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Purification of supercritical-fluid carotenoid-rich extracts by hydrophobic interaction chromatography

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Declarations of interest: none

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 model. 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 well 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, adsorption, Langmuir model, supercritical fluid extract.

LIST OF ABBREVIATIONS

AA: Antioxidant activity

ACar: α-carotene

ADS: Adsorption

BCar: β-carotene

BHT: Butylated hydroxytoluene

BTC: Breakthrough Curve

DES: Desorption

HIC: Hydrophobic Interaction Chromatography

LUT: lutein

PF: Purification Factor

SFE: Supercritical Fluid Extraction

STD: β-carotene standard

TCC: Total Carotenoid Content

NOMENCLATURE

A_{h}: Area lateral to the breakthrough curve

C: Final concentration (µg/mL)

C_0: Initial concentration (µg/mL)

C_{eq}: Concentration in liquid phase (µg/mL)

k: Langmuir dissociation constant

m_{ads}: adsorbent dry mass (mg)

q^*: Resin adsorption capacity (µg carotenoids / mg resin)

q: Adsorbed amount (µg carotenoids / mg resin)

Q: Flow rate (mL/min)

q_{m}: Number of adsorption sites, Langmuir model

q_{max}: Maximum resin capacity (µg carotenoids / mg resin)

t_d: Dead time (min)

V: Volume of the bed (mL)

V_c: Volume of the column (mL)

V_{sol}: volume of solution (mL)

ε: Bed porosity (-)

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 growing interest of the food industry 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. Specifically, *β*-carotene plays a major role in the human body as the main precursor of vitamin A, which is involved in vision, cell differentiation, mucus secretion, reproduction, growth and development of bones [5–7]. Carotenoids have also been linked with a decreased risk of certain types of cancer, cardiovascular diseases, muscular degeneration, cataract formation and have been suggested as being potential inhibitors of Alzheimer’s disease [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 on the valorisation of unexploited natural resources.

The extraction of phytochemicals from vegetable matrices is mostly carried out by organic solvents, due to their ease of use, low cost and wide applicability [11]. However, extractions require several hours to deliver satisfactory recoveries and the obtained extracts are often dilute, which results in the need for additional concentration steps. There are also concerns with regards to solvent toxicity, as methanol, hexane and tetrahydrofuran (THF), being the solvents typically used, can create hazardous issues for both handlers and the environment in terms of management and disposal [12].

With the advent of green technologies, new 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 is regarded as a fast, efficient and clean method for the extraction of natural components from biomass matrices, such as fruit and vegetables [13]. Using SFE for extracting carotenoids is promising approach, and different vegetable waste matrices have already been tested, including banana, grape and tomato peels [14–16], grape, pomegranate and pumpkin seeds [17–19], and apricot bagasse and pomace [20,21].

It is noteworthy that although vegetable extraction via SFE has been previously investigated, only a few number of studies deal with further purification of the extracts in order to obtain the targeted compounds in high purity, 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 found 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 yet 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.

2. MATERIALS AND METHODS

2.1 Extract characterisation

The extract was obtained after the supercritical fluid extraction of 50 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 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 [25].

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 [26]. Briefly, 15 mL of extract samples (in triplicate) were submitted to acid hydrolysis through the addition of 1.2 mL of H_2SO_4 (72%, v/v) and autoclaving at 121 ^oC for 30 minutes. After cooling down, the pH value of the supernatants was adjusted to 5.0 using $CaCO_3$ and the supernatants filtered and analysed by High Performance Liquid Chromatography (HPLC) in an Agilent HPLC system coupled with DAD/RI detectors (Agilent Infinity, 1260 model). The column used was an Aminex HPX-87H (300 x 7.8 mm) (Biorad, UK); the isocratic mobile phase was 0.0005 M H_2SO_4 and the flow rate was 0.6 mL/min. Quantification of sugars was performed according to calibration curves using glucose, xylose, arabinose, and glucuronic acid (all from Sigma-Aldrich UK) as standards.

2.1.2 Total lipid content

The lipid content of the extracts was determined gravimetrically, using the Soxhlet method [27]. 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.

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.

2.1.4 Total carotenoid content (TCC)

The total carotenoid content (TCC) was analysed according to the protocol described by Biehler et al [29]. The SF extracts, obtained in ethanol, were filtered through 0.22 μm membranes and directly analysed in an Agilent HPLC system (Agilent Infinity, 1260 model) using an YMC-C30 silica-based reversed-phase column (250 x 4.6 mm) coupled with a 1260 DAD detector. The mobile phases were (A) methanol/MTBE/water (82:16:2) and (B) methanol/MTBE/water (23:75:2). The gradient started at 100% of A. Solvent B was then linearly increased to 50% (0 - 45 min) and further increased to 100% (46 – 55 min), and then held for 5 minutes, totalling 60 min; the flow rate was kept constant at 1.0 mL/min. Quantification of carotenoids was performed according to calibration curves using α- and β-carotene and lutein (all from Sigma-Aldrich) as external standards. TCC was calculated by summing the concentrations of all carotenoids.

2.1.5 Antioxidant activity

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 (Thermoelection 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).

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 otherwise stated, all experiments were carried out in triplicates.

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, in a 20-mL flask, and mixed with different amounts of resin (25, 50, 100, 200, 300 and 500 mg). 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 follows:

$$q\ (\mu g/mg) = \frac{V_{sol}(C_0 - C_{eq})}{m_{ads}} \qquad (1)$$

where q is the amount of adsorbed carotenoid per mg of resin (μg/mg), C_{eq} is the total carotenoid concentration in the liquid phase (μg/mL) in equilibrium with q, C_0 is the initial concentration in the liquid phase (20 μg/mL), m_{ads} is the adsorbent dry mass (mg) and V_{sol} is the volume of solution (10 mL) in contact with the adsorbent.

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. 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. The adsorption kinetic profiles were established both for individual carotenoids as well as for total carotenoids.

2.2.3 Elution and recovery

To elute the carotenoids from the adsorption resin, acetone was used as a solvent, as it has 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 achieve 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.

2.3 In-column experiments

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 inserted with the aid of the peristaltic pump at a constant *Q* of 2.0 mL/min, 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.

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. 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 under a constant Q was calculated using equation 2 [32]:

$$q^* = C_0 \left[Q . \int_{t_d}^{\infty} \left(1 - \frac{C_{eq}}{C_0}\right) dt . V . \varepsilon \right] \tag{2}$$

where C_0 is the initial concentration and C_{eq} is the total carotenoid concentration in the liquid phase (µg/mL), V is the volume of the packed bed (mL), t_d is the dead time (the time in min required for the fluid to travel through the path of all existing pipes and connections under a constant Q), and ε the bed porosity, calculated according to methodologies previously described [32].

The term $\int_{t_d}^{\infty} \left(1 - \frac{C_{eq}}{C_0}\right) dt$ in the equation is equivalent to the area lateral to the BTC in the graph and corresponds to the amount of total carotenoids adsorbed. The final expression of *Eq*. 2 is as follows:

$$q^* = \frac{C_0 (A_h Q - \varepsilon V_c)}{(1-\varepsilon) V_c} \tag{3}$$

where A_h is the lateral area by the curve.

2.3.3 Adsorption isotherm and mathematical modelling

With the q^* values obtained from the BTCs, the equilibrium isotherm was then built, where q^* is plotted as a function of the concentration in the liquid phase (C_{eq}) for each of the carotenes (α-carotene and β-carotene and also for total carotenoids). The Langmuir model [33] was used to fit the experimental data. It assumes monolayered adsorption and the final equation is as follows:

$$q^* = \frac{q_m k C_{eq}}{1 + k C_{eq}} \tag{4}$$

where C_{eq} is the equilibrium concentration, q_m is the number of adsorption sites and k is the Langmuir dissociation constant (mL/mg), related to the adsorption energy.

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.

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.

3 RESULTS AND DISCUSSION

3.1 Batch adsorption experiments

The main environmental parameters that have been suggested to influence hydrophobic interactions of proteins, which are the molecules extensively studied in HIC, are the type of buffer, buffer ionic strength (i.e., salt concentration), temperature and pH [36]. 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, and these factors can therefore 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 [36]. 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 in carotenoid adsorption.

3.1.1 Resin adsorption capacity and mass optimisation

Figure 1 depicts the relationship between the percentage of adsorption of the individual carotenoids present in the extracts and the mass of resin; pure β-carotene was also tested as a control.

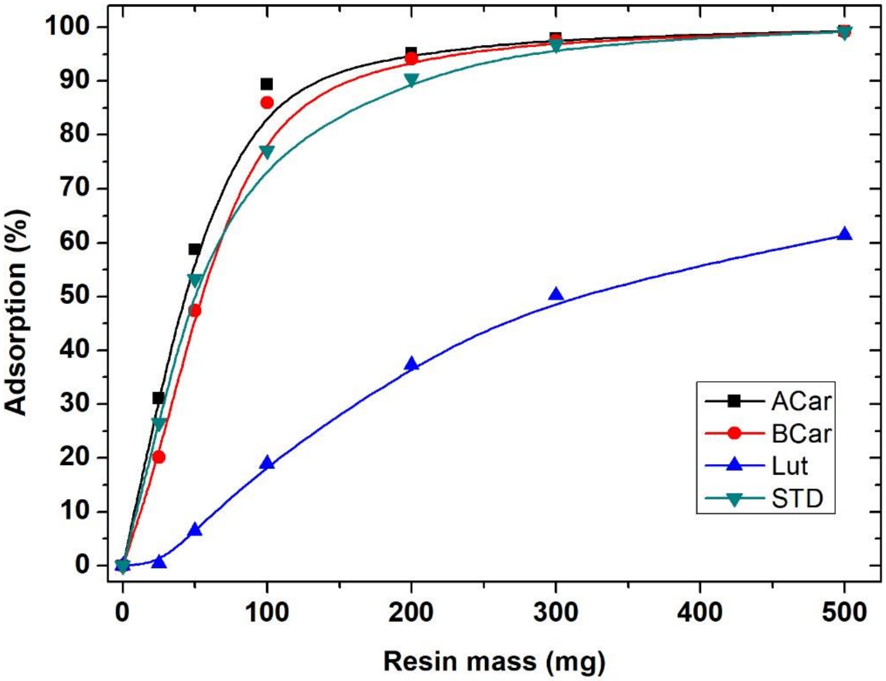

Figure 1. Adsorption percentage of carotenoids adsorbed versus resin amount in batch mode, after 6 hours, at 22 °C (± 2 °C), and pH 6.0. ACar: α-carotene; BCar: β-carotene; LUT: lutein; STD: β-carotene standard.

It can be observed that α-carotene and β-carotene demonstrated a high degree of affinity towards the adsorbent. The Amberlite XAD-1180N resin is a non-ionic hydrophobic cross-linked polymer, available in the form of white beads and regarded as safe for food-related applications. Its adsorptive capability derives from its macroreticular structure (that contains both a polymer phase and a pore phase), high surface area and the aromatic terminations present in its chemical structure (Figure 2). It is usually employed to adsorb large hydrophobic molecules from polar solvents, e.g. proteins [37]. Within the XAD family, this particular adsorbent 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 increasing 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.

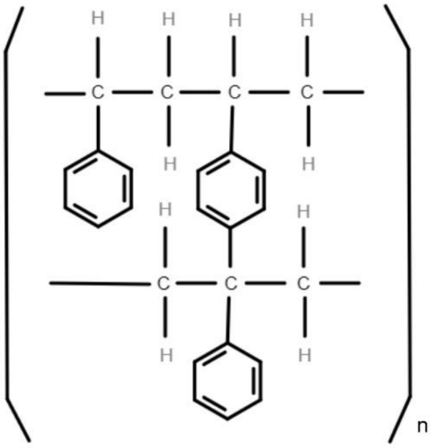

Figure 2. Chemical structure of Amberlite XAD 1180N

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 in Equation 1. 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) compete 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 amount of resin needed for a particular extract with a known carotenoid concentration.

3.1.2 Adsorption kinetics

4 After the optimisation of the resin amount, the next step was to evaluate the adsorption kinetic profile of α-carotene and β-carotene in the extracts (Figure 3).

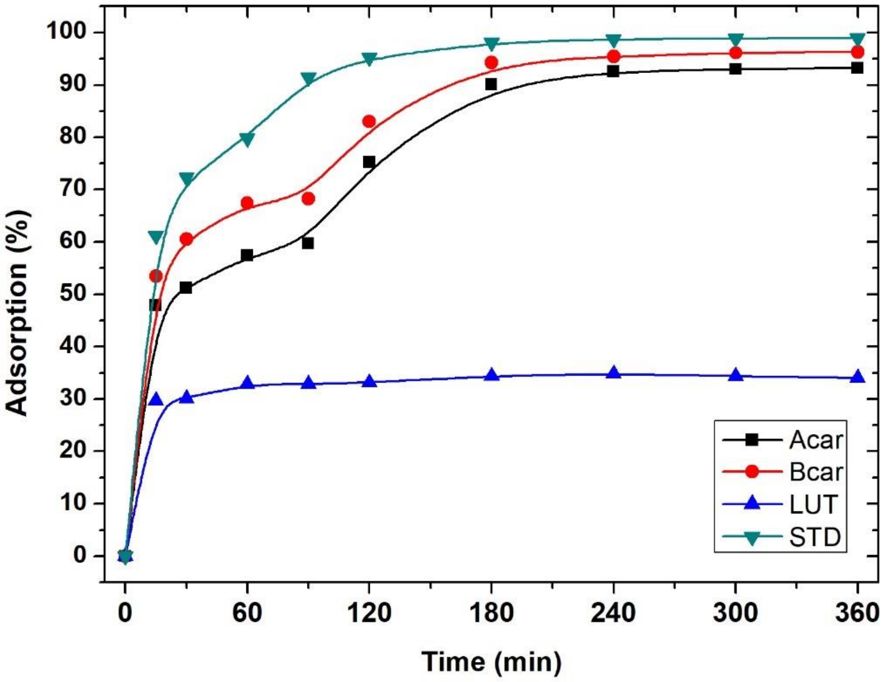

Figure 3. Adsorption kinetics of α-carotene and β-carotene present in extracts in batch mode at 22 °C (± 2 °C), using 560 mg of resin. ACar: α-carotene; BCar: β-carotene; LUT: Lutein; STD: β-carotene standard.

It appears that the binding of a considerable amount of 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. In the case of lutein, the adsorption does not appear to improve considerably with time, starting at 29.4% at 15 min and plateauing at 34.4% after 60 min. Therefore, focusing on the major carotenoids, 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. As to Lutein, the low adsorption

4.1.1 Elution and recovery

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 (data not shown). 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 1); in the case of the standard β-carotene this was lower, i.e. ~ 83%.

Table 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; TCC: total carotenoid content; AA: Antioxidant activity

In addition to carotenoid recovery, the antioxidant activity of the samples during the adsorption/desorption process was measured. 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 in subsequent in-column experiments, the antioxidant agent BHT, was added to minimise degradation.

3.3.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.

3.3.1. Breakthrough curves

The adsorption of individual carotenoids at a flow rate of 2.0 mL/min of extract is represented by the BTCs depicted in Figure 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 said to be saturated when C/C_0 reaches 1.0.

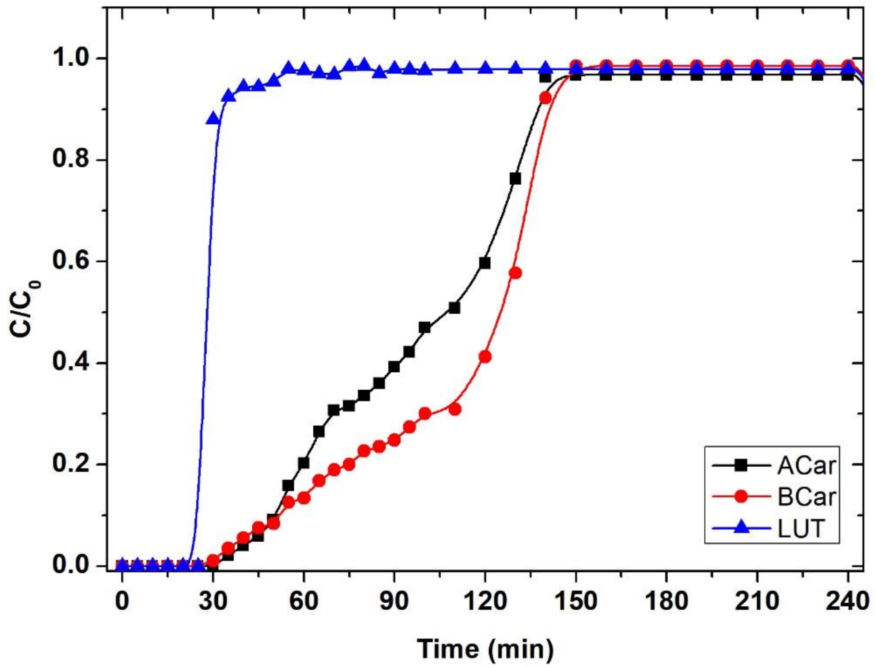

Figure 4. In-column breakthrough curves at 22 ± 2 °C and a flow rate of 2.0 mL/min of extract. ACar: α-carotene; BCar: β-carotene; LUT: lutein. The TCC composition of the extract was: 32.5% α-carotene, 58.9% β-carotene, 8.6% lutein.

In the case of α- and β-carotene, the graphs resembled an "S-shaped" curve, which is characteristic of a well-resolved BTC [38], 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 the 50 min 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 foments 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.

3.3.3 Adsorption isotherm and mathematical modelling

The adsorption of a substance present in a fluid phase onto the surface of a solid particle is governed by a thermodynamically-defined distribution between both phases once the equilibrium is reached. A common way of describing such distribution is through an adsorption isotherm, i.e. by expressing the resin adsorption capacity (q^*) as a function of the adsorbate concentration (C_0) in solution; In this case, the total carotenoid concentration was used. The adsorption isotherm is needed to calculate the in-column q_{max} value, i.e. the maximum amount of adsorbent that the resin is able to uptake. In order to construct this 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, and these are shown in Figure 5.

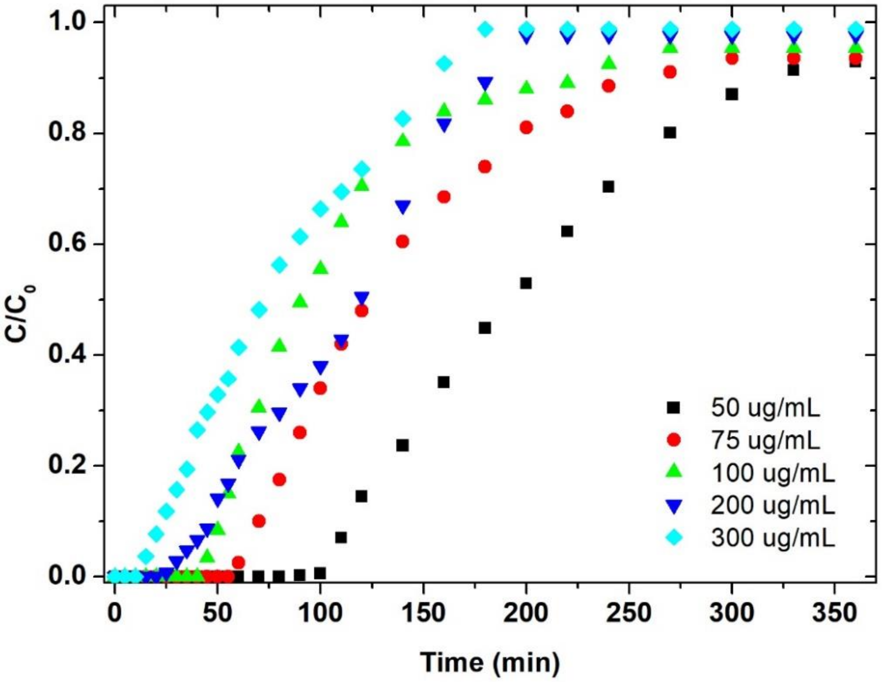

Figure 5. In-column adsorption breakthrough curves at a constant flow rate of 2.0 mL/min of extract, at 22 ± 2 °C, with different inlet concentrations of total carotenoids.

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, data not shown), most likely due do the shorter processing times and consequently lower degradation levels during the process.

The porosity value of the bed (ε) was calculated as 0.42 [32]; this is a reasonable value as it is a manually-packed column and values less than 0.40 are rarely encountered in such columns [39]. 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, on the other hand, causes intense axial dispersion and reduces the interactions between the compounds and the solid phase (0.50 and above) [40]. The dead time was of 5.13 min. The generated experimental data were used to calculate the q^* for each of the BTCs using Equations

2 and 3, under a constant flow rate Q. These were then plotted as a function of total carotenoid concertation, to obtain the adsorption isotherm, and were subsequently fitted to the Langmuir model (Figure 6). This model has been widely used to describe experimental adsorption data involving solutions of a strongly adsorbed component to an adsorbent material [41]. The q_{max} was calculated by taking into account the highest q^* value, after it plateaued.

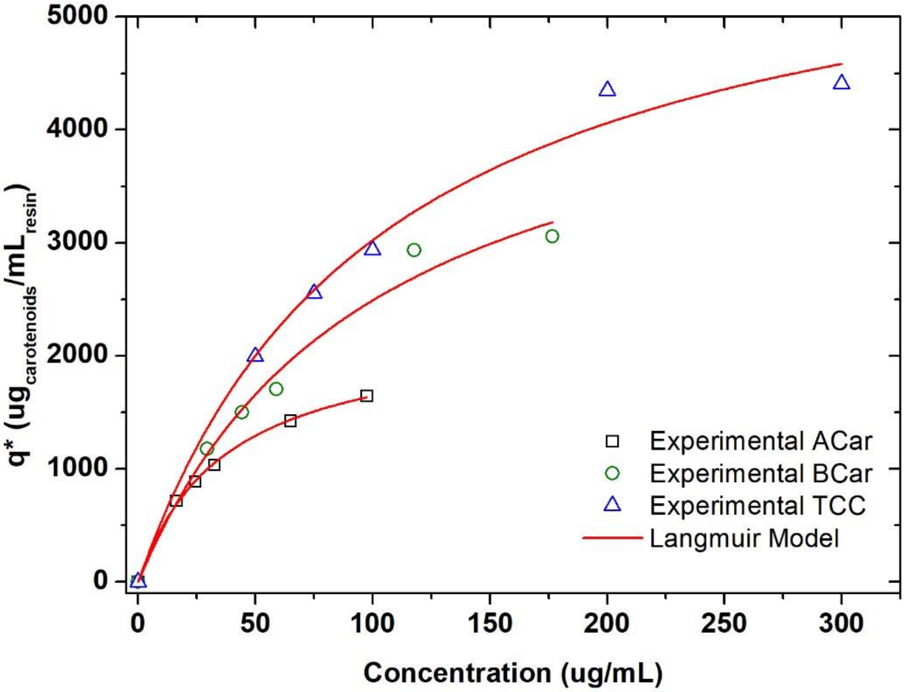

Figure 6. Adsorption isotherm of α-carotene (ACar), β-carotene (BCar) and total carotenoids (TCC, ACar + BCar) in column at 22 ± 2 °C, under a flow rate of 2 mL/min of extract. The experimental data was fitted to the Langmuir model.

The Langmuir model is able to adequately explain the experimental data of solutions at low or moderate concentrations and assumes the existence of a well-defined and localised number of adsorption sites (q_m), where only one molecule is adsorbed per site and no interactions between the adsorbed molecules at the neighbouring sites exist [42]. Based on the model, it is noticeable that the behaviour of both individual carotenes in solution followed a very similar pattern in terms of adsorption performance with concentration. Therefore, if the multi-component solution is taken as single-component by considering only the total carotenoids (TCC) plot and fit, 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). Individually, the q_m and q_{max} values are, respectively, 2253 and 4.57 μg TCC/mg for α-carotene and 4989 and 8.58 μg TCC/mg for β-carotene. 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 magnitudes of 10^{-2} - 10^{-3}, such as that found for this process (2.6×10^{-3} for α-carotene, 9.9×10^{-3} for β-carotene and 9.6×10^{-3} for TCC), are indicators of strong interactions [43].

Given the high R^2 values found for all fits (0.988 for α-carotene, 0.981 for β-carotene and 0.986 for TCC) and the low errors for the model terms and constants, the Langmuir model can be deemed highly efficient for 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. These observations 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 [44]; this, however, was outside the scope of this work.

3.3.4 Elution and carotenoid recovery

Table 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 2. Recoveries of carotenoids and antioxidant activity of recovered samples during in-column adsorption at different flow rates.

Step	Description	Carotenoids						Antioxidant activity	
		ACar (mg)	BCar (mg)	LUT (mg)	Total carotenoids (mg)	Recovery (%)	Global recovery (%)	DPPH (% /mg CAR)	PF
Injection	Initial extract (105.9 µg/mL TCC)	17.27	35.71	1.12	54.08	-	-	1.53	1
Adsorption (2.0 mL/min)	Bound material	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 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 the carotenoid recovery during the elution step, it can be observed that for the lower flow rates (1mL/min and 2mL/min), the recovery was very high (~95%), whereas for the higher flow rate (3mL/min) it was relatively low (~65%). This was most likely due to the fact that at 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 flow rates of 1 mL/min and 2 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 indicated the removal of impurities present in the extracts, including sugars and proteins, which was confirmed later, after the validation runs.

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 others. This way, the rejected fractions could be submitted to the new columns and the recoveries could be maximised by the complete adsorption of the carotenoid fractions. 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.

3.4 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 7.

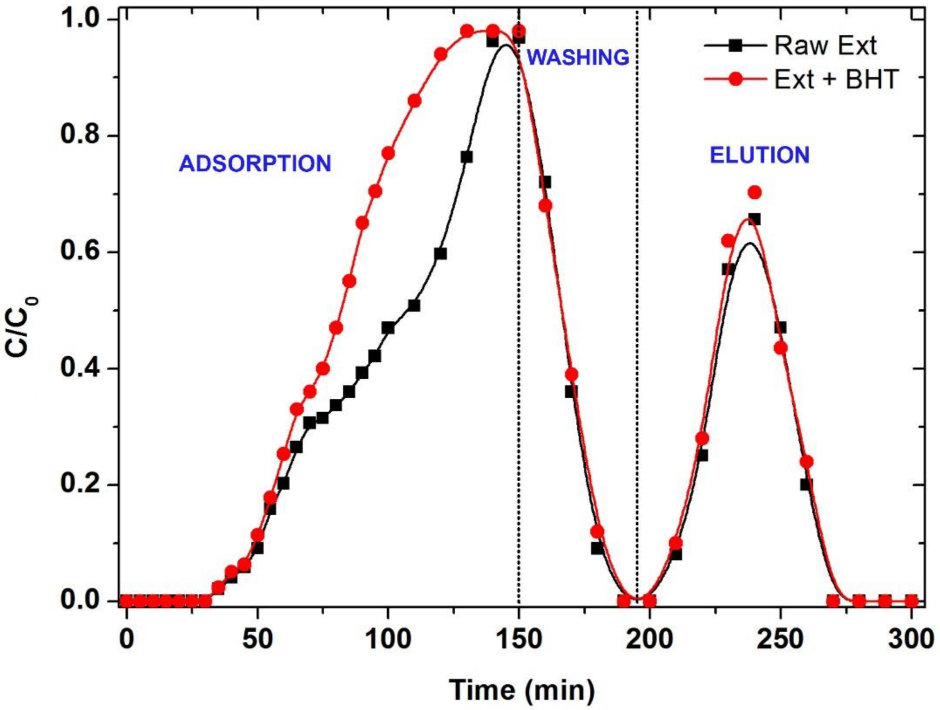

Figure 7. Chromatograms of the in-column validation runs for the purification of carotenoids present in the raw extract (TCC: 105.9 μg/mL) and extract with addition of 1mg/mL of butylated hydroxytoluene (BHT). Process conditions: 450 mL extract, flow rate 2.0 mL/min, temperature 22 ± 2°C, and total processing 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 in 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%).

Table 3 shows the initial composition of the extract and that of the purified fraction following in-column adsorption.

Table 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.

	Composition	Initial extract	Purified extract	PF
-	Total mass (mg, dry weight)	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 extract)	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: Purification Factor (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 contents from the extract, which contributed with ~58% of the total mass in the initial extract. This was most likely due to the fact that carbohydrates are polar molecules and therefore not adsorbed by the hydrophobic resin. In addition, 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 was used, which has an even lower dielectric constant than ethanol, and therefore most of the proteins remained bound to the resin, explaining the significant decrease in their content in the eluate. The 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 and 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. [45] 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 [46]. 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. 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, fomenting 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. The process is potentially scalable and could be implemented for the extraction and purification of carotenoids from natural sources in an industrial setting, as an alternative to the current production via chemical routes. However, intertwined with the future scale-up studies suggested, cost evaluations are mandatory to confirm the full economic viability of the developed protocol for larger-scale production and commercialisation.

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