S-CO_2 extraction process. In this study, it was found that the majority of macronutrients present in the original carrot peel samples, i.e. proteins, carbohydrates and lipids, remained intact within the residual mass. As a consequence, the carotenoid-free residue could be used as starting material for the extraction of those macronutrients, which could be used as natural emulsifiers and stabilisers or transformed via fermentation to biofuels and platform chemicals.

3.4 CONCLUSIONS

The results presented in this study allowed a thorough assessment of the direct influence of process variables on supercritical CO_2 extraction of carotenoids from carrot peels, which enabled its statistical and kinetic modelling, and the assessment of its scalability potential and extract characterisation. The best extraction conditions resulted in a 96.2% of carotenoid recovery, when using the full capacity of the extraction vessel. The kinetic studies demonstrated that supercritical CO_2 can extract carotenoid fractions from carrot peels rapidly, whereas model fitting highlighted the fast extraction trend and desorbing nature of carotenoids, based on the values obtained for the model parameters. The findings of the current study could be applied for other vegetable by-product matrices with similar structure, targeting the extraction of carotenoids.

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CHAPTER 4

Purification of supercritical-fluid carotenoid extracts by Hydrophobic Interaction Chromatography

Authors: Micael de Andrade Lima, Dimitris Charalmpopoulos, Afroditi Chatzifragkou.

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Contributions: Micael de Andrade Lima performed all the analysis mentioned in the text, treated, presented and discussed the outcome data and wrote the first draft of this manuscript. Afroditi Chatzifragkou and Dimitris Charalampopoulos were responsible for all the corrections, input on and proofreading of the main text from its draft status until its final version, now available in press.

PREFACE

After successfully identifying the best extraction conditions for the parameters tested and characterising the carotenoid-rich extracts thereof, we could proceed to tackle the second central objective of this work: setting up and implementing a novel carotenoid-specific purification protocol.

As emphasized in the two previous chapters, SFE is still perceived as an expensive technique. Also, it is known that the downstream processing of raw extracts for the recovery and purification of molecules can easily add up to representing the biggest slice of the overall cost of manufacture, which could potentially compromise the economic viability of a process. Therefore, a fundamental prerequisite to the development of such a protocol was that it demanded relatively low capital and maintenance costs while assuring high recovery yields and retaining most of the extract activity. Conventional technologies to this end, such as the use of membrane technologies or electrophoresis, would probably meet the latter criterion, but not the first.

The idea of using hydrophobic chromatography to purify the carotenoid fractions arose from the results of the characterisation in the last chapter, after observing that the main 'macro-impurities' in the extract were sugars and proteins, which have very different polarity from that of carotenoids. Therefore, separating them by relative hydrophobicity seemed sensible. Not only is it a cheap technique, it is also easy to operate and it does not require very specific apparatus.

Since this had never been attempted before, it was necessary to assess all the fundamental parameters and conditions specific to carotenoid adsorption and desorption, both in batch

and in column mode. Moreover, the final solution had to be tested as to activity and purification factor. Once the protocol was proved efficient, both stages could be unified and the concept of having extraction, recovery and purification of carotenoids from a vegetable matrix in the same work line, concretised.

Purification of supercritical-fluid carotenoid extracts by Hydrophobic Interaction Chromatography

ABSTRACT

Supercritical fluid extraction (SFE) has been widely used for extracting several valuable phytochemicals, including carotenoids. However, there is a scarcity of works dealing with the purification of SFE extracts. The aim of this work was to assess the feasibility and efficiency of a hydrophobic interaction chromatography (HIC) protocol for purifying carotenoid-rich extracts obtained by SFE. Initial batch experiments were carried out to calculate the resin adsorption capacity and adsorption kinetics. Subsequent runs were performed in a manually-packed chromatographic column, using the Amberlite XAD-1180N resin, where breakthrough curves and adsorption isotherms were obtained and fitted to the Langmuir, Freundlich and Langmuir-Freundlich models. The antioxidant activity and carotenoid degradation rates were monitored throughout the processes. In batch, the resin presented a maximum carotenoid adsorption capacity of 1.89 μg/mg, while in column, this value increased to 10.4 μg/mg. The global carotenoid adsorption rate was 93.3% and the elution rate, 94.7%, resulting in a global recovery of 88.4% for total carotenoids and 92.1% for carotenes. The Langmuir model fitted best the experimental data. Analysis of the extracts demonstrated that a 5.5-fold reduction in extract mass was achieved, accompanied by a 4.7-fold and 2.1-fold increase in carotenoid concentration and antioxidant activity, respectively. This work presents a novel process based on preparative HIC for the purification of carotenoid extracts and provides a fundamental understanding on process performance. It

is potentially scalable and can be implemented in extraction and purification of carotenoids from natural sources, as an alternative to their production through chemical synthesis.

Keywords: carotenoids, hydrophobic interaction chromatography, purification, adsorption, Langmuir model, supercritical fluid extract.

4.1 INTRODUCTION

Carotenoids are mainly C_{40} tetraterpenoids formed by eight C_5 isoprene units joined head-to-tail to give a conjugated chain; the two isoprene units at the centre, in turn, are joined head-to-head, granting the molecules a symmetrical structure [1]. The interest of the food in these compounds is primarily driven by their potential use as pigments and as such, they are chemically produced for use as colour additives and supplements [2]. The cosmetic industry also incorporates carotenoids in a diverse range of products, mainly due to their antioxidant properties [3,4]. Additionally, a number of biological functions have been attributed to these compounds [5–7] and they have also been linked with contributing to a decreased risk of certain types of diseases [8–10].

Most of the current commercial carotenoids (e.g. β-carotene, astaxanthin and canthaxanthin) are primarily products of chemical synthesis, but there is considerable interest in producing them via extraction from natural sources, such as fruit, vegetables and microorganisms [1]. The replacement of synthetic pigments, including carotenoids, by natural ones is regarded as advantageous as it minimises the considerable environmental impact of chemical processing and meets the consumers' expectations for natural products. To this end, the extraction and recovery of carotenoids from fruit and vegetable wastes and by-products is a potentially viable alternative, and is in line with current strategies of valorisation of unexploited natural resources.

New greener methods for extracting phytochemicals have been investigated, one of them being supercritical fluid extraction (SFE). Employing CO_2 in supercritical state for extracting molecules of different polarities, usually in conjunction with a co-solvent such as ethanol,

methanol or acetone, the technique has been proved highly efficient for the extraction of natural components from biomass matrices, such as fruit and vegetables [11]. Using SFE for extracting carotenoids is promising approach, and different vegetable waste matrices have already been tested, including banana, grape and tomato peels [12–14], grape, pomegranate and pumpkin seeds [15–17], and apricot bagasse and pomace [18,19].

It is noteworthy that although vegetable extraction via SFE has been previously investigated, only a few studies deal with further purification of the extracts in order to obtain the targeted compounds in high purity [20,21], which would enable specific applications (e.g. in food, nutraceuticals and cosmetics). The extraction of carotenoids by SFE alone is not a selective technique and other compounds (e.g. phenolics, carbohydrates, proteins and lipids) are normally co-extracted. Further purification could potentially be achieved either by well-established techniques such as ultra or nanofiltration [22], or new protocols could be designed using other separation principles.

Taking the above into account, Hydrophobic Interaction Chromatography (HIC) is a chromatographic method that is capable of delivering high product yields at high purity levels, and could be economically viable at a commercial scale. HIC separates biomolecules under relatively mild conditions according to differences in their hydrophobicity; it is primarily used for protein purification as it complements other established methods that separate these molecules according to their charge (ion exchange) or size (gel filtration) [23]. Carotenoids, being highly hydrophobic molecules, are dissolved in polar solvents in supercritical fluid extracts along with carbohydrates and proteins, which are strong hydrophilic and amphiphilic molecules. All these components represent the main

"impurities" found in such extracts, which leads to the hypothesis that HIC could be an excellent choice for a relatively-cheap and efficient purification protocol. An earlier work has reported the use of with HIC for the separation of carotenoids for analytical purposes [24]; however, to the best of our knowledge, there are no works reporting the development of a preparative purification protocol.

Therefore, the aim of this work was to implement a novel approach for the purification of carotenoid-rich supercritical fluid extracts by using preparative Hydrophobic Interaction Chromatography. Batch and in-column experiments of resin adsorption capacity and kinetics were performed to evaluate the adsorption phenomena and assess process performance, while analysis of the antioxidant activity of the extracts as well as carotenoid degradation rates allowed the monitoring of the biochemical changes taking place. Moreover, breakthrough curves and adsorption isotherms were built in order to mathematically describe and subsequently optimise the in-column adsorption process. Finally, validation runs were performed at the optimal conditions to confirm the efficiency of the new purification protocol.

4.2 MATERIALS AND METHODS

4.2.1 Extract characterisation and analytical methods

The extract was obtained following the supercritical fluid extraction of a total of 300 g of freeze-dried Nantes carrot peels (5.6% moisture content, d.b.) in a SF extractor (SciMed UK). The extraction was carried out for 60 minutes at 350 bar, 59.0 °C, 15 g/min of CO_2 flow rate and 15.5% ethanol as co-solvent. These conditions were previously optimised (Chapter 3) and were shown to produce carrot peel extracts with a high carotenoid content dissolved in ethanol, at concentrations of 1.16 mg/g of β-carotene, 0.64 mg/g of α-carotene and 0.17 mg/g of lutein. For the completion of the whole study, around 9,000 mL of extracts were used. They were characterised and kept at -20 °C in the dark until the time of the analyses.

4.2.1.1 Total carbohydrate content

The total carbohydrate content of the extracts was determined according to the protocol developed by the US Renewable Energy Laboratory [25]. The complete procedure can be found in section 3.2.5.

4.2.1.2 Total lipid content

The lipid content of the extracts was determined gravimetrically, using the Soxhlet method [26]. Briefly, 50 mL of extract (in triplicate) were submitted to Soxhlet extraction using pre-weighted round-bottom boiling flasks. Petroleum ether was used as solvent (Sigma-Aldrich UK, 60 °C boiling point) and the extraction carried out for 4 hours. The flasks containing the

lipid residue were oven-dried, placed in a desiccator to cool down, and weighed. Calculation of the lipid content was done by weight difference.

4.2.1.3 Total protein content

The total protein content of extracts was estimated by the Bradford method [28]. The procedure consisted of collecting an aliquot of 0.1 mL of sample and placing it in contact with 1.0 mL of the Bradford reagent (acidified Coomassie Brilliant Blue G-250, Sigma-Aldrich), and leaving the solution in the dark at 25 °C for 10 minutes for colour development. The absorbance was measured at 595 nm; a calibration curve using bovine serum albumin (Sigma-Aldrich) as standard was employed for protein estimation.

4.2.1.4 Total Carotenoid Content (TCC)

The total carotenoid content (TCC) was analysed according to the method described by Biehler et al [29]. The detailed protocol can be found in section 3.2.2.

4.2.1.5 Antioxidant activity (AA)

For the determination of the antioxidant activity of the samples, the DPPH (2,2-diphenyl-1-picrylhydrazyl) method was used [30]. Briefly, 200 µL of the extracts (in triplicate) were mixed with 2 mL of DPPH reagent. The mixture was incubated for 30 min in the dark and the absorbance was measured at 517 nm using a spectrophotometer (Thermo Election Corp., UK). The antioxidant activity values were usually expressed as the percentage of absorbance

change, by comparing the absorbance of samples against the control (200 μL of methanol + 2 mL of DPPH reagent).

4.2.2 Batch adsorption experiments

In order to evaluate the behaviour of carotenoid adsorption to the hydrophobic resin, batch experiments were initially carried out. All experiments were performed at 22 °C (±2 °C) under dim light and, for most of the duration of the assays, extracts were kept inside dark glass containers, to minimise degradation rates. The polymeric adsorbent used was the Amberlite XAD-1180N (Sigma-Aldrich). Prior to its use, the resin was pre-treated with water for 30 minutes to wash out the Na_2CO_3 salts originally present and oven-dried at 60 °C for 6 hours. Unless stated otherwise, all the experiments in this section were carried out in triplicates.

4.2.2.1 Resin adsorption capacity and resin mass optimisation

10 mL of the SF extracts were diluted to a fixed total carotenoid concentration of 20 μg/mL, placed in a 20-mL flask, and mixed with different amounts of resin (25, 50, 100, 200, 300 and 500 mg, each in triplicate). An approximate total of 200 mL of extract was utilised for this assay. A solution containing pure β-carotene was also used as control. The solutions were stirred in an orbital shaker at approximately 50 rpm for 6 hours to ensure maximum saturation. To obtain the total resin adsorption capacity (q^*, μg adsorbate / mg adsorbent), the capacity *(q)* for each run was calculated as described in Equation 2.18.

4.2.2.2 Adsorption kinetics

To evaluate the minimum time required for resin saturation to take place and thus optimise the process time once q^* is known, kinetic studies were carried out. 10 mL of the undiluted extract were mixed with the optimum resin amount (560 mg) and stirred for 6 hours, as described previously. Triplicate samples were taken every 15 min for the first hour, every 30 min for the following two hours and every 60 min for the rest of the experiment, totalling around 350 mL of extract for this experiment. The adsorption kinetic profiles were established both for individual carotenoids as well as for total carotenoids.

4.2.2.3 Elution and recovery

To elute the carotenoids from the adsorption resin, acetone was used as a solvent, as it presentes lower toxicity compared to other hydrophobic solvents (hexane, tetrahydrofuran). Also, it is considered safe for use as an indirect food additive by the US Food and Drug Administration at concentrations between 5 to 8 mg/L [31], and holds a Generally Recognized as Safe (GRAS) status. Moreover, the fact that acetone can be easily removed through evaporation, renders it a suitable solvent for this and other food manufacturing processes.

Following adsorption, the resin material was separated from the remaining solution by vacuum filtration using Whatman paper No. 1 and then left in a desiccator to dry in the dark under room temperature for 1 h. 10 mL of acetone were added and the solution was agitated for 3 hours in an orbital shaker at approximately 50 rpm to promote the elution of the carotenoids. The suspension was centrifuged at 2500 x g and 4 °C, with the supernatant

collected and evaporated under nitrogen. The solid residue was then dissolved in ethanol for HPLC analysis. Also, the antioxidant activity of the extracts obtained at different process stages (in both batch and in-column experiments) were measured, as previously described.

4.2.3 In-column experiments

4.2.3.1 Experimental apparatus and procedure

For the adsorption and desorption tests in fixed bed, a 30 cm x 10 mm *Econo-Column* glass column (BioRad Laboratories, USA), packed with a volume of 16.5 mL (3.5 g, 20 cm bed height) of the adsorbent Amberlite XAD-1180N, was used. At the top of the column, a flow adapter (BioRad) was attached to keep bed dispersion to a minimum and to prevent loss of adsorbent. The flow rate (Q), was regulated by a small variable-speed peristaltic pump (Watson Marlow, USA) and a fraction collector (Watson Marlow, USA) was used to collect samples at constant intervals, usually at every 5 min.

As a standard procedure for the experiments in fixed bed, the column was equilibrated with pure ethanol for 30 min. After conditioning, a variable volume of extract (dependent on the process stage) at different concentration of carotenoids (50 to 300 μg/mL) was injected with the aid of the peristaltic pump at a constant Q of 2.0 mL/min (previously optimised, Appendix F, Figure F.1), for up to 300 min, until complete bed saturation was reached (adsorption step). Subsequently, a washing step was performed by pumping 40 mL of ethanol into the column to remove the non- or weakly-adsorbed fractions from the bed. The elution (or desorption) step with acetone was then carried out, followed by a regeneration step with

0.5% HCl, to remove the very strongly-bound carotenoids and other impurities that were still attached to the resin.

4.2.3.2 Breakthrough curves

Breakthrough curves (BTCs) depicting the ratio between the carotenoid concentration in the column outlet (C) and the column inlet (C_0) as a function of time, were used to describe the progress of adsorption with time. These were constructed as follows: solutions of 350 to 750 mL of SF extracts with a TCC varying from 50 μg/mL to 300 μg/mL (higher concentrations required lower volumes to saturate the column) were injected into the system at a fixed flow rate of 2.0 mL/min, and the total carotenoid concentration was monitored at regular time intervals by HPLC, as described in section 2.1.4. Including the replicates, an approximate total of 7000 mL of extract was needed for this assay. TCC concentrations higher than those in the extracts were obtained by evaporation in a rotavapor (Buchi, UK), whereas lower TCC concentrations were obtained by dilution with ethanol. From the BTCs, the amount of carotenoids adsorbed by the resin (q^*, μg/mg) for each initial TCC concentration was calculated using Equation 2.20.

4.2.3.3 Adsorption isotherm and mathematical modelling

With the q^* values obtained from the BTCs, the equilibrium isotherm was then built, where q^* was plotted as a function of the concentration in the liquid phase (C_{eq}). Three of the different models described in section 2.4.3.4 were used to model the data: Langmuir (Equation 2.21), Freundlich (Equation 2.22) and Langmuir-Freundlich (Equation 2.23). The

Henry model was not included due to being of linear nature and the isotherm obtained being clearly nonlinear, as seen ahead in Figure 4.6.

4.2.3.4 Elution

For eluting the carotenoids from the column during the desorption stage, 100 mL of acetone were used, at different flow rates, namely 1.0, 2.0 and 3.0 mL/min. The eluate was then evaporated to dryness under nitrogen steam, re-dissolved in 50 mL of ethanol and chemically characterised, in order to calculate the global recoveries and antioxidant activities post-purification.

4.2.4 Validation runs

A complete in-column run, performed in duplicate under the optimal conditions of resin mass, process time and elution flow rate, was carried out for validation purposes. Moreover, another in-column purification run was carried out after adding 1 mg/mL butylated hydroxytoluene (BHT) to the extracts. These final experiments consumed around 1600 mL of extract.

4.2.5 Process flowchart

Figure 4.1 shows the process flowchart to enable a visual aid to the stages followed in this work.

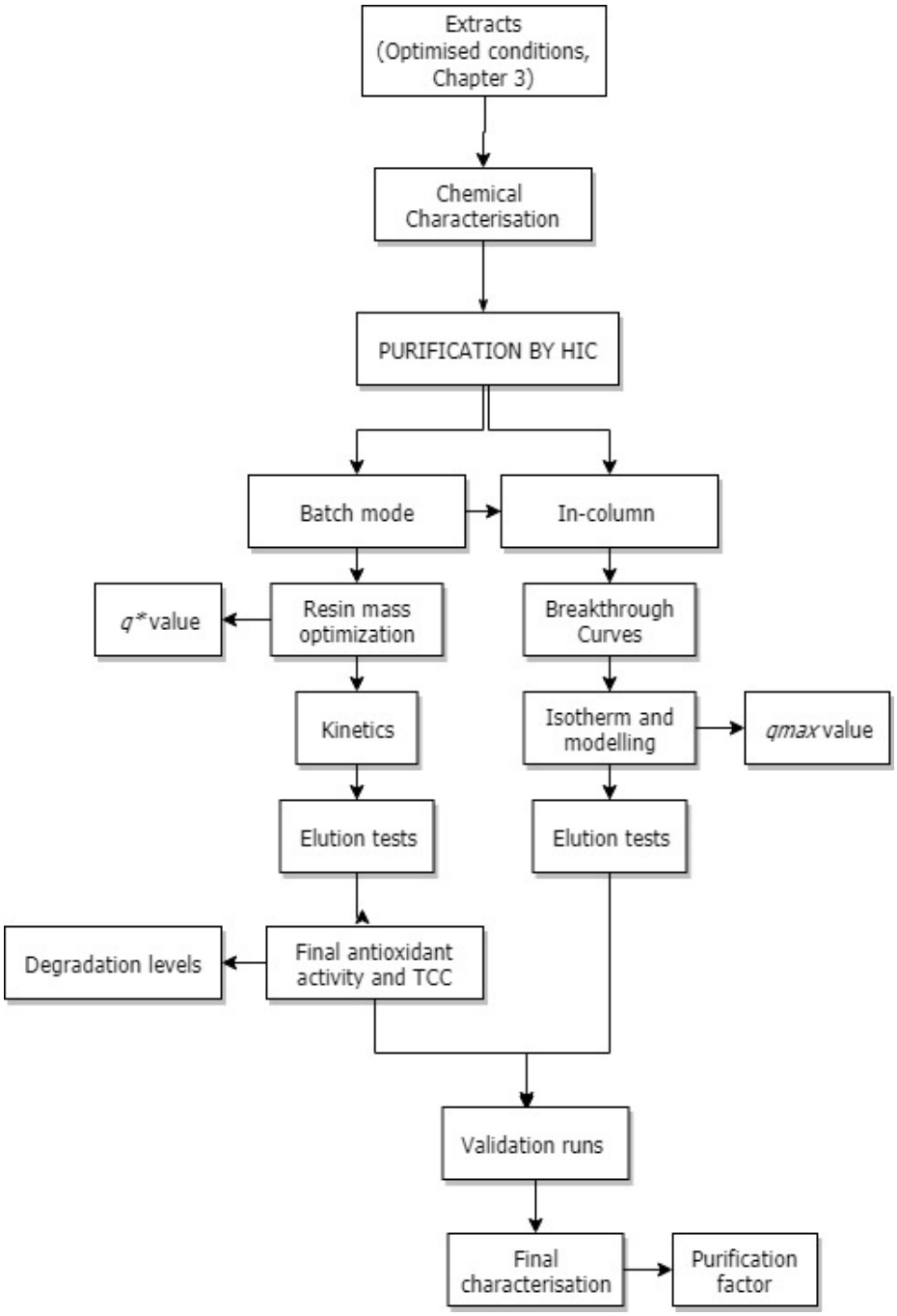

Fig. 4.1. Process flowchart for the purification protocol

4.3 RESULTS AND DISCUSSION

4.3.1 Batch adsorption experiments

As discussed in section 2.4.3, the main environmental parameters reported to influence hydrophobic interactions of proteins, which are the molecules extensively studied in HIC, are the type of buffer, buffer ionic strength (i.e., salts concentration), temperature and pH [27]. The first two are due to the fact that proteins are amphipathic compounds and therefore, under high salt concentrations, their hydrophobic terminations are exposed on the surface, favouring their binding to the adsorbent. This does not apply to carotenoids, which are purely hydrophobic molecules, so these factors can be neglected in this case. As hydrophobic interactions are temperature-dependent, temperature considerably affects the adsorption process. While high temperatures (30 - 45 °C) usually have a positive influence on adsorption (in the case of proteins), lower temperatures (below room temperature) are known to reduce considerably the resin binding capacity [27]. Since carotenoids are extremely heat sensitive, room temperature (22 °C ± 2 °C) was selected as an appropriate processing temperature. For all experiments, the pH was maintained at 6.0, the same pH as the original extracts. More acidic conditions (pH 4.0 and below) are known to trigger carotenoid degradation [1] and there is no evidence to justify the need for the further fine-tuning of this parameter for carotenoid adsorption.

4.3.1.1 Resin adsorption capacity and mass optimisation

Figure 4.2 depicts the relationship between the percentage of adsorption of the individual carotenoids present in the extracts and the mass of resin. Pure β-carotene (STD) was also tested as a control.

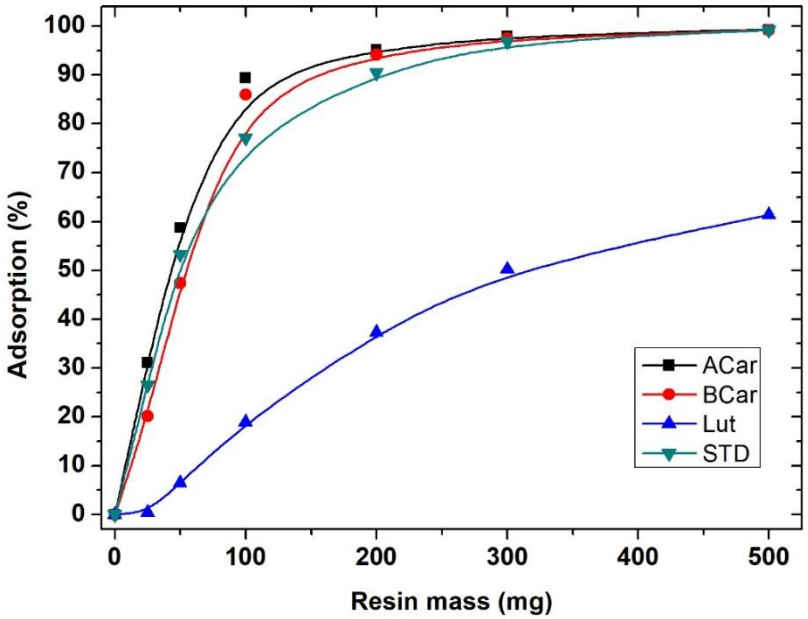

Fig. 4.2. Adsorption percentage of carotenoids adsorbed versus resin amount in batch mode, after 6 hours, at 22 °C (± 2 °C), and pH 6.0. ACar: α-carotene; BCar: β-carotene; LUT: lutein; STD: β-carotene standard.

It can be observed that α-carotene and β-carotene demonstrated a very high degree of affinity towards the adsorbent. The Amberlite XAD-1180N resin is a non-ionic hydrophobic cross-linked polymer, which is safe for food-related applications and usually employed to adsorb large hydrophobic molecules from polar solvents, e.g. proteins [28]. Within the XAD family, this particular resin has the largest pore diameter (300 Å), which makes it ideal for

the binding of very large molecules, such as carotenoids. It can be observed that the adsorbent seemed to have a slightly higher affinity for α-carotene as opposed to β-carotene when used in lower amounts, but this became less pronounced with the increase in the resin mass. The adsorption of α-carotene and β-carotene was directly proportional to the amount of resin used up to 100 mg of resin, resulting in ~85% adsorption. Using 200 mg of resin resulted in an adsorption rate of ~95%, whereas further increases in the mass of adsorbent did not improve the % adsorption any further.

Lutein, belonging to the xanthophyll class of carotenoids, is more polar than carotenes [1] and this reflects the lower adsorption percentage. It can be observed that once the resin started to saturate with carotenes, the adsorption of lutein increased, most likely due to adsorbent excess. It must be noted however, that lutein accounts only for ~5-7% of the total carotenoids content (TCC) in the extract and therefore the overall process efficiency is more dependent on the recovery of the carotenes.

On the basis of these in-batch data, the total resin adsorption capacity (q^*) for each carotenoid and for total carotenoids can be calculated using Equation 2.18. The q^* was ~1.89 μg of TCC per milligram of resin in the case of the extract and ~2.13 μg/mg in the case of the β-carotene standard. The q^* was higher in the latter due to the fact that in the supercritical-derived extract, hydrophobic molecules other than carotenoids (primarily lipids) might be competing for the adsorption sites and hence the overall yield decreases, as opposed to the pure β-carotene standard. The resin adsorption capacity is critical knowledge to assess the binding efficiency of the adsorbate to the adsorbent and to calculate the optimum amount of resin needed for a particular extract with a known carotenoid concentration.

4.3.1.2 Adsorption kinetics

After the optimization of the resin amount, the next step was to evaluate the adsorption kinetic profile of α-carotene and β-carotene in the extracts (Figure 4.3).

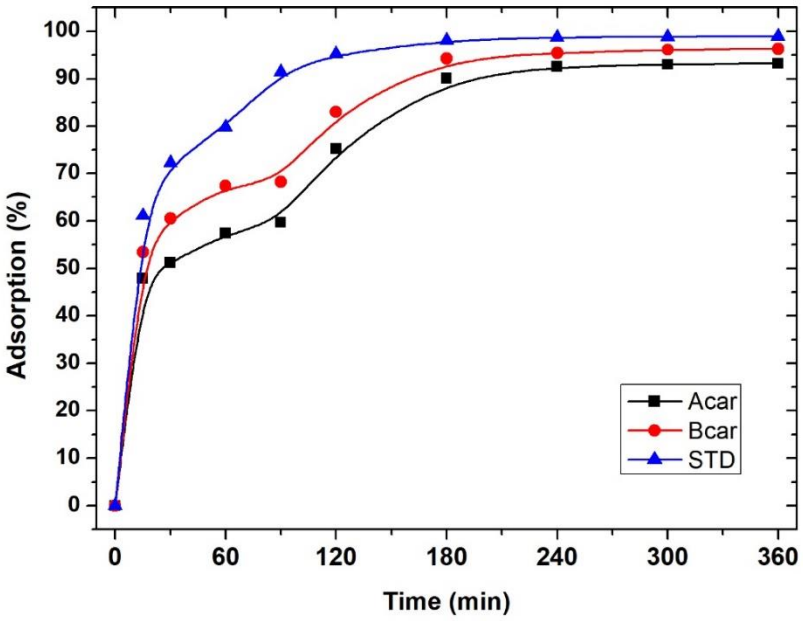

Fig. 4.3. Adsorption kinetics of α-carotene and β-carotene present in extracts in batch mode at 22 °C (± 2 °C), using 560 mg of resin. ACar: α-carotene; BCar: β-carotene; STD: β-carotene standard.

It appears that the binding of a considerable amount of the carotenoids took place almost instantaneously when in contact with the resin (~48% for α-carotene in extract, ~53% for β-carotene in extract and ~61% for β-carotene standard) and then slowly increased with time. The adsorption of the standard β-carotene was faster than in the case of the carotenes in the extract, most likely due to the presence of lipids in the latter, which compete for the adsorption sites. The optimum time selected for adsorption was 180 min, at which time point

~ 90% of α-carotene and ~94% of β-carotene had already been successfully bound to the resin.

4.3.1.3 Elution and carotenoid degradation

In this work, acetone was used as the solvent to desorb the carotenoids due to the reasons already discussed and also due to the much better yields found in preliminary experiments when comparing it to more polar solvents, e.g. methanol, and to its lower toxicity compared to other nonpolar eluents, e.g. hexane (Appendix F, Table F.1). For acetone, high recoveries were achieved during the desorption process, i.e. ~91.6% for α-carotene, ~96.6% for β-carotene, ~90.6% for lutein and ~94.2% for total carotenoids (Table 4.1); in the case of the standard β-carotene, this was lower (~ 83%).

In addition to carotenoid recovery, the antioxidant activity of the samples during the adsorption/desorption process was measured (Table 4.1). It was shown that the antioxidant activity of the eluted sample was ~93% of that in the original extract, whereas that of the standard β-carotene was again lower, i.e. ~87%. The higher antioxidant activities in the eluates of the raw extracts can be attributed to the presence of lipids, which have been shown to exert a protective effect on carotenoids against degradation under adverse environmental conditions, such as exposure to high temperatures for extended periods of time, light and oxygen [1]. It needs to be noted, however, that although the antioxidant activities of the samples during adsorption/desorption were high, these values are based on measurements in the liquid phase. Therefore, the potential contribution of carotenoid oxidation to these decreases, rather than solely to incomplete adsorption, cannot be excluded. To circumvent

this phenomenon in the subsequent in-column experiments, the antioxidant agent BHT was added to minimise degradation.

Table 4.1. Carotenoid concentration and antioxidant activity of samples in the extract (t = 0), after 3 hours of adsorption (ADS) and after 3 hours of desorption (DES), in batch.

	Carotenoid	**t = 0**	**ADS, 3h**	**DES, 3h**	**% (Final/Initial)**
Standard	BCar (μg/mL)	102.2	94.7	84.8	83.0
	AA (%)	54.9	52.8	47.5	86.5
Extract	ACar (μg/mL)	34.0	32.6	31.1	91.6
	BCar (μg/mL)	61.2	60.1	59.1	96.6
	LUT (μg/mL)	11.7	10.9	10.6	90.6
	TCC (μg/mL)	106.9	103.7	100.8	94.2
	AA (%)	32.5	31.7	30.1	92.6

ACar = α-carotene in extract; BCar = β-carotene in extract; LUT = lutein in extract; TCR = Total Carotenoid Recovery; AA = Antioxidant Activity.

4.3.2 In-column adsorption experiments

Having optimised the conditions in batch mode, the adsorption of the carotenoids present in the extract to the hydrophobic adsorbent was investigated in a fixed-bed column. This is important in order to evaluate the feasibility of operating the process under a semi-continuous mode, which is advantageous from an industrial perspective, and calculate key

process parameters that can be used to build mathematical models to predict process performance and assist in scaling up.

4.3.2.1 Breakthrough curves (BTCs)

The flow rate used for the adsorption experiments in column had been previously optimised and, among the values tested (1.0, 2.0 and 3.0 mL/min), the intermediate configuration showed the best adsorptive behaviour (Appendix F, Figure F.1). Therefore, the adsorption of individual carotenoids at the flow rate of 2.0 mL/min of extract is represented by the BTCs depicted in Figure 4.4, in which C/C_0 is plotted as a function of time. The breakpoint is the time point where C/C_0 starts to increase. The column is regarded as saturated when C/C_0 reaches 1.0.

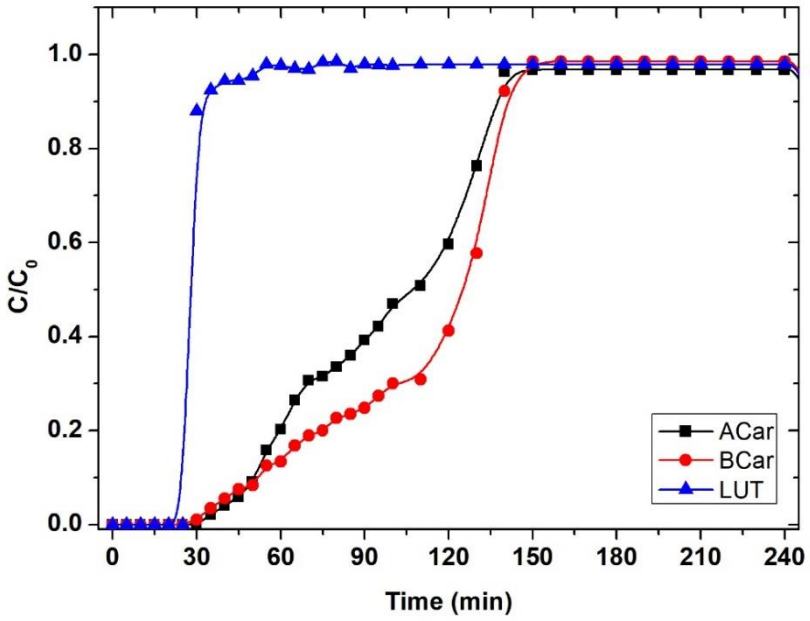

Fig. 4.4. In-column breakthrough curves at 22 ± 2 °C and a flow rate of 2.0 mL/min of extract. ACar: α-carotene; BCar: β-carotene; LUT: lutein. The TCC composition of the extract was: 32.5% α-carotene, 58.9% β-carotene, 8.6% lutein.

In the case of α- and β-carotene, the graphs resembled an "S-shaped" curve, which is characteristic of a well-resolved BTC [29], and the breakpoint was assigned at 35 minutes for both compounds. For the first 50 minutes, the adsorption of α-carotene and β-carotene was simultaneous, but between 50 and 110 min, α-carotene demonstrated a faster adsorption rate than β-carotene. Nevertheless, for both compounds, maximum saturation was achieved at the same time point, i.e. 140 min, corresponding to a 96% adsorption and a resin adsorption capacity (q^*) value of 10.4 μg/mg, 5.5-fold higher than the value obtained in batch mode. This can be attributed to the typical design of a chromatographic column, i.e. adsorption takes place continuously since the extract is constantly fed into the column under

a steady flow rate, the number of theoretical plates are much higher than that of a batch process due to the column dimensions, and the bed is fixed; the latter prevents particle dispersion and allows more efficient contact between the liquid phase and the adsorbent surface, which favours interactions and explains the significant increase of the q^* value. In the case of lutein, on the other hand, an almost-immediate saturation of the column was observed, confirming once again that this particular adsorption/desorption process is not suitable for relatively polar compounds, as already observed in batch experiments.

4.3.2.2 Adsorption Isotherm and mathematical modelling

In order to construct the adsorption isotherm, BTCs were initially constructed to describe the adsorption of total carotenoids (within the extracts) at different concentrations, from 50 µg/mL to 300 µg/mL. The process is explained in section 2.4.3.3. and the curves are shown in Figure 4.5.

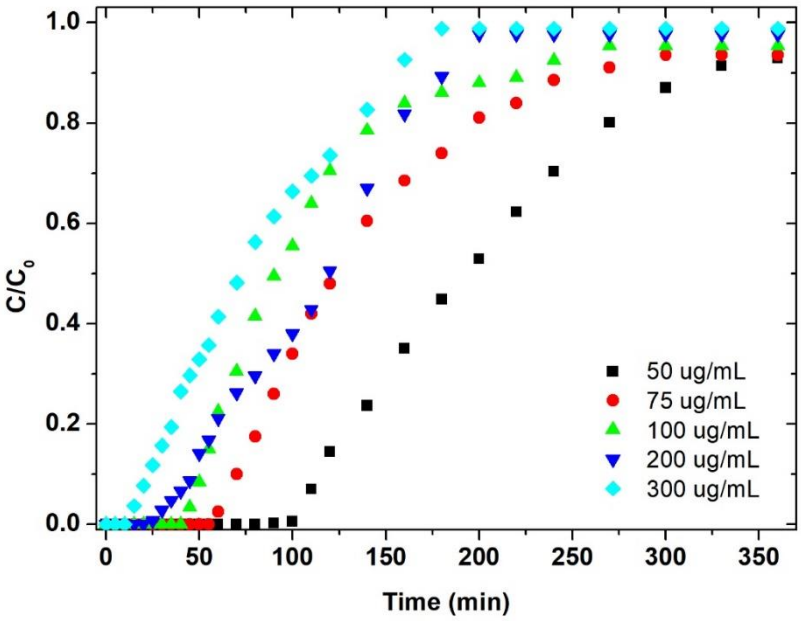

Fig. 4.5. In-column adsorption breakthrough curves at a flow rate of 2.0 mL/min of extract, at 22 ± 2 °C, with different inlet concentrations of total carotenoids.

The breakpoints were observed at earlier time points for higher concentrations than for lower, ranging from 15 min (at 300 μg/mL) to 100 min (at 50 μg/mL). Also, higher concentrations seemed to result in slightly better adsorption (which ranged from 92% for 50 μg/mL to 98% for 300 μg/mL), most likely due do the shorter processing times and consequently lower degradation levels during the process.

The results on the hydrodynamic characterization of the bed can be found on Appendix F (Table F.2). The porosity value of the bed (ε) was calculated as 0.42 [30]; this is a reasonable value as it is a manually-packed column and values less than 0.40 are rarely encountered in such columns [31]. Peristaltic pumps do not inflict enough pressure to pump solutions

through very compact beds and to avoid backlashing of fluids, the column should not have very low ε (<0.30). Very high ε (0.50 and above), on the other hand, causes intense axial dispersion and reduces the interactions between the compounds and the solid phase [32]. The dead time was of 5.13 min. This experimental data generated were used to calculate the q^* for each of the BTCs using Equations 2.19 and 2.20. These were then plotted as a function of total carotenoid concertation, to obtain the adsorption isotherm, subsequently fitted to three different models: the Langmuir model, the Freundlich model, and the Langmuir-Freundlich model (Figure 4.6). These have been widely used to describe experimental adsorption data involving solutions of a strongly adsorbed component to an adsorbent material [33]. The q_{max} was calculated by taking into account the highest q^* value, after it plateaued.

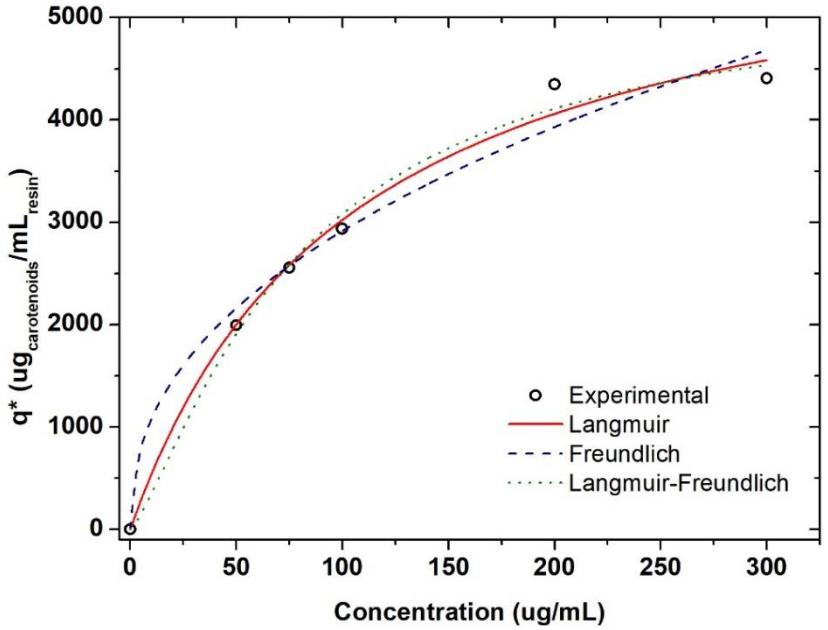

Fig. 4.6. Adsorption isotherm of carotenoids at 22 ± 2 °C fitted to different models

All the adjusted model parameters can be found in Appendix G, Table G.1. Based on the Langmuir model, the number of adsorption sites (q_m) was estimated as ~6173 and the highest q^* value as around 4600 μg of total carotenoids per mL resin, corresponding to a maximum adsorbent capacity (q_{max}) of ~12.3 μg TCC/mg, close to the actual q^* value attained experimentally (10.4 μg/mg). In practical terms, this indicates that 1.0 L of extract at 100 μg/mL of TCC will only require a resin amount of 8.1 g to be treated in one single run, which demonstrates the potential economic viability of the developed protocol. Also, the constant k in the model indicates how strong the compounds are adsorbed to the solid phase, with lower values indicating a stronger interaction between the adsorbent and adsorbate; k-values in the range of 10^{-2} and 10^{-3}, such as that found for this process (9.6×10^{-3}), are indicators of strong interactions [34].

In the case of the Langmuir-Freundlich model, the q_m value was estimated as ~5344, with a q_{max} value of ~13.3 μg TCC/mg resin. For the Freundlich model, the b-value, which is the Freundlich isotherm constant related to adsorption capacity, was estimated at ~401. Both models also gave high R^2 values (0.974 and 0.977, respectively), but the standard errors of the equations' terms and constants (q_m, k, b, n) were higher (ranging from 15 to 22%), than in the case of the Langmuir model (Appendix G, Table G.1). Overall, given the higher R^2 (0.986) and the lower error values for the constants of the latter model (6.8 and 12.6%) compared to the other two, the Langmuir model was considered the best in describing the adsorption isotherm of carotenoids. Therefore, it can be concluded that the carotenoid adsorption follows a monolayer distribution on the surface of the particles and that the interactions are considered strong. All these point to the need for optimising the elution

conditions and that the adsorbate take-up of carotenoids is independent of concentration. The influence of some factors such as extract concentration, and internal and external mass transfer coefficients is often negligible in model development at small scales, but for larger scale operations, these are crucial. In this regard, the data generated in this work can be used for calculating key scale up parameters including the mass transfer coefficients, the adsorption rates and the specific *Bi* and *Sh* numbers [35]; this, however, was outside the scope of this work.

4.3.2.3 Elution and recovery

Table 4.2 shows the carotenoid recovery (%) and antioxidant activity (% of activity per mg of total carotenoids) of the recovered samples throughout the process, i.e. after the adsorption and the elution steps, under three different elution flow rates. Moreover, the global carotenoid recoveries were calculated in relation to the initial carotenoid content of the extract.

Table 4.2. Recoveries of carotenoids and antioxidant activity of recovered samples during in-column adsorption at different flow rates.

Step	Description	TCC						AA	
		ACar (mg)	BCar (mg)	Lut (mg)	Total (mg)	Rec (%)	Global Rec (%)	DPPH (% /mg CAR)	PF
Injection	Initial extract	17.27	35.71	1.12	54.08	-	-	1.53	1
Adsorption	Bound fractions	12.03	23.25	0.22	35.48	65.6	-	-	-
Elution	1 mL/min	10.87	23.02	n.d.	33.89	95.4	62.6	2.96	1.93
	2 mL/min	10.74	22.92	n.d.	33.66	94.7	62.2	3.21	2.10
	3 mL/min	7.99	15.25	n.d.	23.24	65.5	43.0	2.12	1.39

ACar = α-carotene; BCar = β-carotene; LUT = lutein; n.d. = not detected, PF = purification factor (ratio between the elution and injection steps).

It is important to highlight the apparent lower % adsorption recovery value (~66%) compared to those reported for the batch experiments. This is due to the fact that in the experiments performed in column, the runs were allowed to progress for much longer than their breakpoints, i.e., until saturation was reached. For global calculations, the ratio of adsorbed carotenoids relative to the total amount injected (~65.6% in this case) is used; this then does not account for the amount of carotenoids that bypass the resin after the breakpoint and before bed saturation (~34.4%) and hence, the lower values compared to those obtained in batch experiments. In terms of carotenoid recovery during the elution step,

it can be observed that for the lower flow rates (1.0 mL/min and 2.0 mL/min), the recovery was very high (~95%), whereas for the higher flow rate (3.0 mL/min) it was relatively low (~65%). This was most likely due to the fact that under that flow rate the eluent did not have enough time to interact with the resin and desorb the bound carotenoid fractions. The global recoveries achieved were ~62% for the flow rates of 1.0 mL/min and 2.0 mL/min, which demonstrate that the process is efficient and potentially economically viable. Moreover, it is interesting to note the increase in the antioxidant activity of the extract after the purification steps, especially at 2.0 mL/min, where a 2.1-fold increase in antioxidant activity was obtained. This indicates the removal of impurities present in the extracts, including sugars and proteins, which was confirmed later (section 4.3.4).

One alternative to overcome the lower overall recovery rates caused by allowing the adsorption step to run up until complete column saturation is to stop the adsorption process just after the breakpoint, i.e., at approximately 30 min, and then proceed to the washing and elution steps. This should increase the recovery rates and also prevent additional losses of carotenoids. On the other hand, this approach would require longer processing times due to the fact that more runs would be needed to process the same amount of extracts. Another option would be the inclusion of two or three columns in series, where the outlet of one is connected to the inlet of the other. This way, the rejected fractions could be submitted to the new columns and the recoveries could be maximised by the complete adsorption of the carotenoids. Both setups will naturally imply on extra capital costs but, after economical evaluations, the investment might pay back. Therefore, a cost analysis should be carried out to assess each of the above scenarios separately and decide on the preferred approach that ensures the economic viability of the process at a larger scale while maximising yields.

4.3.3 Validation runs

Two additional runs (in duplicates) were performed in order to validate the optimum conditions for the purification of the carotenoids, i.e. 450 mL of extract at 105.9 μg TCC/mL, under a flow rate of 2.0 mL/min, temperature of 22 ± 2°C, and process time of 210 min (adsorption: 140 min, washing: 20 min, desorption: 50 min). The second run was carried out at the same settings, with the only difference being that butylated hydroxytoluene (BHT) was added in the extract at a concentration of 1.0 mg/mL. BHT is a strong antioxidant agent widely used in the food industry, which in this case could potentially minimise the likelihood of carotenoid degradation during the purification process. The process chromatograms are shown in Figure 4.7.

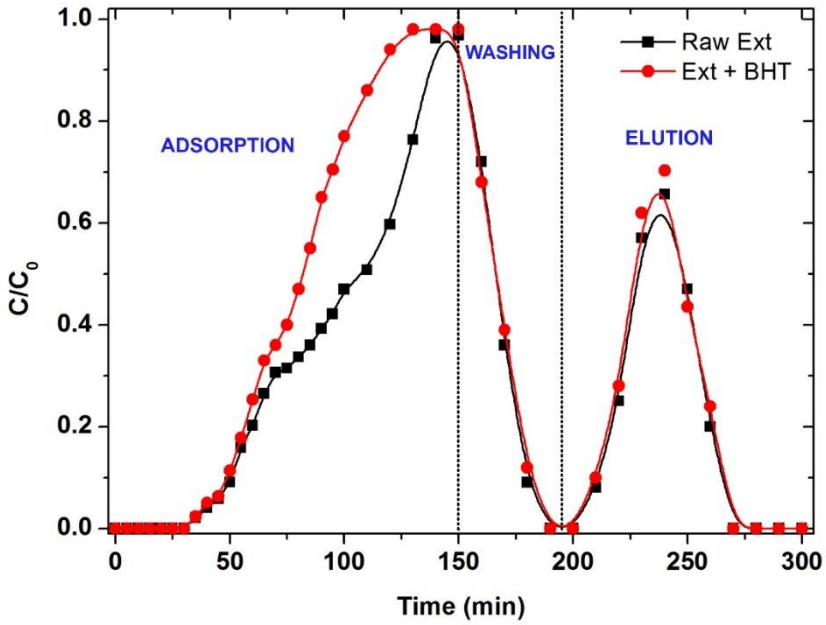

Fig. 4.7. Chromatograms of the in-column validation runs for the purification of carotenoids present in the raw extract (TCC: 105.9 μg/mL) and extract with addition of 1mg/mL of butylated hydroxytoluene (BHT). Process conditions: 450 mL extract, flow rate 2.0 mL/min, temperature 22 ± 2°C, and total processing time of 210 min. C and C_0 correspond to final and initial TCC, respectively.

Using a flow rate of 2 mL/min of extract, which had a total carotenoid concentration of ~106 μg/mL, the chromatographic bed was saturated within 145 minutes (C/C_0 = 0.96) and within 130 minutes, in the case of the extract with added BHT (C/C_0 = 0.981). A difference was also noticed in the adsorption curve profile of the latter, which demonstrated a faster rate of adsorption. The above suggest that there is likelihood that the presence of BHT reduced carotenoid degradation during the process, which was nevertheless low, as also shown by

the previous data. In line with these observations, the global carotenoid recovery was also slightly higher in the case of the extract with BHT (~70% vs 65%).

4.3.4 Final extract characterisation

Table 4.3 shows the initial composition of the extract and that of the purified fraction following in-column adsorption.

Table 4.3.. Chemical composition of the initial extract with 1mg/mL BHT and of the purified fraction following in-column adsorption. Process conditions: 450 mL extract at 105.9 µg TCC/mL, flow rate 2.0 mL/min, temperature 22 ± 2°C, and total processing time of 210 min.

	Compound	**Initial extract**	**Final extract**	**PF**
-	Total extract mass (mg, d.b.)	2690	505	0.18
	Antioxidant activity (%)	26.4	55.4	2.09
Macronutrients	Total Protein (mg/g extract)	258.4	362.4	1.40
	Total Lipids (mg/g extract)	162.3	631.0	3.89
	Total Carbohydrates (mg/g ext)	576.0	n.d	0.00
	Glucose	307.0	n.d.	-
	Xylose	108.5	n.d.	-
	Arabinose	35.2	n.d.	-
	Galacturonic acid	126.4	n.d.	-
Micronutrients	Total Carotenoids (mg/g extract)	1.97	9.27	4.71
	α-carotene	0.64	3.65	5.70
	β-carotene	1.16	5.61	4.84
	Lutein	0.17	n.d.	0.00
	Total Phenolics (mg/g extract)	n.d	n.d	-

PF = (ratio between values in the purified extract and those in the initial), n.d.: not detected.

The mass of the extract following purification (eluate) decreased by 5.3-fold compared to the initial mass. This is in line with the complete removal of all the carbohydrate content from the extract, which contributed with ~58% of the total mass in the initial extract. This was likely due to the fact that carbohydrates are polar molecules and therefore not adsorbed by the hydrophobic resin.

Additionally, the process resulted in the removal of a large percentage of the proteins and, to a lesser extent, of the lipids present in the extract. Following purification, the eluate, besides carotenoids, consisted of lipids (~63%, 4.0-fold increase) and proteins (~36%, 1.4-fold increase). According to the mass balance, the mass of proteins and lipids decreased in the eluate by 74% and 28%, respectively. Proteins are amphipathic molecules and can respond differently depending on the properties of the solvent used. Ethanol, used as the solvent in the adsorption step, has a small dielectric constant and therefore reduces the solubility of the proteins, resulting in stronger interactions between the proteins and the hydrophobic resin. In the desorption stage, acetone, which has an even lower dielectric constant than ethanol, was used and therefore, most of the proteins remained bound to the resin, explaining the significant decrease in their content in the eluate. Lipids, on the other hand, due to being hydrophobic and having high affinity for both the resin and acetone, behave similarly to carotenoids and, as a result, a significant amount of the lipids present in the initial extract was recovered in the eluate. From a product development perspective, the presence of high amounts of lipids in the purified extract could be desirable as they can protect against carotenoid degradation [1].

The purification factor in the case of total carotenoids was 4.71 or 5.27 if only α-carotene and β-carotene are taken into account, which coupled with the compositional data,

demonstrate that the purification process generates extracts with high levels of purity. The extract was also analysed for phenolic compounds; however, no amounts were detected. This indicates that carotenoids are most likely the compounds primarily responsible for the antioxidant activity in the extract and the purified fraction, which is also supported by the observed increase in the antioxidant activity of the purified fractions by a factor of ~2.1.

The use of hydrophobic interaction chromatography for the purification of carotenoids from vegetable extracts is a novel approach and as such, there are no data in the literature to enable direct comparisons. However, carotenoid purification has been assessed before at a preparative scale using size-exclusion separation by membrane technologies. Gomez-Loredo et al. [36] studied the purification by ultrafiltration (UF) of a microalgae-derived fucoxanthin extract obtained from a two-phase aqueous system. The authors used cellulose UF membranes with a 10-kDa molecular weight cut-off (MWCO). The maximum recovery was 63%, with a concomitant reduction in protein-related impurities by 16%. Moreover, a fungus-derived-canthaxanthin extract was extracted by different solvents (hexane, acetone, methanol and ethanol) and purified by nanofiltration and nonporous membranes. The membrane that showed the best performance had a 0.25 kDa MWCO and the maximum recovery was 84% when the extract was dissolved in methanol [37]. The present work advances the knowledge in the downstream processing of carotenoid-rich extracts and proposes a potentially scalable and economically viable process for the extraction and purification of carotenoids from natural sources that can be used as an alternative to the production of carotenoids through chemical synthesis.

4.4 CONCLUSIONS

This work developed a preparative method based on hydrophobic interaction chromatography to effectively purify carotenoids from a carotenoid-rich extract produced after supercritical fluid extraction of carrot peels. In the batch process, the total resin adsorption capacity (q^*) was ~1.9 μg of total carotenoids per milligram of resin whereas in the in-column process, this was ~10.4 μg/mg, most likely due the improved hydrodynamic conditions at the particle surface. Mathematical modelling of the adsorption isotherm (q^* as a function of the total carotenoid concentration), with the q^*-values generated from in-column breakthrough curves, demonstrated that the Langmuir model was able to adequately describe the adsorption process, and generated an estimated q_{max} value, i.e. the maximum amount of adsorbent that the resin is able to uptake, of ~12.3 μg/mg. This is close to the experimental q^*-value achieved and can be said to be very high, confirming the economic viability potential of the process.

The global recovery of carotenoids in the batch process measured after elution was 89.9%, whereas in the in-column process this dropped to 62.2%. This was due to the fact that the column was allowed to run up to complete saturation. The validation runs performed demonstrated that there is most likely a small level of carotenoid degradation taking place during the process, although the global carotenoid recoveries achieved were still high (~ 65%) and can be increased if the antioxidant BHT is added (~70%). The purity of the final eluate was also high, as the carotenoid concentration increased by ~5-fold compared to the raw extract, whereas all of the carbohydrates were removed (originally ~58% in the extract), most likely due to the fact that carbohydrates are polar and therefore have no affinity with the hydrophobic adsorbent. Also, the actual mass of proteins decreased by 74%

most likely due to the low dielectric constant of acetone used for the elution, indicating that the majority of the protein remained bound to the resin through strong interactions. On the other hand, the mass of lipids decreased by only 28%, most likely due their high affinity for both the adsorption resin and the eluent acetone. From a product development point of view, the presence of high amounts of lipids in the purified extract could be desirable as they can protect against carotenoid degradation.

This work presents an efficient novel process based on preparative hydrophobic chromatography for the purification of carotenoid-rich extracts and provides a fundamental understanding on process performance. Such process is potentially scalable and can be implemented for the extraction and purification of carotenoids from natural sources, as an alternative to the production of carotenoids via chemical routes.

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CHAPTER 5

Extraction of carotenoids from vegetable waste matrices: generalisation of optimised conditions

PREFACE

As demonstrated in Chapters 2 and 3, throughout the course of the last two decades, there has been a number of works in the literature reporting the supercritical fluid extraction (SFE) of carotenoids from fruits, vegetables, algae, micro-organisms and even from crustaceans. Although undoubtedly providing interesting insightful information regarding the SFE for this specific application, the procedures, methodologies and, most importantly, the results presented are not always homogeneous or complete. This precludes a direct comparison among their outcomes and a consensus on what conditions, if any, would be characteristic or common to carotenoid extraction inside the same type of matrix.

In this last experimental chapter, assuming that there might be a general set of conditions that could be employed to extract carotenoids from different fruits and vegetables, we decided to extrapolate those previously optimised in Chapter 3 onto other matrices in this group to assess how well they would reflect and replicate the results obtained for carrots. Also, with the data on chemical composition found in the literature for each specific matrix, we could potentially understand how the vegetable structures and other inherent parameters may influence the extraction behaviour.

There are clear limitations that impeded us from making stronger claims, such as the lack of information on mass spectra or advanced microscopy analyses. Nevertheless, the results obtained by testing these conditions on other fruits and vegetables certainly allow interesting insights on the subject, apart from confirming that the parameters optimised for carrots can be safely used to other matrices as a very efficient set of conditions to this end.

Extraction of carotenoids from vegetable waste matrices: generalisation of optimised conditions

ABSTRACT

The aim of this work was to assess whether the developed mathematical model for predicting the recovery of carotenoids from carrot peels by supercritical fluid extraction (SFE) as a function of co-solvent concentration, temperature and pressure, can be applied to other fruit and vegetable matrices. To this end, fifteen carotenoid-rich matrices, including the flesh and peels of sweet potato, tomato, apricot, pumpkin and peach, and the flesh and wastes of green, yellow and red peppers, were submitted to SFE under the optimised conditions and the obtained extracts were characterised as to total carotenoid content and antioxidant activity, whereas calculations of total carotenoid recoveries were also performed. The total carotenoid recovery was in most cases higher than 90% w/w, with β-carotene being the most abundant and successfully extracted compound (with recoveries ranging from 88% to 100% w/w), most likely due to its low polarity; more polar carotenoids, such as lutein and lycopene, were less well extracted. Taking into account the literature data on the composition of various fruit and vegetable matrices, it seems that the carotenoid extraction depended, to some degree, on the composition of the matrices, with those with a very high carbohydrate content demonstrating a slightly less efficient carotenoid recovery. The high recoveries indicate that the developed model can be used as a general model for the extraction of carotenoids from various fruit and vegetable matrices by SFE. Moreover, the process was capable of extracting carotenoids from a mixed sample of fruit and vegetable

matrices, with high recovery levels (74% – 99% w/w). These findings demonstrated that SFE can be used as a viable alternative to conventional solvent-based extraction techniques and potentially as a viable method for adding value to fruit and vegetable waste streams by generating carotenoid-rich extracts from these matrices.

Keywords: carotenoid extraction; supercritical CO_2; process optimisation; vegetable waste.

5.1 INTRODUCTION

Carotenoids are molecules especially ubiquitous in red- and orange-coloured fruits and vegetables. They are a central component of human nutrition due to the important biological functions which they are involved in [1–3]. They are vastly used as food colourants, have potent antioxidant activities, and can also be employed as precursors of aroma or flavour compounds [4].

Due to the above, there is a clear interest by the food, chemical, pharmaceutical, cosmetics, personal care and nutraceutical sectors in utilising carotenoids for various applications, as functional and/or bioactive compounds. As a consequence, the global carotenoid market is projected to surpass the USD 2,000-million figure by 2023, registering an annual growth rate of 4% from 2018 to 2023. Europe is the main world market, representing 42% of the total, followed by North America and Asia, accounting for 25% and 20% of the world total, respectively [5]. Although most of the current commercial carotenoids are produced via chemical synthesis [4], having encompassed 76% of the market in 2014 [6], there is a significant trend towards extracting these compounds from natural sources, such as fruits and vegetables [7], and also from biomass derived from microbial fermentation processes [8]. In 2014, 24% of the global production derived from these sources [6].

Due to the numerous disadvantages inherent in extracting carotenoids by conventional organic solvents, new methods for extracting these phytochemicals have been investigated, among which is supercritical fluid extraction (SFE). The technique has been successfully used to extract carotenoids from a range of vegetable matrices, such as pumpkin, carrot, tomato and watermelon [9–14], and also from vegetable and fruit waste matrices including

banana, grape and tomato peels [15–17], grape, pomegranate and pumpkin seeds [18–20], as well as apricot bagasse and pomace [21,22].

The main aim of these studies was to optimise the process conditions in order to achieve maximum carotenoid recovery, similarly to what was done in Chapter 3 of this thesis. This is normally approached using various statistical designs and methods (e.g. Central Composite designs, Rotable designs, One-Way ANOVA) or non-statistical methods (e.g. sequential optimisation). The key parameters that are investigated include the type and concentration of the co-solvent used (e.g. ethanol, methanol, hexane, acetone, isopropanol, as well as sunflower, hazelnut and canola oils), temperature, pressure, CO_2 flow rate, sample particle size and sample moisture content. It can be argued that the results in the literature are not presented uniformly across the different studies and are sometimes difficult to compare. While a few authors indicate the amount of carotenoid recovery (% w/w) in the extracts in relation to the initial sample load, others limit themselves to presenting the carotenoid concentration in the extracts or their antioxidant activity, with no conclusions drawn with regards to the effectiveness of the applied method in terms of carotenoid recovery, or its efficiency compared to conventional solvent extraction methodologies. As a result, a direct comparison between different matrices, techniques and kinetic models becomes challenging.

In Chapter 3, the extraction of carotenoids from carrot peels was optimised by Response Surface Methodology, using a Non-Factorial 2^3 Central Composite Design of Experiments. Kinetic experiments carried out at lab scale, which were appropriately validated and modelled, enabled the optimisation of the extraction time and subsequently a study assessing the scalability potential of the method was conducted using a 10-fold higher

amount of sample. Supercritical CO_2 (S-CO_2) derived extracts were characterised in terms of their composition and correlations were established between the conditions of extraction and the composition of the final extracts. The optimum settings for carotenoid extraction were identified as: 59 °C, 350 bar, 15 g/min CO_2, with 15.5% (v/v) ethanol as co-solvent, with a 30 min run. Under these conditions, the total carotenoid recovery was of 87.0% (against the 86.1% predicted by the model) and, in the larger-scale experiments, this value reached a 96.2% mark which, to the best of the authors' knowledge, is the highest ever reported in the literature for vegetable wastes.

The aim of this work was then to assess whether the previously developed model for carrot peels can be applied to other fruit and vegetable matrices, with the view to be used as a general predictive model for the extraction of carotenoids from fruit and vegetable matrices by SFE. To this end, fifteen carotenoid-rich samples, including the flesh and peels of sweet potato, tomato, apricot, pumpkin and peach, and the flesh and wastes of green, yellow and red peppers, were submitted to SFE under the optimised conditions and the obtained extracts were characterised for their total carotenoid content and antioxidant activity, whereas calculations of the total recovery were also performed.

5.2 MATERIAL AND METHODS

5.2.1 Sample preparation

Fifteen matrices of carotenoid-rich fruits and vegetables were tested, all purchased from a local supermarket chain in Reading (UK). These included the flesh and peels of sweet potato (*Ipomoea batatas*, var. Beauregard), red tomato (*Lycopersicon esculentum Mill.,* var. Sungold), apricot (*Prunus armeniaca*, var. Moorpark), pumpkin (*Cucurbita pepo*, var. Cinderella) and peach (*Prunus persica*, var. freestone); green, yellow and red bell peppers (*Capsicum annuum*) and their waste residues (seeds and stems), as well as a mix of all these different matrices (using the same amount of each vegetable) to simulate an industrial scenario of a fruit and vegetable processing establishment.

All vegetables were washed and peeled manually. The samples were then frozen at -20 °C for 36-48 h, freeze dried in a lyophiliser (VirTis SP Scientific, UK) for 72 h, milled with a home grinder for 2 min and sieved to cut off particles greater than 750 μm in diameter. The samples were then stored in containers away from light and kept at -20 °C until further analysis.

5.2.2 Supercritical Fluid Extraction

For each run, 5.0 g of freeze-dried samples were placed in a supercritical fluid extractor (SciMed, UK). 95.0 g of inert glass beads (Sigma-Aldrich, UK) were added to fill the vessel volume in order to avoid dispersion effects and the samples submitted to a CO_2 flow rate of 15 g/min and the dynamic extraction time was fixed at 30 minutes. These operating conditions were previously optimised for carrot peels via a Central Composite Design of

Experiments (Chapter 3) and included: temperature of 59.0 °C, pressure of 350 bar and 15.5% (v/v) of ethanol as co-solvent. Runs were performed in duplicates and the results are presented as the average value for all measurements. The extracts were collected dissolved in ethanol and stored at -18 °C in dark glass containers until further analysis.

5.2.3 Moisture content

The moisture content in the samples was measured by a halogen moisture analyser (Mettler Toledo, UK). The apparatus was equipped with an oven operating at 105 °C and a precision scale, which determined the water content by gravimetry from the sorption isotherms plotted by the equipment.

5.2.4 Carotenoid analysis

The carotenoid content of the initial fruit and vegetable samples were analysed according to the protocol described by Biehler et al [23]. The full step-wise procedure is described in section 3.2.2.

5.2.5 Antioxidant Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) method was used to measure the antioxidant activity with some modifications [27]. Briefly, 200 μL of the extracts (in triplicate) were mixed with 2 mL of DPPH reagent. The mixture was incubated for 30 min in the dark and the absorbance was measured at 517 nm using a spectrophotometer (Thermoelection Corp., UK). The antioxidant activity values were expressed as the percentage of absorbance change,

by comparing the absorbance of individual samples against that of the control (200 µL of methanol + 2 mL of DPPH reagent).

5.3 RESULTS AND DISCUSSION

5.3.1 Sample characterisation

The moisture and carotenoid profiling of each vegetable sample, as well as their total carotenoid concentration (TCC), calculated as the sum of β-carotene, α-carotene, lutein and lycopene concentrations and analysed based on the solvent extraction method presented in section 3.2.2, is shown in Table 5.1. In addition to these experimental data, literature data presenting the typical chemical composition of the vegetable samples in terms of total carbohydrate, protein and lipid content were collected. These compositional data are scarce and, for some matrices, not available at all. Both the experimental and the literature figures are presented on dry weight basis to enable direct comparisons.

Table 5.1. Moisture and carotenoid composition of samples (experimental data) and chemical macro-composition (literature data).

Sample	Moisture (% w/w)	Carotenoid concentration (µg/g dry weight basis) - Experimental data					Compositional data (% g/g, dry weight basis) - Literature data			
		BCar	ACar	Lut	Lyc	TCC	Carbs	Protein	Lipid	Reference
SPF	5.3	383.7 ± 21.0	-	46.9 ± 6.7	-	430.6 ± 27.7	80.5	12.2	4.1	[24]
SPP	4.9	144.2 ± 9.4	-	20.9 ± 5.2	-	165.1 ± 14.6	85.5	5.1	4.4	[25]
TMF	8.1	91.0 ± 8.4	-	25.4 ± 5.1	113.2 ± 9.3	229.6 ± 22.8	45.3	25.9	14.5	[26]
TMP	6.2	154.4 ± 9.7	-	16.6 ± 4.0	82.5 ± 7.3	253.5 ± 21.0	93.2	1.9	1.6	[27]
APF	7.2	132.7 ± 9.1	-	-	-	132.7 ± 9.1	89.9	2.4	2.9	[28]
APP	6.8	212.4 ± 14.0	72.7 ± 10.3	-	-	285.1 ± 24.3	-	-	-	-
PKF	5.9	239.6 ± 13.6	-	-	145.7 ± 12.0	383.3 ± 23.6	88.3	6.1	1.6	[29]
PKP	5.3	49.3 ± 9.6	-	-	92.7 ± 8.4	142.0 ± 18.0	86.3	12.2	5.3	[29]
PCF	6.6	20.4 ± 5.2	-	-	-	20.4 ± 5.2	-	-	-	-
PCP	7.0	47.2 ± 6.3	-	12.3 ± 3.9	-	59.5 ± 10.2	-	-	-	-
GPF	7.9	56.7 ± 6.6	-	262.3 ± 15.6	-	319.0 ± 22.2	55.5	10.3	5.1	[30]
YPF	7.7	31.9 ± 4.9	-	205.2 ± 11.4	-	237.1 ± 16.3	55.8	12.3	6.0	[30]
RPF	7.9	-	-	66.7 ± 7.1	-	66.7 ± 7.1	55.4	13.5	5.1	[30]
XPW	5.2	18.3 ± 4.2	-	90.9 ± 8.4	-	109.2 ± 12.6	61.7	29.1	4.5	[31]
MIXED SAMPLE	6.1	133.9 ± 11.3	-	46.2 ± 7.0	23.3 ± 7.6	203.4 ± 25.9	79.2	15.9	4.9	This work

SPF = sweet potato flesh; SPP = sweet potato peels; TMF = tomato flesh; TMP = tomato peels; APF = apricot flesh; APP = apricot peels; PKF = pumpkin flesh; PKP = pumpkin peels; PCF = peach flesh; PCP = peach peels; GPF = green pepper flesh; YPF = yellow pepper flesh; RPF = red pepper flesh; XPW = pepper wastes. BCar = β-carotene; ACar = α-Carotene; Lut = lutein; Lyc = lycopene

The most abundant carotenoid in the samples was β-carotene, which was found to be absent only in the flesh of red peppers. An earlier work reported the presence of β-carotene in red peppers [32], but at a relatively low concentration of 5.4 μg/g on a fresh weight basis (corresponding to 49.1 μg/g on a dry basis); the reason for this disparity is not clear but could be due to differences in variety and/or environmental factors. On the other hand, α-carotene was only detected in apricot peels. There are reports of α-carotene in sweet potatoes [33] and in pumpkins [34], but in our samples, these only appear as traces, below the confidence interval of the standard curves. It is important to highlight that the carotenoid content in fruits and vegetables is highly variable even within the same variety owing to the fact it is dependent on various external factors, such as type of soil, season of harvest, sun exposure, as well as state of ripening [4]. The latter can influence the carotenoid profiling [35], since some of these molecules are only formed in the last stages of ripening. Another work reported the presence of β-carotene, lutein and β-cryptoxanthin in the flesh of peach [36], however in this study only β-carotene was detected and even so, at very low concentrations. This signifies the importance of ripening, as in our case, this specific fruit was visibly not fully ripened at the time of analysis.

Lutein was the second most abundant carotenoid in the samples, and was absent only in pumpkin and apricot samples (flesh and peels), and in the flesh of peach. The concentrations were much lower than β-carotene, except in the case of peppers, where lutein was the major carotenoid among the four analysed. In the tomato samples, lycopene was present at high concentrations, and in the particular case of tomato flesh, it was the most abundant carotenoid. These observations are similar to other published works [37,38].

Apart from the aforementioned discrepancies, all carotenoids analysed in this work are also reported in the literature to be present in the same samples that were tested in this study, at similar concentrations [30,39–41]. In addition to the four carotenoids, a number of other peaks were also observed, indicating the presence of other carotenoids. Although the quantification for these was hindered due to the lack of external standards, the identification of some of the peaks was made possible tentatively by comparison with chromatographic data available in the literature; these are presented and discussed in the next section.

In terms of macronutrients (carbohydrate, protein and lipid) contents in the fruit and vegetables, it is clear that they can differ considerably. However, carbohydrates overall represent the main macronutrients in all the vegetables matrices tested in this study, ranging from 55% in peppers up to 93% in tomato peels. These carbohydrates include free monomeric sugars (e.g. glucose, fructose, sucrose), fibres (e.g. cellulose, hemi-cellulose, pectin) and polysaccharides (e.g. starch). The fibre content (both the type and the concentration) is critical, as it is a key component of the fruit and vegetable cell wall and due to its complex and rigid structure, it can often make the extraction of targeted molecules by SFE challenging by limiting their dissolution into the solvent phase. The literature data show that the proteins and lipid contents also vary considerably, with the former ranging from 1.8% in tomato peels to 29.1% in pepper wastes and the latter from 1.62% in pumpkin flesh to 14.5% in tomato flesh.

5.3.2 Carotenoid extraction by SFE

Table 5.2 presents the recoveries percentage on a dry weight basis of individual and total carotenoids from the fruit and vegetable matrices by SFE using S-CO_2 at 15 g/min and the following previously-optimised processing conditions: 60 °C, 349 bar and 15.5% (v/v) of ethanol for 30 min. (Chapter 3). The data presented in Table 5.1 were used to obtain the initial carotenoid concentration of the individual carotenoids and the total carotenoid concentration (TCC) in the different samples. The total carotenoid recovery (TCR) (dry weight of TCC in the extracts / dry weight of TCC in the original sample), was calculated based on the α-carotene, β-carotene, lycopene and lutein concentrations. In addition to these, some of the unknown carotenoid peaks were tentatively identified based on chromatographic data available in the literature, for the relevant matrix, and their recoveries calculated using the ratio of the specific peak area in the extracts to the peak area found in the characterisation runs, as in Table 5.1.

Table 5.2. Recovery (%, w/w d.w.b.) of individual and total carotenoids from different fruit and vegetable matrices in the SFE extract

Sample	BCar	ACar	Lut	Lyc	ChlA*	ChlB*	Vlx*	Cap*	Zea*	TCR**	AA (%)
SPF	99.4±2.6	-	79.9±3.7	-	-	-	-	-	-	97.4	36.6±2.0
SPP	99.8±2.9	-	68.2±3.8	-	-	-	-	-	-	95.9	20.7±1.8
TMF	99.0±2.8	-	36.2±6.2	98.5±2.1	-	-	-	-	-	91.8	30.4±1.7
TMP	96.9±1.7	-	29.5±5.8	92.5±2.2	-	-	-	-	-	91.0	87.9±1.6
APF	99.0±2.6	-	-	-	-	-	-	-	-	99.0	39.2±2.2
APP	98.7±3.1	97.9±2.7	-	-	-	-	-	-	62.9±3.2	98.5	51.9±2.2
PKF	92.4±3.5	-	-	87.4±3.1	56.7±4.5	-	-	-	-	89.1	42.3±1.1
PKP	88.2±3.6	-	-	83.2±2.5	51.0±3.0	-	-	-	-	84.9	77.1±1.0
PCF	99.8±3.1	-	-	-	-	-	-	-	-	99.8	7.0±5.1
PCP	99.2±2.6	-	75.3±3.9	-	-	-	-	-	-	94.2	34.1±3.1
GPF	98.6±2.1	-	99.8±1.1	-	198±7.2	290±9.4	98.2±1.9	-	-	99.6	17.5±4.6
YPF	99.8±2.5	-	99.6±1.8	-	-	-	-	-	-	99.6	49.7±0.9
RPF	-	-	98.1±2.2	-	96.5±3.0	-	97.6±2.2	75.0±2.3	-	98.1	46.5±2.7
XPW	96.7±1.8	-	94.5±2.1	-	285±6.9	292±9.1	283±8.7	37.2±5.8	-	94.9	19.0±3.4
MIX	98.9±2.4	96.3±3.9	73.9±3.0	91.0±2.5	145±4.0	34.0±1.6	102±4.1	-	-	92.5	57.7±2.5

Process conditions: T = 59 °C, Pressure = 350 bar, EtOH = 15.5%, CO_2 flow rate = 15 g/min, run time: 30 min; TCR: total carotenoid recovery, AA: antioxidant activity. BCar = β-carotene; ACar = α-Carotene; Lut = lutein; Lyc = lycopene; ChlA = Chlorophyll A; ChlB = Chlorophyll B; Vlx = Violaxanthin; Cap = Capsanthin; Zea = Zeaxanthin; (-) = not detected.

SPF = sweet potato flesh; SPP = sweet potato peels; TMF = tomato flesh; TMP = tomato peels; APF = apricot flesh; APP = apricot peels; PKF = pumpkin flesh; PKP = pumpkin peels; PFC = peach flesh; PCP = peach peels; GPF = green pepper flesh; YPF = yellow pepper flesh; RPF = red pepper flesh; XPW = pepper wastes; MIX = sample mix.

*Identified indirectly, by comparison with data in the literature; ** Averaged total carotenoid recovery as a function of the first four carotenoids only.

It is readily noticeable that the processing conditions used for SFE are highly optimised for the extraction for α-carotene and β-carotene. In the vast majority of the matrices tested, the recovery values for both these molecules were higher than 95%. In the case of β-carotene, which was the most abundant carotenoid in the matrices, the lowest recovery values were obtained for pumpkin flesh and pumpkin peels (92.4% and 88.2%, respectively), which could be probably attributed to their more complex structures. The very high carbohydrate content of pumpkin flesh and peels (>86%, Table 5.1) indicate the presence of high levels of cellulose, hemicellulose and potentially pectin in its matrix, which might have hindered the diffusion of the carotenoid molecules into the CO_2 fluid phase. Taking this into account, in cases where the fruit and vegetable tissues are rich in complex polysaccharides, the SFE process could potentially benefit from an extended extraction time, to ensure the dissolution of the remaining carotenoid molecules trapped in sites which are not easily accessible by the solvent; this happens in the later stages of the extraction and is primarily governed by diffusive mass transfer phenomena [42]. Another alternative would be to employ a slightly higher solvent flow rate, which would facilitate the extraction of these compounds.

The sample of tomato peels was the only matrix with a carbohydrate content higher than those of pumpkins, but in this case, the recovery of β-carotene (>96%) was higher in the former than in the latter. Similarly, lycopene, which is a major carotenoid in tomato flesh and peels (49.3% and 32.5% of TCC, respectively) and in pumpkin flesh and peels (65.3% and 47.7%, respectively), was also recovered more efficiently in the case of the pumpkin matrices (~95% vs ~85%). These differences could be due to the slightly higher moisture content of tomato flesh and peels (8.1% and 6.2%, respectively) than that of pumpkin (5.9% and 5.3%), which in some previous works, was shown to result in increased TCC recoveries [43,44].

However, in order to understand to which extent the moisture content of the samples could have influenced the recovery results, further work including a detailed compositional analysis of these matrices and a study on their morphology is needed.

Lutein is a carotenoid that belongs to the xanthophyll group and their main difference compared to α-carotene and β-carotene is the presence of oxygen atoms, either as hydroxyl groups and/or epoxides attached to the rings in the molecule terminations, which makes them considerably more polar. This can explain the noticeably lower recovery values in the extracts compared to α-carotene and β-carotene for most matrices. An exception to this trend was the pepper samples, where lutein was almost completely recovered. This might be due to the comparatively low carbohydrate content of peppers (Table 5.1), reflecting a less rigid cell wall structure, which could have resulted in higher mass transfer rates and consequently, higher recoveries. The estimated recovery values for the other xanthophylls present in the matrices tested, such as zeaxanthin, violaxanthin and capsanthin, were similarly low, which seems to confirm the fact that the model used to identify the optimal SFE conditions is much more suitable to nonpolar molecules, such as α-carotene, β-carotene and lycopene, rather than to molecules of higher polarity.

Chlorophylls, which were present in the samples of pumpkin and pepper, are much more polar than carotenes and xanthophylls. In the case of the pumpkin samples, the estimated recovery values (51-57%) were in line with the above hypothesis on the influence of polarity on SFE. One noteworthy point to be made however, is that the estimated recovery values for chlorophyll A and chlorophyll B for pepper flesh were much higher than 100% (198-290%). This is most likely due to two reasons: on one hand, ethanol, a polar solvent, was used as a co-solvent during SFE and this would facilitate the extraction of more polar substances, such

as chlorophylls; on the other hand, the reference method used to extract carotenoids from the matrices (section 3.2.2), on which all the calculations for carotenoid recovery were based, is highly specific for carotenoids and involves non-polar solvents to achieve extraction, including hexane and acetone, thus leaving other polar molecules behind. The amount of co-solvent used in the SFE was considerably high (15.5%), and could explain the apparent higher extraction yields observed for chlorophylls, when in fact, this is probably only a consequence of the SFE process being a more efficient chlorophyll extraction protocol than the conventional solvent extraction procedure for carotenoids. In order to confirm this hypothesis, a targeted analysis needs to be carried out to specifically measure the chlorophyll content of the raw materials and of the supercritical fluid extracts.

The sample mix was prepared by mixing an equal amount of each of the fourteen vegetable samples and was tested in order to simulate a real industrial scenario or a conceptual SFE process for treating fruit and vegetable waste. The rationale behind this experiment lays on the fact that it is likely that an SFE waste extraction plant could be applicable and economically feasible within an industrial establishment/processor that aims to recover and exploit value-added components from multiple vegetable- and fruit-derived by-products and waste streams. The aim was to evaluate the potential of the established SFE extraction protocol to deliver high yields of carotenoids from a mixed source, instead of a specific vegetable matrix. Apart from capsanthin and zeaxanthin, all previously identified carotenoids were likely present in the mix and their recoveries were considerably high (74% – 99%) (Table 5.2). Significantly, this indicates that the efficiency of the process was not affected by the simultaneous extraction of mixed fruit and vegetable matrices. Taking this into account and also the fact that the total carotenoid recovery and particularly that of β-

carotene (the most ubiquitous carotenoid in the vast majority of the matrices) was, for all matrices, above 85% and in many, above 95% TCR (including the sample mix), a strong argument can be made for establishing SFE as a preferable unit operation for the industry. The findings demonstrated that SFE is a viable alternative to conventional extraction techniques, and potentially a viable method for adding value to vegetable waste streams by generating carotenoid-rich extracts from these.

The antioxidant activity of the extracts (Table 5.2) was highly variable, ranging from approximately 7% in peach flesh to 88% in tomato peels. With the exception of sweet potatoes, the antioxidant activity in the fruit and vegetable peels was in all cases higher than that in the flesh, despite the fact that the carotenoid concentration would often follow the opposite trend. This is likely to be associated with the higher amount of phenolic compounds in the peels, as they have been shown to contain these molecules in higher amounts than the flesh [7,45,46], since their role is to act as physical and chemical barriers to deterioration caused by mechanical injuries or fungal infections. The use of ethanol as a co-solvent in SFE increased the polarity of CO_2 and this has possibly led to the extraction of phenolic compounds; these are also known to exert high levels of antioxidant activity, sometimes even higher than that of carotenoids [7].

An important finding of this work is the fact that the mathematical model developed to predict the optimum conditions for maximum extraction of carotenoids from carrot peels was applicable for a variety of fruit and vegetable matrices, including a mix thereof. The ability of the model to assess the recovery of total carotenoids from each individual matrix as well as in the mix sample is presented in Table 5.2. The model took into account three parameters to predict the total carotenoid recovery, namely temperature, pressure and co-

solvent (ethanol) concentration. The high descriptive ability of the model indicates that these parameters are most likely the most important to be considered.

In order to better understand the probable reasons behind this, data from optimisation studies for carotenoid extraction by SFE from different fruit and vegetables carried out in the last 15 years were compiled and are presented in Table 5.3. The table is very similar to the previous Table 1.2, but here, information more relevant to the current study were included. While in the first we focused on the working range selected by the authors and the different kinetic models used to guide our optimisation experiments, in the current one, we focus more on the optimum conditions and carotenoid concentration in the extracts to help us make inferences about the extraction behaviour. In all these studies, a number of parameters were investigated (temperature, pressure, solvent flow rate, co-solvent concentration, extraction time, mass loading, etc), and various responses measured (yields or recoveries, total carotenoid concentration), whereas the statistical methods employed to develop models varied as well. A direct comparison of the results obtained in this work to these in the literature is challenging, as their presentation can vary considerably. Some authors report the carotenoid data as concentration – the carotenoid mass in the extract per extract mass – or as the carotenoid mass in the extract per raw (dried or wet) sample mass. However, a more efficient way to present the results would be as % recovery (% of total of carotenoids extracted from the total carotenoid mass originally in the raw samples), as this would enable a direct comparison between different samples, processes and extraction protocols. Unfortunately, not all authors report such data.

Table 5.3. Literature data on optimal process parameters, carotenoids recoveries and optimisation methods for the extraction of carotenoids from various fruit and vegetable matrices by SFE.

Food matrix	Target Compd.	Mass load (g)	Optmised Extraction Parameters					Carotenoid Recovery (%, w/w)	Carotenoid content (mg/g ext)	Statistical models / Var. of influence	Refer.
			CoSol (%)	T (°C)	P (bar)	Q CO_2 (g/min)	t_E (min)				
Apricot bagasse	BCar	2.0	-	60	304	0.85	90	-	-	ANOVA / Temp, Press, PSize, Temp	[21]
Apricot pomace	BCar	1.0	27.4% EtOH	69	311	1.6	90	-	0.098	Central Composite / Temp, Press, CoSol	[22]
Carrot	TC	2.0	5% Canola oil	70	551	1847	240	-	1.91	ANOVA / Temp, Press	[10]
Carrot Peel	TC	50	15.5% EtOH	59	350	15	30	96.2%	-	Central Composite / Temp, Press, CoSol	[47]
Citrus press cake	TC, BCrip	1.0	5-20% EtOH	60	310	2.1	20	38.0 (TC) / 73.0 (BCrip)	0.550	Central Composite / Press, CoSol	[48]
Citrus waste	TC	100	-	45	252	27	120	-	1.98	Box-Behnken / Temp, Press, Ratio	[49]
Paprika	TC	720	-	80	500	415	-	85.0	1.15	Press, Moist	[50]
Pitanga	TC	5.6	-	60	250	4.1	120	55.0	5.47	-	[51]
Pumpkin	TC	0.4	0-10% EtOH	70	350	1.24	-	74.0	0.110	Central Composite / Temp, Press, CoSol	[52]
Pumpkin	ACar, BCar, LUT	2.0	10%EtOH + 10% Olive Oil	50	250	1.25	-	76.0	0.472	One-way ANOVA / CoSol, Temp	[9]
Pumpkin flesh, seeds	BCar	100	6.0 g EtOH	48	300	212	60	-	0.205	Box-Behnken	[53]

Food matrix	Target Compd.	Mass load (g)	Optmised Extraction Parameters					Carotenoid Recovery (%, w/w)	Carotenoid content (mg/g ext)	Statistical models / Var. of influence	Refer.
			CoSol (%)	T (°C)	P (bar)	Q CO_2 (g/min)	t_E (min)				
Red bell pepper (flesh)	CAP	5.0	-	50	400	2.5	210	15	0.100	-	[54]
Red bell pepper (waste)	BCar	30.0	-	60	240	25.8	120	68.1	-	Temp, Press, PS	[55]
Spinach	BCar, LUT	500	-	40	350	60	360	-	17.2	-	[56]
Tomato	LYC	24	Enzyme-aided	86	500	3.44	270	38.0	-	/ Enzyme activity	[11]
Tomato	LYC	-	10% Hazelnut oil	65	425	230	480	72.5	11.6	-	[57]
Tomato	BCar, LYC	10	5% Canola oil	40	400	478	720	-	6.60	-	[12]
Tomato juice	LYC	15	-	80	350	1.7	180	77.0	-	One-way ANOVA / Temp, press, CoSol	[58]
Tomato peel	LYC	1.2	14.0% EtOH	62	450	3175	30	33.0	-	Central Composite Rotatable / Temp, Press	[17]
Watermelon	LYC	0.5	15.0% EtOH	70	207	1.0	35	-	38.0 (w.b.)	Temp	[14]

CoSol = Co-solvent concentration; EtOH = Ethanol; MeOH = Methanol; QCO_2 = Solvent flow rate; t_E = Extraction time; TC = Total carotenoids; ACar = α-carotene; BCar = β-carotene; LYC= lycopene; LUT = Lutein; CAP = Capsanthin; BCrip = β-cryptoxanthin; Var. of inf. = variables of influence; CoSol = Co-solvent; Temp = Temperature; Press = Pressure; Moist = Moisture; PSize = Particle Size; w.b. = wet basis.

Despite the aforementioned limitations, a deeper assessment of the literature data in relation to that generated in this work, still allows for interesting conclusions. For instance, the most common co-solvent employed is ethanol, which usually ranges from 5 to 15% v/v. The preference for this particular entrainer is due to its low price and toxicity and also to its ability in increasing the polarity of CO_2, compared to other polar solvents, such as methanol or acetone. In addition to these solvents, some authors used vegetable oils as co-solvents (e.g. canola, olive, hazelnut, sunflower oil), due to their positive effect on the solubility of carotenoids, since these molecules are highly lipophilic. The apparent solubility of lycopene was calculated for a solvent mixture of consisting of supercritical CO_2 and canola oil in an earlier work [12] and it was concluded that the solubility of carotenoids when oil was used as a co-solvent was higher than when ethanol was used or only pure supercritical CO_2 was employed. Another work also reported very good yields when using canola oil as a co-solvent for extracting carotenoids from carrots [10], due to penetration of the oil into the cell structure of dried carrots, which in turn caused its swelling and enabled the diffusion of supercritical CO_2 through the matrix. However, these studies did not report the % recovery of total carotenoids, and it is therefore difficult to compare these to the results of this study. The ethanol concentration used here (15.5% v/v), which was predicted by the model as the optimum value for SFE of carrot peels (Chapter 3), can be classified at the upper end of the concentration ranges usually used for SFE and is likely to have improved carotenoids recoveries compared to lower ethanol concentrations or no ethanol, probably not only due to a significant increase in polarity but primarily, by facilitating the dissolution of larger molecules, such as carotenoids, in the supercritical solvent. This is also most likely one of the

reasons contributing to the high carotenoid recoveries obtained for all the different fruit and vegetable matrices tested here as well.

Pressures usually used in SFE range from 127 to 507 bar, while temperatures range from 40 to 90 °C. The use of high temperatures is limited due the thermolability of carotenoids, as high temperatures are known to cause their degradation and isomerisation [59]. The parameters of pressure and temperature together dictate the solvation power of CO_2 and are usually considered to significantly influence the extraction process. From the data in Table 5.3, it can be observed that mid- to high temperatures (between 55 °C and 70 °C) and high pressures (between 300 and 450 bar) usually result in higher carotenoid recoveries. The general consensus is that the pressure is a more influential parameter than temperature, and pressures above 250 bar have been shown to positively influence carotenoid extraction. This can be explained by the fact that higher pressures result in a higher solubility of carotenoids in the solvent due to increased solvent density. The settings used in this work, i.e. pressure 350 bar and temperature 59 °C, which were predicted by the model as the optimal values for carotenoid extraction from carrot peels, were within these boundaries, which again demonstrates the applicability of the model to a wide range of fruit and vegetable matrices. Some studies found that when the pressure was increased above 350 bar, the recoveries of lycopene, α- and β-carotene decreased compared to lower pressures were employed [51,60,61]. This may be due to the increased polarity of the supercritical fluid solvent mixture (CO_2 and polar solvents) caused by a significant density increase, which decreased the affinity of the solvent for non-polar molecules, consequently diminishing its selectivity [59].

The extraction time (t_E) and solvent flow rate (Q) have also been reported to influence the SFE process for fruit and vegetable matrices. The values reported in the literature vary significantly (Q from 0.85 to 3175 g/min and t_E from 30 to 720 min). The general trend is that longer extraction times can lead to decreased carotenoid recovery, possibly due to degradation reactions [62,63]. Therefore, an increased solvent flow rate (e.g. higher than 10 g/min) should be used to decrease the extraction time [10]. In this work, a flow rate of 15 g/min was used, enabling the SFE protocol to be completed within 30 min, which is advantageous from the perspective of an industrial process, since shorter processing times imply lower energy consumption and higher productivities. As these two parameters seem to be influenced by the type of the food matrix, optimisation studies are required to pinpoint these parameters for particular types of matrices.

The influence of other process variables on the recovery of carotenoids from fruit and vegetable matrices are more difficult to predict. For instance, the amount of sample loading in the supercritical fluid extractor varies greatly in the literature (0.5 to 720 g) due to a number of reasons. The most apparent is equipment dimensions, since extraction vessels are available in a range of different volumes, limiting the sample mass to a certain amount. Some of the works cited were performed in lab-scale equipment, where factors such as particle aggregation and bed geometry are not so important and therefore negligible, whereas others were performed using pilot-scale extraction vessels, where these factors have a considerable influence to the final results.

Finally, in terms of total carotenoid recovery (TCR), it can be seen that the recovery values obtained in this work for a number of fruit and vegetable matrices are higher than those reported in the literature. The extraction of pumpkin flesh by SFE was reported to result in

a TCR of 74% at 70 °C and 350 bar [52] and 76% at 50 °C and 250 bar [9], while in the current study, this was 89%. The application of an intermediate temperature (59 °C) compared to the temperature used in these published works seems to have increased the yields considerably. For tomatoes, the TCR values reported in the literature were of 72% for flesh [57] and 33% peels [17], whereas in this work, the TCR for tomato flesh was 92% and for peels 91%. One possible reason for the low recovery observed for the tomato peels in the case of the published work could be the very high flow rate employed (the highest flow rate used amongst all published works), which most likely resulted in a very low residence time for the solvent within the extraction vessel and consequently, in low mass transfer rates.

5.4 CONCLUSIONS

Fifteen fruit and vegetable matrices, including flesh, peels and wastes, were submitted to supercritical fluid extraction using the processing parameters previously identified through mathematical modelling to be optimal for the extraction of carotenoids from carrot peels. The total carotenoid recovery was in most cases higher than 90% w/w, with β-carotene being the most abundant and most successfully extracted compound. Taking into account literature data on the composition of various fruit and vegetable matrices, it seems that the carotenoid extraction depended, to a considerable extent, on the composition of the matrices, with those with a very high carbohydrate content demonstrating a slightly lower carotenoid recovery. The high carotenoid recoveries indicate that the developed model can be used as a general model for the extraction of carotenoids from various fruit and vegetable matrices by SFE. Moreover, the SFE process was able to extract carotenoids from a mixed sample of fruit and vegetable matrices, with high recovery levels (74% – 99% w/w). These findings demonstrated that SFE can be used as a viable alternative to conventional solvent-based extraction techniques and potentially as a viable method for adding value to fruit and vegetable waste streams by generating carotenoid-rich extracts from these matrices.

5.5 REFERENCES

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUDING NOTES

6.1 SUMMARISING REMARKS

The work presented in this thesis involved the supercritical fluid extraction (SFE), with subsequent purification, of carotenoids from carrot peels and the application of the optimised conditions to other fruit and vegetable matrices. Firstly, an in-depth work was carried out on the optimisation of critical extraction parameters including temperature, pressure, co-solvent concentration and extraction time, and the obtained mathematical model was statistically and experimentally validated (Chapter 3). Subsequently, the carotenoid-rich extracts were submitted to purification by hydrophobic interaction chromatography (HIC) using an adsorption resin. To this end, the key parameters required to develop an efficient process were assessed. The purification led to maximum carotenoid recovery of approximately 88% (w/w) in batch and 70% (w/w) in column and resulted in extracts with high levels of purity (Chapter 4). Finally, the applicability of the developed mathematical model predicting the extraction of carotenoids from carrot peels was assessed onto various types of fruit and vegetable matrices. The experimental data, along with supporting literature data, allowed for some interesting conclusions on how the matrix composition and the polarity of different carotenoids can potentially influence carotenoid recovery (Chapter 5). In brief, the extraction of carotenoids, particularly of α-carotene and β-carotene, under the optimal extraction conditions predicted by the model was more efficient for fruit and vegetable matrices with simpler structures. In this last section, a general overview of the impact of the obtained results is given whereas the challenges, potential applications and recommendations for future work in this area are also discussed.

6.2 NOVELTY AND SCIENTIFIC CONTRIBUTION

The work performed advances the knowledge in waste valorisation by utilising green alternatives for obtaining valuable extracts with a high degree of purity. The results have already been extensively compared and contrasted to those available in the literature in Chapters 3 to 5 and hence, only the implications thereof on a broader spectrum are discussed in this chapter.

The data obtained in Chapter 3 bring into context the possibility of using a residue from a vegetable processing plant for the extraction of natural pigments by a supercritical fluid. This proposed methodology, although already tested, had not been approached in such detail in other works thus far, linking together process optimisation studies, mathematical modelling and statistics, mass transfer kinetics and extract compositional characterisation to advance our understanding on the supercritical fluid extraction of carotenoids from vegetable wastes.

Chapter 4 goes even further by proposing a new purification method for carotenoid-rich extracts based on hydrophobic interaction chromatography, with the use of a hydrophobic resin. The purification method was shown to be highly efficient, relatively easy to set up and, as it did not require specialised equipment, has a relatively low capital cost compared to more widespread methods, such as ultra- or nanofiltration. The purified carotenoid-rich extracts could be directly employed as ingredients for the manufacturing of functional products, such as foods, food supplements or cosmetics. Overall, these upstream and downstream processes proposed constitute an environmental-friendly alternative to the current carotenoid production processes via chemical synthesis routes.

Chapter 5 constitutes another important addition to the existing knowledge in the literature. Specifically, by successfully applying the optimal conditions for carotenoid extraction from

carrot peels by SFE found in Chapter 3 to other fruit and vegetable matrices, it provides a set of conditions that can be used almost universally for carotenoid extraction by SFE. This is useful for developing holistic biorefinery processes where carotenoid extraction can potentially constitute one of the process steps, or when the feasibility of carotenoid extraction from underutilised matrices and fruit and vegetable waste is pursued. From an environmental perspective, this work has potential for the re-utilisation of low value by-products from fruit and vegetable processing plants, and as such, this approach could decrease the negative impact of waste disposal on the environment and stimulate the circular bioeconomy concept.

6.3 LIMITATIONS AND FUTURE WORK

Despite the promising results, it is necessary to acknowledge that this work presents some limitations which require further work in order to be addressed. These are listed and discussed in the next subtopics.

6.3.1 Effect of particle size

One parameter that has not been assessed regarding the SFE of carotenoids was sample particle size. In Chapter 2 (section 2.2.2.6) it was briefly discussed that this variable has been reported as having previously showed a considerable influence on extraction. Due to equipment limitations in this work, the particle size (around 250 μm) was kept as low as possible by using the home grinder at its highest setting, since lower particle sizes result in higher solvent diffusivity and surface contact area. Very low sizes, on the other hand, can cause particle and bed dispersion, disfavouring extraction. Therefore, despite the high recoveries obtained without optimising this parameter, its fine-tuning could yield even better results or impart shorter process times, both of which would increase overall productivity.

6.3.2 Scale up

A particular and crucial study that needs to be carried out involves the scalability of the proposed SFE protocol and subsequent HIC purification process. In the case of SFE, mathematical models that describe and predict the extraction process, can often

overestimate the process efficiencies and recoveries compared to larger scale operations. This is due to process parameters which may not be accounted for during lab-scale SFE, such as biomass aggregation, blockage of piping or bed geometry. However, in pilot and industrial scales, the effect of the aforementioned parameters should be investigated in order to ensure good process scalability, and provide input into optimal equipment designs and process economics. In the case of the HIC purification process, examples of important scale up factors that need to be considered include the initial extract concentration, as well as the internal and external mass transfer coefficients. Overall, scale up studies on the proposed integrated process is paramount in order to assess its economic viability for commercial applications [1].

Although various scale-up methodologies have been proposed in the literature for SFE, there is still a debate on which process parameters need to be investigated [2]. The most commonly-used methodology involves performing scale up trials where extractor volumes increase while certain parameters are maintained constant [3]. Also, with regards to the extrapolation of the optimal extraction conditions to other fruit and vegetable matrices which was attempted in Chapter 5, it is clear from the results that the developed extraction method was targeting more nonpolar compounds, especially β-carotene, which was the most abundant carotenoid molecule in the matrices tested. However, these extraction conditions might not be ideal in cases where more polar compounds are targeted in particular matrices, as shown by the results obtained for lycopene (present in pumpkin) or lutein (present in sweet potato, tomato and peach).

6.3.3 Cost Assessments

The market for natural carotenoids is clearly promising. Synthetic carotenoids are sold for between $250 and $2,000/kg, while natural carotenoids sell for between $350 and $7,500/kg [4]. The vast price range is due to the fact that some carotenoids have now become commodities (e.g. lutein and beta-carotene), while others (e.g. lycopene, zeaxanthin) have maintained their very high added value, justifying the investment on research and new technologies for the extraction and purification of these molecules.

Although the perception of SFE as a costly technique has been gradually changing and many industrial processes are already well implemented (e.g. coffee decaffeination), the process is still considered capital intensive due to the investment costs linked with equipment acquisition and installation [2]. This is perhaps one of the most important limitations of the proposed process, but cost-reducing steps throughout the overall process could potentially contribute towards its commercial implementation. For instance, for pre-treating carrot peels and the other fruit and vegetable materials, freeze-drying was selected in order to preserve the stability and activity of the highly thermolabile carotenoids, as well as to ensure that external influences to the accuracy and reproducibility of the data (e.g. moisture content, carotenoid degradation) were minimised. In future work, evaluating other drying methods for the raw materials, such as oven-drying, could be useful. Within the context of an industrial fruit and vegetable processing plant, the drying of the processing-derived by-products should ensure their stability, regardless of their final use (e.g. animal feed or further processing for carotenoid extraction). While some degree of carotenoid degradation is likely to occur during, for example, oven or air drying, the decrease in the overall costs could potentially justify changing the drying method.

Intertwined with the suggestions for scale-up experiments, final Cost of Manufacture (COM) calculations should be performed in order to assess the feasibility of the proposed method on a commercial scale. Once important scale-up parameters of the process are estimated through mathematical modelling (e.g. t_{CER} and t_{FER} – the times of each extraction phase – mass transfer rates, yields and recoveries), cost estimations can be calculated with confidence, which should include the capital cost of all equipment, utilities and raw materials involved in the extraction and purification stages. Moreover, energy costs during sample pre-treatment (freezing and/or drying), extraction (heat exchangers, chillers, extraction rig, etc.), extract post-treatment (evaporation, freezing), and purification (pumps, etc.) should also be considered. Finally, direct and indirect labour and transportation costs as well as the market price of the extracts, with the latter being directly influenced by the purity of the final carotenoid extracts and their potential applications, need to be included in the COM calculations.

6.3.4 SFE-HIC in line

In this work, the SFE extraction and HIC purification of carotenoids showed very good results and, as a consequence, both processes can be employed in tandem as a unified protocol to this end. One way to take this knowledge even further and open up other novel possibilities is to test them in line, i.e., the possibility of having these two stages happen simultaneously inside the extraction vessel.

As extensively explained in Chapter 2 (section 2.2.2), the S-CO_2, acting as the solvent, penetrates the solid particles and extracts the molecules of interest, which remains disperse

in the mobile phase until it suffers a pressure drop or a temperature increase. These cause the CO_2 to leave its supercritical state and the carotenoid molecules to precipitate. The rationale behind the in-line approach is that the S-CO_2 could act as both the SFE extracting agent and the HIC mobile phase. Regarding the procedure, the adsorbent material could be placed on the top of a fine filter just above the extraction bed, so that the carotenoid-rich CO_2 phase would interact with it and adsorption could take place.

There are no records in the literature dealing with liquid-phase supercritical-fluid adsorption and therefore, predicting the behaviour or the outcome of this protocol is a cumbersome task. The HIC process and all of its parameters would certainly have to be re-assessed due to the very different environmental conditions of pressure and temperature imposed and the unknown pathways that the adsorption phenomena would follow under such circumstances. Nevertheless, if the proposal proves successful, costs would be cut considerably due to the lower demand on time, energy and equipment.

6.3.5 Residue valorisation

One final element to consider with regards to future work is the SFE residue left after the extraction of carotenoids. The proposed SFE method leads to the generation of a dry solid vegetable matrix (94% w/w of the initial sample), that is potentially free from solvents. These residues, being very rich in organic matter (particularly fibre and pectin but also polyphenols and micronutrients) may be used as a soil enhancer or as a fibre-rich animal feed. They can also be employed as ingredients by the food industry offering a number of functionalities, e.g. as emulsifiers, thickening agents and stabilisers [5], or alternatively as

substrates for microbial fermentations [6]. Taking into account the composition of the residue after supercritical fluid extraction of the carrot peels (20.5 mg/g total protein, 12.2 mg/g total lipids, 592 mg/g total carbohydrates), the production of a dietary fibre powder by hot-air drying and blanching [7] or of fibre extracts [8] are viable options. Within this biorefinery framework, the extraction of phenolic compounds, especially chlorogenic acid which accounted for around 95% of the total phenolic content in the carrot peel samples and was not extracted by SFE (3.68 mg/g in the residues), could also be considered. Potential methodologies that could be used for the extraction of chlorogenic acid from the residue include conventional solvent-based extractions but also green technologies, such as microwave-assisted extraction, subcritical-water or pressurised liquids extraction; the latter have been shown to extract phenolic compounds efficiently [9,10] and especially those of a large molecular weight, as is the case with chlorogenic acid [11].

6.4 CONCLUDING CONSIDERATIONS

The food processing industry is facing challenging times and is under pressure by governments, global organisations and consumers to develop efficient waste management strategies that minimise environmental impact, valorise natural resources and stimulate the circular bioeconomy. In this respect, there are great opportunities for SFE to be used as a core technology for the valorisation of food processing waste and low-value by-products. Despite the inherent advantages of SFE, there are still hurdles to be overcome, particularly those related to its relatively high cost, which prevent the technology from becoming fully widespread across the industry. After more than three decades of research and development in SFE, there are still no commercial SFE plants in places where the food industry is one of the key industrial sectors, for example in South American countries. However, considering the need to valorise abundant natural resources and the environmental concerns associated with the use of harsh organic chemicals in manufacturing, the work contained in this thesis enable a strong argument that can be made torwards the implementation of SFE as a core green technology within the agri-food industry.

6.5 REFERENCES

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APPENDICES

APPENDIX A – HPLC CALIBRATION CURVES

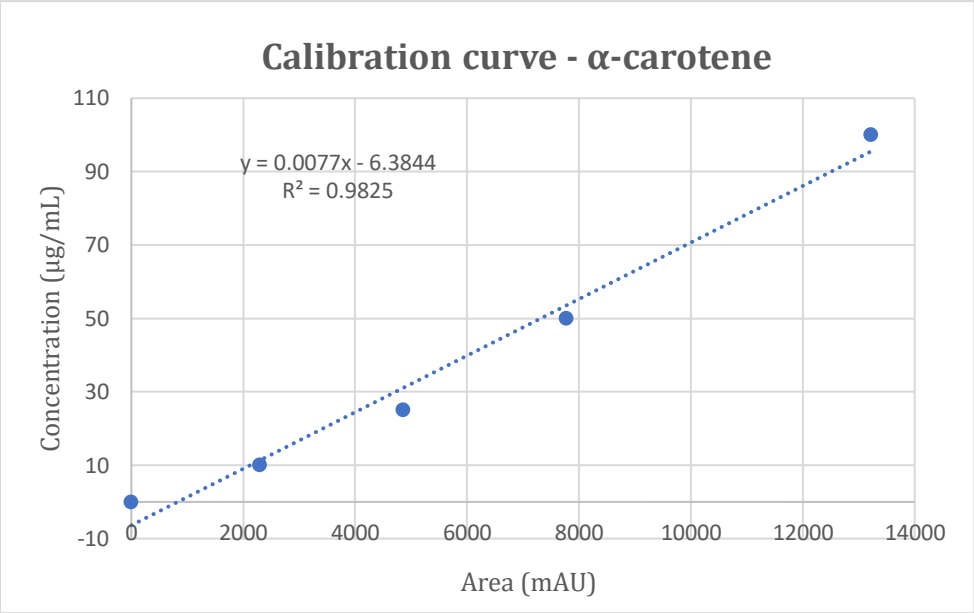

Figure A.1. α-carotene calibration curve

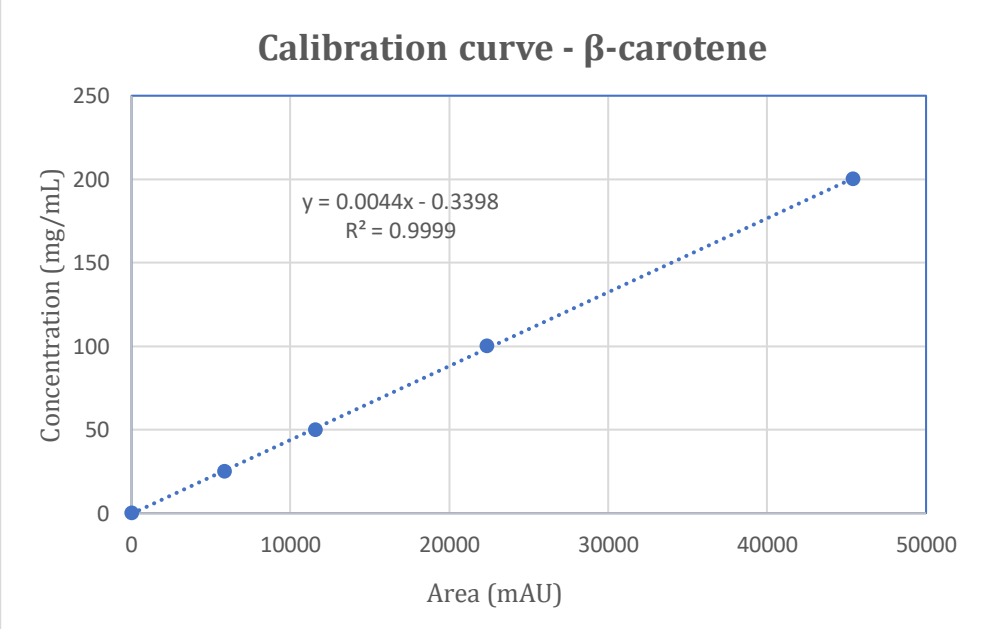

Figure A.2. β-carotene calibration curve

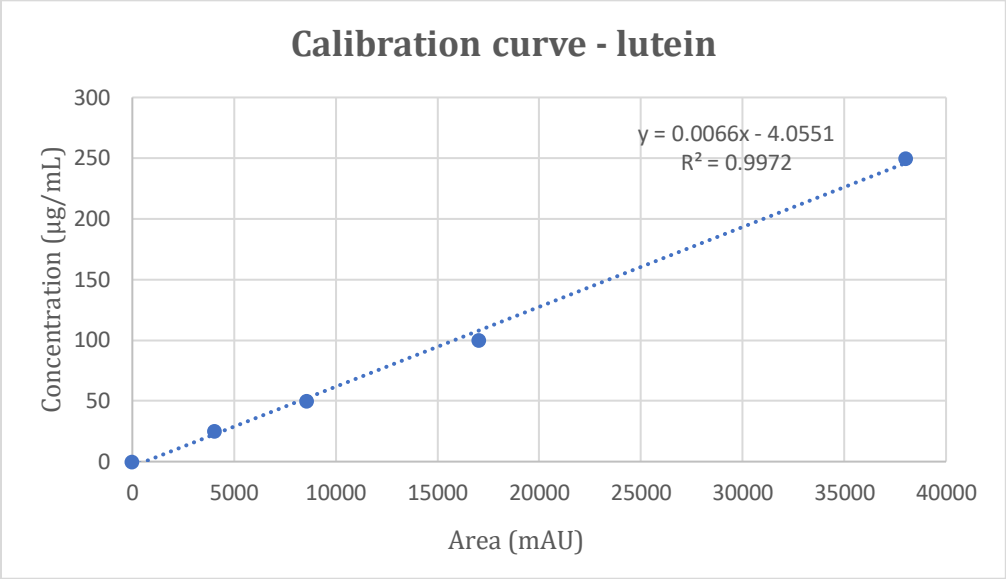

Figure A.3. lutein calibration curve

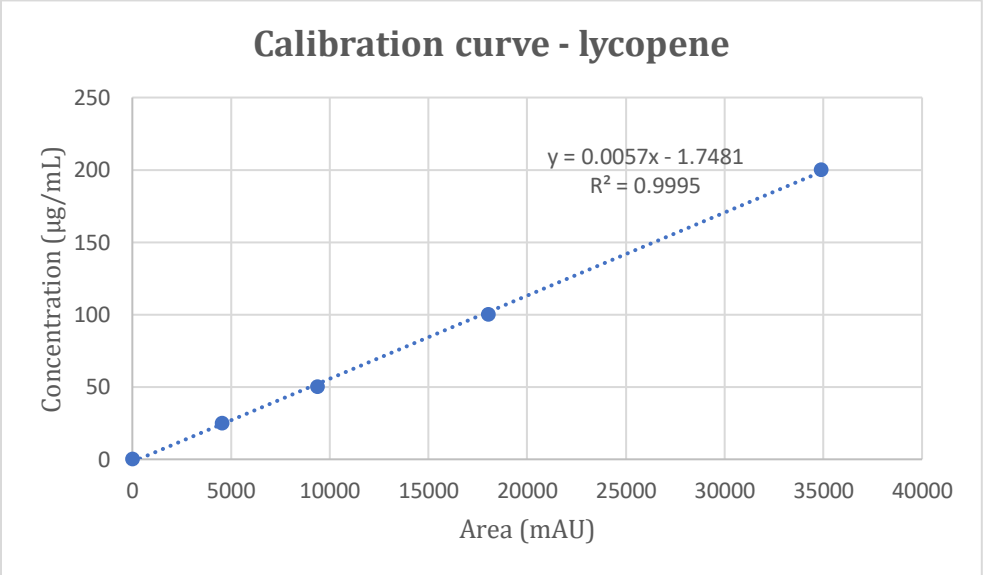

Figure A.4. lycopene calibration curve

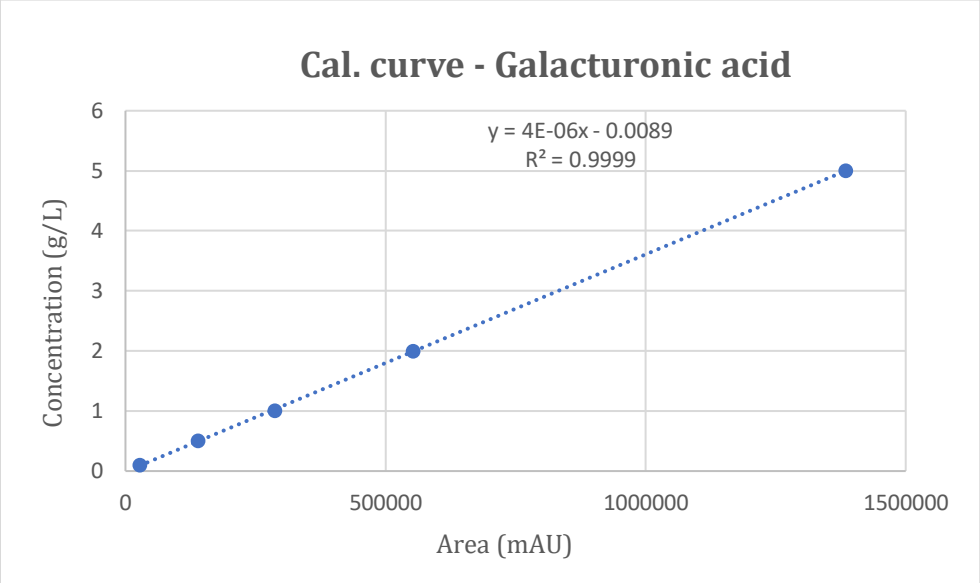

Figure A.5. Galacturonic acid calibration curve

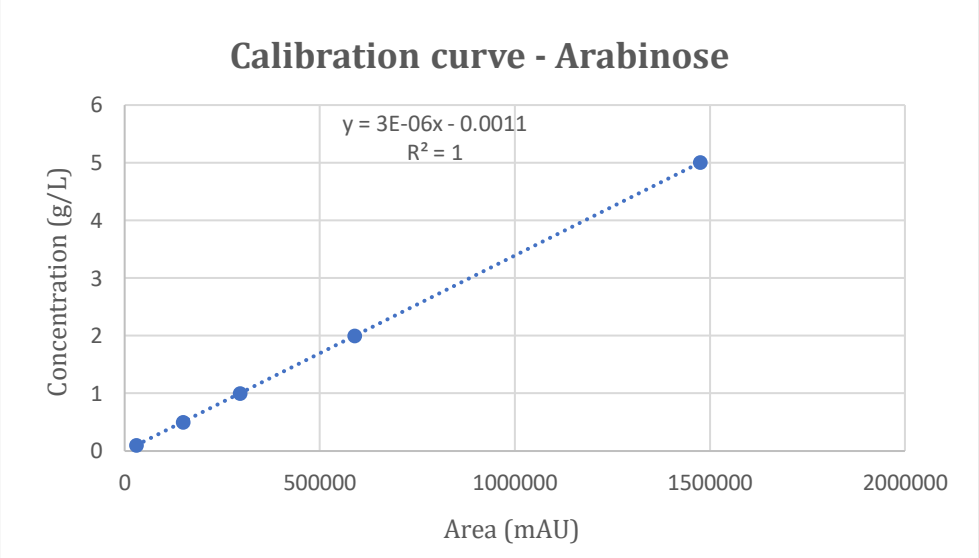

Figure A.6. Arabinose calibration curve

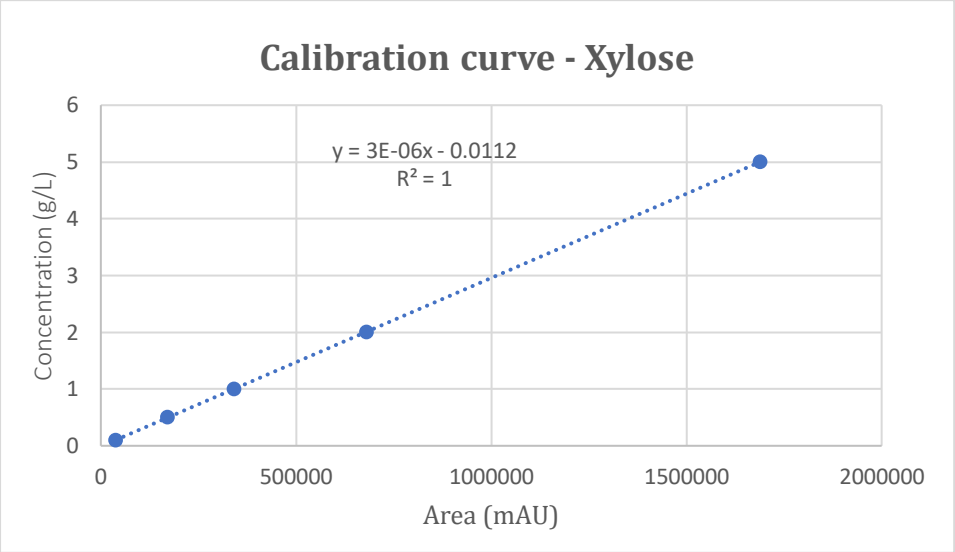

Figure A.7. Xylose calibration curve

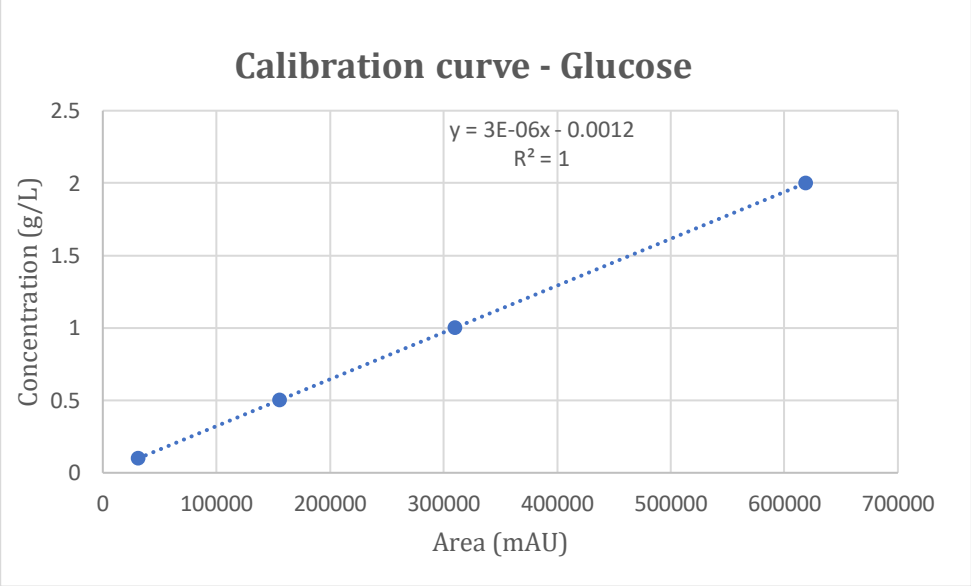

Figure A.8. Glucose calibration curve

APPENDIX B – PRELIMINARY SCREENING (SFE)

Table B.1. Process conditions for each preliminary run. MeOH = methanol, EtOH = ethanol. R05 was a multi-stage run where MeOH was used as a co-solvent in the first stage (40 mins) and EtOH in the second (40 mins).

Run #	Sample Load (g)	Extraction time (min)	CO_2 flow rate (g/min)	Pressure (Bar)	Temperature (°C)	Co-solvent
R01	40	60	20	300	60	0
R02	40	60	20	300	60	2% MeOH
R03	20	60	15	300	60	5% EtOH
R04	20	80	15	300	70	5% MeOH
R05	20	40/40	15	300	50/70	2% MeOH/5% EtOH

Table B.2. Total Carotenoid Content (TCC) and Total Phenolic Content (TPC) of the extracts obtained in each preliminary run.

Run	TCC (HPLC)			TPC (Folin-ciocalteu)		
	Initial content (mg)	Recovered (mg)	Recovery (%)	Initial content (mg)	Recovered (mg)	Recovery (%)
R01	10.2	3.45	33.8	134.55	2.1	1.6
R02	10.2	4.40	43.1	134.55	38.76	28.8
R03	5.1	3.15	61.8	66.90	3.24	4.9
R04	5.1	1.52	29.9	66.90	15.39	23.0
R05	5.1	3.50	68.7	66.90	25.2	37.7

APPENDIX C – CAROTENOID PROFILING OF CARROT SAMPLES

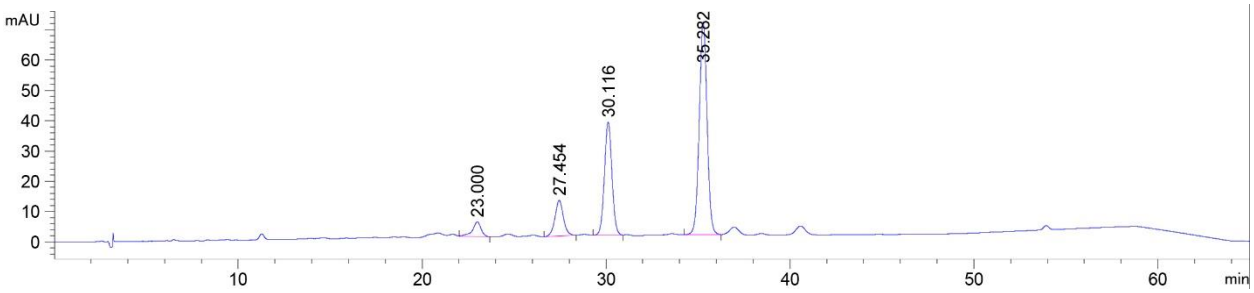

Fig. C.1. Carotenoid profiling of carrot flesh samples. From left to right: lutein (t_r ~ 23.0 min), lycopene (t_r ~ 27.5 min), α-carotene (t_r ~ 30.1 min), β-carotene (t_r ~ 35.3 min).

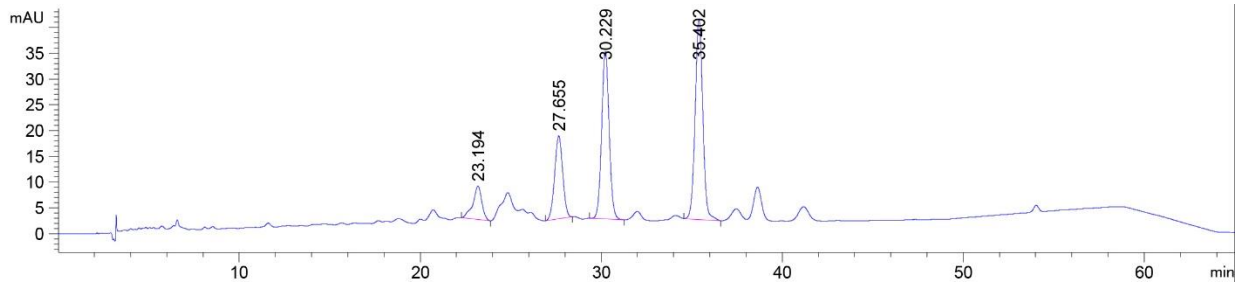

Fig. C.2. Carotenoid profiling of carrot peel samples. From left to right: lutein (t_r ~ 23.2 min), lycopene (t_r ~ 27.7 min), α-carotene (t_r ~ 30.2 min), β-carotene (t_r ~ 35.4 min).

APPENDIX D – DOE STATISTICS

Table D.1. Design matrix evaluation (ANOVA) of the response surface model for total yield.

Var. source	Square Sum	Degrees of Freedom	Mean Square	F-value	P-value	R^2
Regression	34.72	9	3.86	3.06	-	0.807
Residual	8.80	7	1.26	-	-	-
Total	43.52	16	-	-	-	-
F_{tab} (95%)	-	-	-	2.72	0.05	-

Table D.2. Design matrix evaluation (ANOVA) of the response surface model for total carotenoid recovery.

Var. source	Square Sum	Degrees of Freedom	Mean Square	F-value	P-value	R^2
Regression	33.18	9	0.0369	5.26	-	0.870
Residual	4.94	7	0.0070	-	-	-
Total	38.12	16	-	-	-	-
F_{tab} (95%)	-	-	-	2.72	0.05	-

APPENDIX E – CARBOHYDRATE DATA

Table E.1. Carbohydrate profiling (as to relative percentage) of raw carrot peels and extracts of four selected SFE conditions (section 3.3.5).

	Raw sample	**EXT1**	**EXT2**	**EXT3**	**EXT4**
Glucose (%)	53.3±2.7	46.6±2.2	48.8±2.1	46.3±2.5	44.3±1.8
Xylose (%)	18.8±1.5	20.2±1.5	19.9±1.7	20.7±1.5	21.6±1.8
Arabinose (%)	6.1±0.80	7.1±0.90	6.9±0.90	7.4±1.1	7.6±0.80
Galacturonic Acid (%)	21.8±1.9	26.1±2.0	24.4±1.9	25.6±1.3	26.5±1.3
Total (%)	100	100	100	100	100

APPENDIX F – ADSORPTION DATA

Table F.1. Desorption by different solvents in batch mode at a flow rate of 2.0 mL/min and 22 °C ± 2 °C. n.d. = not detected. Global REC refers to the recovery including both adsorption and desorption yields.

Solvent	Carotenoid	Elution (%)	TCC elution (%)	Global REC (%)
Methanol	ACar	2.4	3.2	2.7
	Bcar	3.1		
	LUT	7.2		
Acetone	ACar	94.5	84.4	70.1
	Bcar	91.7		
	LUT	n.d.		

Table F.2. Bed characterization data

	Parameter	Value
Process conditions	Flow rate (Q)	2.8 mL/min
	Bed height (H_B)	21 cm
	Bed volume (V_B)	16.5 mL
	Dead time (t_d)	5.13 min
Hydrodynamic data	Blue dextran average retention time (t_{r1})	7.61 min
	Acetone average retention time (t_{r2})	9.10 min
	Total porosity (ε_T)	0.42
	Bed porosity (ε)	0.64
	Particle porosity (ε_P)	0.38

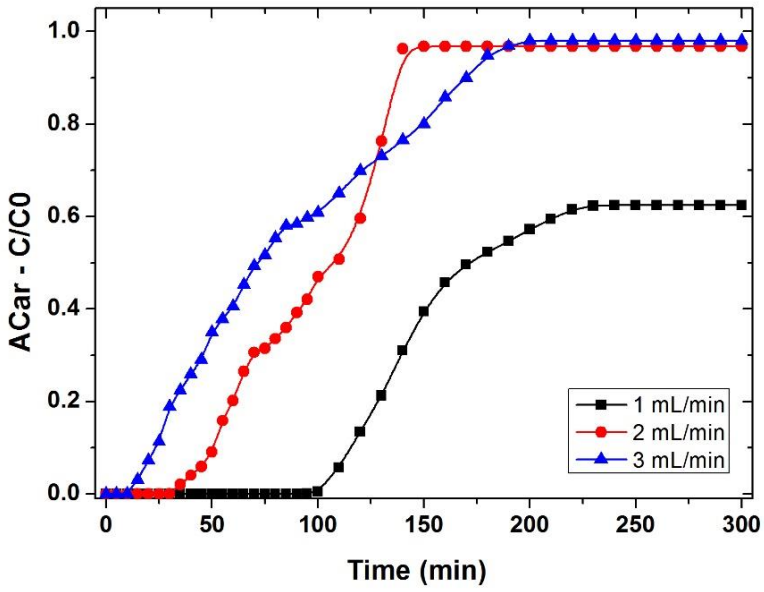

Figure F.1. In-column breakthrough curves of α-carotene adsorption under different flow rates. The curve profile for β-carotene is very similar and therefore, has not been included. The flow rate of 2 mL/min presented the highest adsorption levels within the shorter time frame.

APPENDIX G – ADJUSTED PARAMETERS (ADSORPTION MODELS)

Table G.1. Nonlinear adjusted parameters of the adsorption models.

Model	Parameter	Physical meaning	Value	Error (±)
Langmuir	q_m	Number of adsorption sites	6173	424.7
	k	Langmuir dissociation constant	0.0096	0.001
	R^2	-	0.986	-
Langmuir-Freundlich	q_m	Number of adsorption sites	5344	853.2
	k	Langmuir dissociation constant	0.828	0.015
	b	Adsorption capacity constant	1.28	0.44
	R^2	-	0.974	-
Freundlich	b	Adsorption capacity constant	401	119.1
	n	Adsorption energy	2.32	0.31
	R^2	-	0.977	-