Removal of Added Value Alkaloids from Food Aqueous Streams

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Abstract

Today we face significant threats to our own and other species existence, the increasing population and consumption are having unprecedented demands on agriculture and natural resources. This thesis focuses on two food products, lupin beans and bitter oranges, that could help release some of that pressure. These two products as crops have low water consumption and are very resistant to temperature changes and soil acidity, also lupin beans are a natural fertilizer and pesticide. Both have good nutritional values but they have in their composition toxic compounds that make them not edible. Large amounts of fresh water are used by food processing industry, to remove lupanine a toxic alkaloid that confers a bitter taste to the lupin beans, to make their consumption safe. Bitter orange fruits have high quantities of synephrine a toxic alkaloid, so they are not being used by the food industry, manly the peals are being used to produce food additives, everything else is wasted. These two compounds, lupanine and synephrine, are added value compounds that are used in the pharmaceutical industry, so the present work considers the use of adsorptive separation methods to remove and recover these toxic compounds with pharmaceutical properties, allowing the safe consumption of lupin beans and bitter orange juice while decreasing it's environmental impact. Lupanine binding percentage for commercial polybenzimidazole (PBI) polymer and three different PBIs, that suffered thermal treatment and pH conditioning, were studied for lupanine pure stock solutions and lupin beans effluent (pure and basified). The treated PBIs showed improved results compared to the commercial PBI and the basified effluent also improved the binding of lupanine. Adsorption isotherms and binding kinetics were studied for the basified effluent treated with the thermally treated PBI (PBI-T). Several Recovery Solvents were tested to recover lupanine after the binding for the several PBI treatments and the best recovery solvent from the ones tested was THF. Dried bitter oranges were were extracted with water and with ethanol, the extracted compounds were analysed and quantified in the HPLC obtaining a concentration of 493 ppm of synephrine and 345 ppm of naringin (one of the main bitter oranges flavonoids). A preliminary binding assay was performed with a synthetic mixture of bitter orange juice (40 ppm of synephrine; 50 ppm naringin pH 2.65) mixed with 25 mg of resin (2 acidic resins, 2 basic resins and 2 polymeric adsorbers), from which the acidic resins were the best to bind synephrine while keeping most of the healthy flavonoid (naringin) in solution.

Keywords: Food production, sustainability, lupin beans debittering wastewater, lupanine, bitter orange, synephrine, naringin, resin, polybenzimidazole polymer (PBI)

1. Introduction

The rising temperatures, extreme weather, lack of potable water and biodiversity loss are some of the many problems that will continue to affect human life and food production in the following years. Even though climate change will affect the food industry, it is itself responsible for causing it. For example, over 70% of all freshwater withdrawals from rivers, lakes, and aquifers are necessary to irrigate agricultural fields[1, 2]; also, animal production causes up to 18% of the total human-induced greenhouse emissions[3]. To reduce these numbers, we cannot affect production as it is crucial for human life, so we need to keep producing food while reducing its environmental impact. This work focuses on exploring the sustainability potentials of two food products, Lupin Beans (*Lupinus Albus* L. and Bitter Oranges (*Citrus Aurantium*, CA). We selected these because as crops they require scant amounts of water to grow[4, 5], as they don't need irrigation. They are also resilient to high temperatures and changes in soil pH [6, 7], growing even under climate change challenging conditions. Also, lupin beans are natural fertilisers[8], natural pesticides[9], and have a high protein content being an alternative to meat and could reduce the need for animal protein consumption[10, 11]. The

fundamental problem with these products in the food industry is the natural toxic alkaloids which extraction is required before consumption [12]. So, the food industry does not use bitter orange fruits today, mainly the peals are used to produce food additives and flavours, the rest is lost [13, 14]. Even though its fruits are full of healthy antioxidant flavonoids, it's alkaloid (synephrine) prevents it's consumption [15]. On the other hand, the food industry already widely uses lupin beans, but it's necessary to remove their main alkaloid (lupanine) from the beans that implies the use of huge amounts of water [16], so this process needs to be optimized to extract lupanine while reducing water consumption .

To extract alkaloids from lupin beans, successive extractions with water have traditionally been used (debittering process), since most alkaloids are water-soluble [12]. This process involves the consumption of large amounts of water which is eventually discarded with the effluent wastewater at the end of each leaching batch [16].

The industrial lupin beans debittering process comprises four stages: Swelling, cooking, debittering and extensive washing. This process lowers the alkaloid levels to approximately 0.04% and then they can be consumed in human food [12].

To reduce some of the amount of water spent in the debittering process, nanofiltration could be used to treat the effluent so it could be reused, as it was first suggested by T. Esteves, et al.[17]. At the same time a retentate, comprising the debittering and the washing phases of the lupin beans industrial debittering process, is obtained with a lupanine concentration of approximately 3 g/L. This stream is the focus of this thesis for the development of a process for lupanine isolation by adsorption and recovery for further purification and valorisation in the pharmaceutical industry.

These toxic compounds, that need to be removed from the orange fruits and lupin beans, are added-value compounds. They are starting materials used by the pharmaceutical industries. By isolating only synephrine, from the bitter orange juice, it can be sold to the pharmaceutical industry and keep the antioxidant properties of the juice unchanged. Another approach would be to also isolate these healthy flavonoids and used them as additives to produce new antioxidant and possibly antiviral and anti-inflammatory food products.

So, our main goal is to decrease waste and water consumption while removing the toxic added value compounds to make lupin beans and bitter orange sustainable and healthy food products.

Pharmaceutical properties : Lupin beans have health benefits, they can decrease hypercholesterolemia [18, 19] and may also have some anti-inflammatory effects, while lupine hydrolysates may help prevent diseases related to chronic inflammation [20]. Quinolizidine alkaloids from Lupinus species increase insulin secretion, having potential use in the treatment of type 2 diabetes [21].

Citrus Aurantium (CA) was traditionally used as a medicinal material in China because of its various pharmacological activities [22], with multiple therapeutic potentials, such as [15]: Anticancer [23, 24], Antianxiety [25, 26], Antiobesity [27, 28], Antibacterial [29], Antioxidant [30] and Antidiabetic activity [31].

Flavonoids are some of the major bioactive constituents of bitter orange fruits, such as naringin, hesperidin, neohesperidin, naringenin and hesperetin. The characteristics of these flavonoids have been investigated intensively, and they have shown to possess antioxidant [30], antiviral, anti-allergic [32], vasoprotective [33] and anticarcinogenic properties [33]. Citrus Aurantium (CA) extract also showed potential pesticidal activity [34].

Naringin and hesperidin are the most abundant flavonoids in CA reaching a concentration of 299.2 $\pm$ 0.5 mg/kg and 210.3 $\pm$ 1.3 mg/kg of dry fruit, respectively [35].

Other bioactive compounds commonly found in citrus Aurantium are the amines with adrenergic activity such as octopamine, tyramine, N-methyltyramine, hordenine, and synephrine, with the last one being the most abundant amine in CA [36]. Synephrine acts on several adrenergic and serotonergic receptors and has activity on trace-amine-associated receptors [37]. This adrenergic stimulation results in weight loss [38].

Added Value compounds extractions : Adsorption methods are the most used to treat wastewaters [39]. Synthetic resins are used in water and wastewater treatment, food processing and medicinal applications. They form an integral part of many food processing units, mainly for the removal of unwanted compounds [40]. Commercial resins are not expensive, do not require complicated procedures and are commonly used in adsorption separation processes because of their hydrophobicity or ionic selectivity [41, 42]. Also, one of the main advantages of using resins is that they can be reused upon regeneration [43].

Resins are classified into two types: ion exchange resins or polymeric adsorbents. Ion exchange resins act based on the interchange of ions between two phases, the insoluble phase (the resin) and the solution phase [41, 44]. With polymeric adsorbents, the interactions between the compounds and the resin are based on hydrogen bonding, hydrophobic or van der Waals interactions [42]. Alkaloids, also

known as organic bases, are widely distributed in many plant materials, and most of them have significant physiological activities. Its alkalinity can be characterised by the value of its conjugated acid pKa (synephrine has a pKa of 9.8 [45] and lupanine has pka of 9.1 [46]).

Polybenzimidazoles (PBI, Figure 1) is an extremely heat-resistant heterocyclic thermoplastic that is being used for various applications, in particular, for high-temperature applications, fibre spinning, and reverse osmosis and organic solvent nanofiltration membranes (allowing high-temperature filtrations[47]), because of its excellent thermal and chemical tolerance and film-forming capability [48]. PBI has a pka of 5.5 [49] and proton donor (-NH-) and a proton acceptor (-N=) hydrogen-bonding sites (1) which exhibit specific interactions with protic and aprotic polar solvents[50].

PBI has been subject to some modifications (structural and ionic) and its adsorption potential has been assessed for the removal of genotoxic impurities from solutions containing active pharmaceutical ingredients [51]. In these studies, the following modifications were performed: PBI with thermal treatment (PBI-T) to create rough porous surfaces increasing the surface area of the polymer, PBI with thermal and acid or basic treatment (PBI-TA and PBI-TB respectively) to induce ionic interactions between the polymer and the adsorbate.

The synthesis and processing of active pharmaceutical compounds are usually carried out in organic solvents, so PBI is an interesting polymer because it is compatible with these solvents. The purification of lupanine is also interesting for the same reason, although the adsorption process with PBI will be performed in an aqueous solution (effluent) the recovery process can be performed using organic solvents, allowing to explore, if necessary, harsher chemical conditions and organic solvents. This is something that cannot be done with commercial resins.

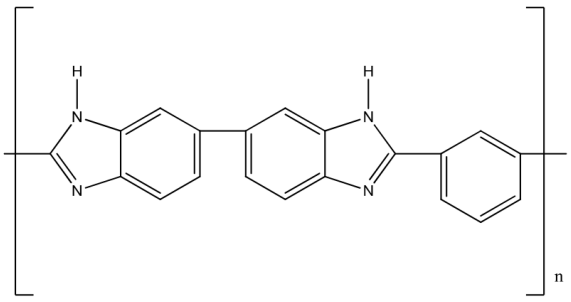

Figure 1: PBI Molecular Structure [103]

Adsorption Modeling Methods : Knowing how to model the experimental data from the adsorption processes can help us predict the mechanisms of the various adsorption systems and design low-cost adsorbents for the detoxification of effluents [52].

Having a good interpretation and understanding of the adsorption equilibrium information is the most important piece of information needed to improve the adsorption mechanism pathways and effectively design the adsorption process [53].

Binding and Recovery : The percentage of compound bound to the adsorbers was calculated from equation 1 where C_0 (mg/L) is the initial concentration and C_f (mg/L) is the final concentration in solution.

$$\text{binding } (\%) = \frac{[C_0 - C_\text{f}]}{C_0} \times 100 \tag{1}$$

The adsorption capacity of each adsorber was calculated from equation 2 where Q (mg/g) is the amount of compound bound to the adsorber, C_0 (mg/L) is the initial concentration, C_f (mg/L) is the final concentration in solution, V (L) is the volume of solution used, and M (g) is the adsorber mass.

$$Q = \frac{V \times [C_0 - C_\text{f}]}{M} \tag{2}$$

Adsorption isotherms : Adsorption isotherms are used to describe the equilibrium relationships between adsorbent and adsorbate.Adsorption isotherms linear regressions usually correlate the quantity adsorbed and that remaining in solution at a fixed temperature [52].

The Freundlich (equation 4) and the Langmuir models (equation 3) are often used at the same time to compare and choose the better one according to the determination coefficient (R^2) [54]. q (mg/g) is the amount of compound bound to the adsorber in a monolayer and q_m (mg/g) is the maximum amount of compound bound to the adsorber in a monolayer for the Langmuir model, whereas K_L and K_F are equilibrium constants (L/mg) for the Langmuir and Freundlich models, respectively, and n is a parameter related with the surface layer heterogeneity [51]. The Freundlich isotherm can be used for non-ideal adsorption on heterogeneous surfaces and the Langmuir isotherm assumes monolayer adsorption on a homogenous surface [52].

$$\frac{q_\text{e}}{q_\text{m}} = \frac{K_\text{L} C_\text{e}}{1 + K_\text{L} C_\text{e}} \tag{3}$$

$$q_\text{e} = K_\text{F} C_\text{e}^{1/n} \tag{4}$$

Adsorption Kinetics : Adsorption kinetics measures the rate of adsorption, which determines the time required to reach equilibrium for the adsorption process [55]. There are a variety of different models to fit the adsorption rates, these models describe the rate of retention of adsorbate from a solution to the solid-phase interface at a given adsorbent dose, temperature, flow rate and pH [56].

The pseudo-first-order (equation 6) and pseudo-second-order (equation 5) kinetic models are the most commonly used empirical models for alkaloid adsorption [54]. Where q_e and q_t (mg/g) are the adsorption capacities at equilibrium and at time t (min), respectively, and k_1 (min^{-1}) and k_2 (g/(mg·min)) are the pseudo-first-order and second-order rate constants for the models [51]. The pseudo-first-order kinetic model, also known as the Lagergren model, is more accurate to fit, the initial stage of adsorption, for a high initial concentration of adsorbate. When the initial concentration is low, the pseudo-second-order kinetic model is more suitable for fitting the subsequent stage of adsorption [54].

$$\frac{t}{q_t} = \frac{1}{k_2 \cdot q_e^2} + \frac{t}{q_e} \tag{5}$$

$$\ln(q_e - q_t) = \ln(q_e) - k_1 t \tag{6}$$

2. Materials and Methods

Reagents Ethyl acetate (EtOAc), tetrahydrofuran (THF),isopropanol (IPA), Ethanol (EtOH), Hexane, dichloromethane (DCM), methanol (MeOH),glacial acetic acid (AcOH) and acetonitrile (MeCN) HPLCgrade, HCl 37% solution,potassium hydroxide (KOH) pellets and NaOH pellets were purchased from Fischer Scientific. DMSO was purchased from Carlo Erba Reagents S.A.S and 1-Butanol was purchased from Merck KGaA. Dowex AG50W-X8 was purchased from BDH Chemicals Ltd Poole England; Purolite PD206 was purchased from Purolite ion exchange resins; Amberlite IRA68, Amberlite XAD-4 and Amberlite IRA458 were purchased from Rohm and Haas France S.A.S; Amberlite XAD-16 was purchased from ThermoFisher (Kandel) GmbH. Hesperidin (CAS 520-26-3) and Synephrine (CAS 94-07-5) were purchased from TCI Chemicals Europe N.V and Naringin (CAS 10236-47-2) was purchased from Alfa Aesar. Pristine polybenzimidazole (PBI) polymer 100 mesh powder was purchased from PBI Performance Products Inc. (USA).

Raw materials Industrial lupin beans effluent, with a lupanine concentration of 3 g/L (debittering phase), was kindly provided by Tremoceira M. Ferreira Bastos Lda. (TMFB) Portugal.

Lupanine was kindly provided by the Faculty of Pharmacy of the University of Lisbon.

Mature bitter orange fruits were collected at Instituto Superior Tecnico (IST) Alameda, Lisbon, Portugal in 2020 (16 of December).

Equipment Quantification of lupanine was performed on a Hitachi LaChrom High Performance Liquid Chromatography (HPLC) with a Kinetex 5 µm EVO C18 100A LC column (250 mm x 4. 6mm). The HPLC was constituted by two pumps pumps (L-7100), an interface module (D-7000), an autosampler (L7250) and a UV detector (L-7400, $=220\lambda$).). The mobile phase consisted of 15% of MeCN and 85% of aqueous Na_2HPO_4 buffer (pH 10.5) at a flow rate of 1 ml/min, injection volume of 20 µl and 30 min of run time.

Quantification and detection of synephrine, naringin and hesperidin were performed on a VWR Hitachi Chromaster HPLC equipped with a Luna 10 µm C18(2) 100 A , (250 mm x 4.6 mm) LC Column, a UV-Vis Detector (5420), a Column Oven (5310) and na autosampler (5260). For synephrine and naringin in the bitter orange juice synthetic mixture the UV detection was 225nm for synephrine and 283 nm for naringin, the injection volume was 10 μL, with a flow rate of 1 mL/min and the mobile phase consisted of 50%Water (0.6% acetic acid) and 50% Methanol. For synephrine, naringin and hesperidin on bitter orange dried extracts the UV detection was 225 nm, the injection volume was 10 μL, with a flow rate of 0.6 mL/min and the mobile phase consisted of 60%Water (0.6% acetic acid) and 40% Methanol.

Bitter Orange samples were lyophilised on a CHRIST Alpha 1-2 LD plus lyophilizer.

2.1. Lupin Beans Methods

2.1.1 PBI derived adsorbers

PBI thermal treated (PBI-T) [51] : Was obtained by dissolving pristine PBI polymer in DMSO (15% w/w) by heating, under air, at 163 °C for 3 h with magnetic stirring and further 100 °C for 24 h. The solution was then cooled to 50 °C and precipitated with water. The resulting solid was crushed, filtered, and successively washed with water (40 mL/g polymer), MeOH (20 mL/g polymer) and DCM (20 mL/g polymer) for 3 min each with magnetic stirring (3 times for each solvent). The solid obtained was then dried under vacuum.

Ph Conditioning [51] : PBI-T was pH conditioned with HCl 0.25 M (PBI-TA) or NaOH 0.1 M (PBI-TB) solutions by washing. The polymers were immersed for 3 min in 20 mL of acidic or basic solution per gram of polymer with magnetic stirring. After this, the polymers were successively washed

by magnetically stirring for 3 min in solutions of water (40 mL/g polymer), MeOH (20 mL/g polymer) and DCM (20 mL/g polymer) (3 times for each solvent) and dried under vacuum overnight.The polymers were removed from each solution by simple filtration and transferred to the next solvent.

2.1.2 Lupanine Binding Assessment

Binding assays were performed by adding to 2 mL Eppendorf tubes, 100 mg of each PBI polymer, and 1 mL of solution (1 mL of lupanine stock solution at 3g/L or lupin beans effluent regular pH or basified). The tubes were allowed to stand overnight at room temperature, under agitation (100 rpm) with magnetic stirring. Each polymer was tested in duplicate. After this, the tubes were centrifuged at 10,000 rpm for 3 min, and the supernatant was recovered and basified with KOH pellets (pH between 13 - 13.5). After this, the samples were centrifuged again, at 10,000 rpm for 3 min and the supernatant was filtered to HPLC vials and analysed for lupanine quantification. Duplicate samples of the stock solutions (pure lupanine and effluent) were also analysed by HPLC for lupanine quantification. The effluent sample was processed as previously described.

A stock solution of lupanine was prepared by dissolving 0.1 g of pure lupanine in 10 mL of Milli-Q water (10 g/L). Aliquots of the stock solution were pipetted in consecutive dilutions into 14 volumetric flasks to obtain solutions of lupanine with concentrations between 10 g/L and 0.005 g/L The lupanine calibration curve was used to obtain the lupanine concentration in the samples.

Binding Adsorption Isotherm Experiments : Binding adsorption isotherm experiments were performed at room temperature. 1 mL of basified effluent was added to different amounts of PBI-T, from 10 mg to 100 mg. The mixtures were left overnight stirring at 100 rpm under magnetic agitation. The experimental data were fitted to Langmuir (equation 3) and Freundlich (equation 4) respectively.

Binding Kinetics Experiments : Binding kinetics experiments were performed at room temperature for 100 mg of PBI-T and 1mL of basified effluent, left stirring at 100 rpm and collected after certain time intervals (2, 5, 10, 15, 60, 120, 180, 240, 360, 420, 1380, 1440 and 1620 min).

The experimental data were fitted to pseudo first- and pseudo-second-order kinetic models equation 6 and 5 respectively:

Recovery Assays : After binding experiments, it was added 1 mL of each recovery solvent to the PBI pellet (HCl 0.1M in water, HCL 0.1M in methanol, DCM, EtOH, THF and Ethyl acetate). The resuspended mixture was left at 100 rpm at room temperature for 24 h. After this time, the mixtures were centrifuged at 10,000 rpm for 3 min for PBI separation. The organic solvents were evaporated at room temperature and the volume was refilled with water and basified with KOH (pH between 13 - 13.5) before the lupanine HPLC analysis protocol.

2.2. Bitter Orange Methods

Mature biter oranges fruits were collected at IST Alameda on December 16^{th} of 2020, and were dried to constant weight on a vacuum over and grinded.

Amine Extraction [57]: 3.5 g of grinded dried bitter orange was extracted with 10 mL of water for 30 min under magnetic agitation. The samples were then centrifuged and the supernatant was filtered under vacuum. The obtained residue was extracted again using the same procedure and the supernatant was frozen and lyophilised. This was repeated 3 times. The lyophilised samples were redissolved in 20 mL of methanol and analysed in the HPLC.

Flavonoids Extraction [57] : 0.4 g of dried bitter orange was extracted with 25 mL of ethanol (80%) for 2 h at 90 ^{o}C under magnetic agitation. The samples was then filteredand the supernatant was concentrated to dryness under vacuum. The solid residue was extracted again using the same procedure for 3 times. The dried samples were redissolved in 12.5 mL of methanol and analysed in the HPLC.

Synephrine, Naringin and Hesperidin quantification and calibration curves A stock solution of each compound (synephrine, naringin and hesperidin) was prepared by dissolving 25 mg of the pure compounds in 250 mL of methanol (100 ppm). The stock solution was then used to obtain solutions of the pure compounds with concentrations between 0.5 ppm and 100 ppm. A 1.5 mL sample of each solution was then filtered (PTFE syringe filter) and transferred to HPLC vials for HPLC analysis .

2.2.1 Preliminary Binding Assesment

To test which resin was better for adsorbing synephrine and not the flavonoids, a synthetic mixture of the bitter orange juice was prepared comprising synephrine and naringin as a representative

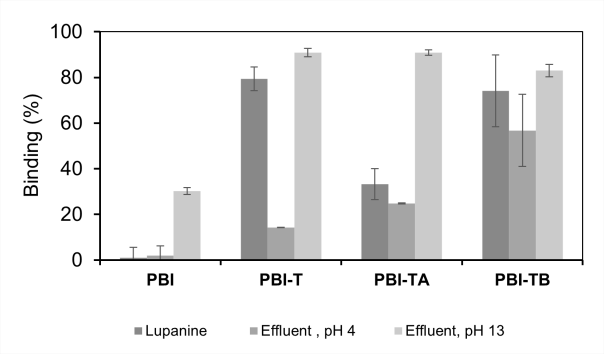

Figure 2: Lupanine binding for 100 mg of different PBI polymers in 1 mL of solution at 3 g/L (pure lupanine; effluent at pH 4 or basified with KOH at pH 13) after 17 h of magnetic agitation (100 rpm). PBI: raw material, PBI-T: PBI raw with thermal treatment, PBI-TA: PBI-T with acid treatment, PBI-TB: PBI-T with basic treatment.

flavonoid. Based on the quantification of these compounds in bitter orange extracts (synephrine and naringin) by Pellati et al. [57] and on the concentration of these compounds being much lower in the juice than in the peel [58], concentrations of 50 ppm of naringin and 40 ppm of synephrine were mixed in water and the final pH was adjusted to around 2.65 with HCl 4M, as this is the pH reported in the literature for the juice [29].

Binding assays were performed by adding to 2 mL Eppendorf tubes, 25 mg of each resin, and 1 mL of the synthetic bitter orange juice. The Eppendorf tubes were left overnight at room temperature, under agitation (100 rpm) with magnetic stirring. Each resin was tested in duplicate. After this, the tubes were centrifuged at 10,000 rpm for 3 min, and the supernatant was recovered, lyophilised, redissolved in methanol and analysed by HPLC for synephrine and naringin quantification.

Duplicate samples of the stock solutions (synthetic bitter orange juice) were also analysed by HPLC for synephrine and naringin quantification.

3. Results and Discussion

3.1. Bindings for different PBI conditioning

The adsorption of lupanine from the effluent is important not only to further valorise it in the pharmaceutical industry but also by decreasing its concentration in the effluent we can reuse its water for new extractions. We chose PBI as an adsorber because it showed a good performance when it was previously used by our group to adsorb amines and sulphates [51].

We evaluated whether the changes made by F. A. Ferreira et al. [51] thermal treatment or different pH conditioning of PBI (pka = 5.5 [49]) polymer could influence the binding of lupanine (pka = 9.1 [46]).

From Figure 2 it is clear to see that the thermal treatment and pH conditioning improved significantly, the binding of the commercial PBI for lupanine, whether in pure solution or in the effluent. Without basifying the effluent, the higher binding percentage of lupanine was around 50% (for PBI-TB), but higher binding percentages ($> 80\%$) were only obtained after basification of the effluent.

Basified effluent treated with PBI-T had the highest lupanine binding percentage of 91.18 ± 1.69, and if we take a closer look at the results obtained for the basified effluent, the pH conditioning of PBI-T slightly diminished the binding percentage of lupanine, reaching 81.95% for PBI-TA and 83.69% for PBI-TB. If further polymer processing does not improve considerably lupanine binding, it shows to not be feasible to explore these two adsorbers. For this reason, the following experiments only consider the basified effluent and PBI-T.

3.2. Binding Adsorption Isotherm Experiments

To perform the binding adsorption isotherm experiments, 1 millilitre of basified effluent was added to different amounts of PBI-T, from 10 mg to 100 mg at room temperature and left stirring for 17 h. The experimental data was then plotted according to each model's linearised equations 4 and 3 and the parameters for each model were calculated (Table 1) and used to obtain the theoretical amount of lupanine bound to the adsorber PBI-T (q values) for the experimental concentration of lupanine in solution (C_e) and the results are represented in Figure 3.

From the Figure 3, it is possible to verify that for lupanine concentrations in solution (C_e) below 1500 ppm, both models fit the experimental data, but for higher concentrations, it becomes unclear which model fit's best, so it was necessary to use another common statistical technique used in regression analysis to determine the dispersion of data points, the sum of squares (Equation 7).

For a set X of n items:

$$\text{Sum of squares } = \sum_{i=0}^{n} \left(X_i - \bar{X}\right)^2 \qquad (7)$$

The sum of squares is a measure of deviation from the mean, the distance between each data point and the line of best fit is squared and then summed up. The best fit is the linearisation with the smallest value. By comparing the calculations for the sum of squares for each model (Table 1 it is clear that the best fit for our experimental data is the Freundlich model indicating that the adsorber presents a multilayer heterogeneous binding site distribution.

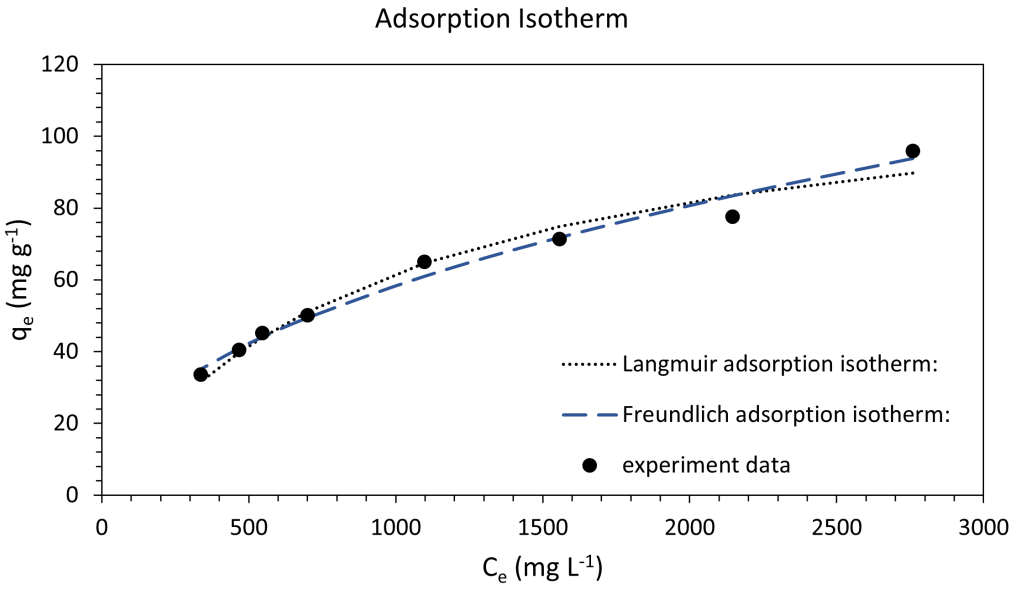

Figure 3: Binding adsorption isotherm experiments at room temperature, for 1 mL of basified effluent added to different amounts of PBI-T (10 mg - 100 mg).

Langmuir			**Freundlich**		
Intercept	0.00829	± 0.00054	**Intercept**	0.46818	± 0.02269
Slope	7.89263	± 0.78743	**Slope**	0.83210	± 0.15616
qm (mg/g)	120.69158		**n**	2.13592	
KL (L/mg)	0.00105		**Kf (L/mg)**	2.29813	
Sum of squares	372.33587		**Sum of squares**	0.88664	
R^2	0.97509		**R^2**	0.98610	

Table 1: Langmuir and Freundlich parameters obtained from the linear trend lines.

3.3. **Lupanine Binding Kinetics Experiments**

To obtain the binding kinetics experiments 1mL of basified effluent was added to 100 mg of PBI-T and left stirring at 100 rpm at room temperature, the reaction was stooped after certain time intervals and lupanine quantified, and the adsorption capacity for each time point was calculated from equation 2. The experimental data were fitted to pseudo first- and pseudo-second-order kinetic models' equations 5 and 6 respectively, only the pseudo second-order model gave a linear plot representation for the data. The physical parameters were determined andu sed to calculate the theoretical amount of lupanine bound to PBI-T (q_t values) for each time (t) and the results are represented in Figure 4.

Although we could obtain a good linearization of the data using the pseudo-second-order kinetics model with a good R^2 (Table 2, we could observe some dispersion of the experimental data that was noticeable by the value of the sum of squares (equation 7) presented in Table 2, this was caused by the lack of experimental data for a long period (overnight) the sum of squares is much lower if we take only in consideration the data obtained in the first 420 minutes (with a value of 290.5).

Pseudo-second order kinetics		
Intercept	0.6037	± 0.3924
Slope	0.0301	± 0.0005
qf (mg/g)	33.171	
K2 (g/ (mg·min)	0.0015	
R^2	0.9954	
Sum of squares	4144.1	

Table 2: Parameters obtained for the pseudo-second order kinetics

3.4. **Recovery Assays**

In the binding assays, PBI-T presented the highest binding percentages of lupanine, but another important aspect to consider is the recovery of lupanine from the adsorber for further valorisation. So, the first recovery assays were performed not only for PBI-T but also for PBI-TA and PBI-TB using six different solutions: HCl 0.1M in water, HCL 0.1M in MeOH, DCM, EtOH, THF and EtOAc.

The use of HCl solution aims to explore ionic in-

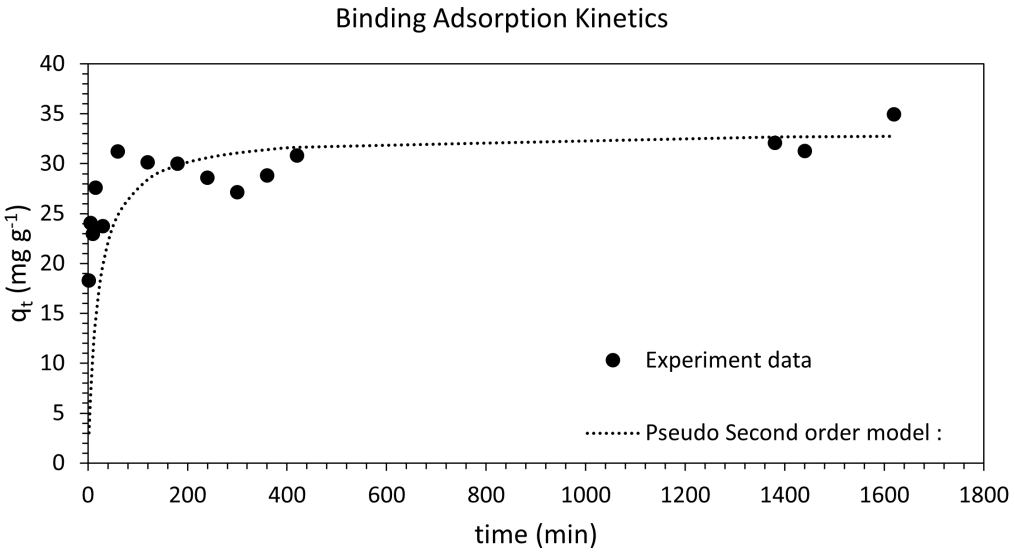

Figure 4: Binding kinetics for the basified effluent and PBI-T and theoretical representation of the pseudo-second-order model obtained from the parameters calculated for the experimental data.

teractions, the use of alcohols such as MeOH and EtOH (polar protic solvents) aims to break down hydrogen bonds that form during binding and explore the use of weaker interactions (hydrophobic or dipole-dipole), so the combination of MeOH and HCl (HCL 0.1M in MeOH) combine these characteristics. Also, some moderately polar aprotic solvents (DCM, THF and EtOAc) were also choosen to study if they were able to disrupt lupanine/PBI interactions.

From Figure 5 we observe that the highest recovery (equal or higher than 80 %) for PBI-T was obtained when THF (also the solvent with the highest recovery percentage). For PBI-TB ethanol and THF gave the highest recoveries (around 80 %) and, for PBI-TA it was DCM (79 %).These solvents were able to disrupt the weak interactions between lupanine and PBI releasing lupanine into the medium.

Considering these results, and the finding that PBI-T showed the best lupanine binding performance, from an economical point of view, the best strategy to isolate lupanine would be to use PBI-T for the binding experiment and THF for the recovery step. Furthermore, for PBI-TA and PBI-TB there is also the need of spending more solvents and time in the processing of the polymer for pH conditioning.

The worst recovery solvents were ethyl acetate and HCl 0.1M in water. With the last one being the only aqueous solution assessed and it had a low concentration of acid. An aqueous solvent based recovery would be the ideal for the recovery step because it would be more environmentally friendly.

So, after these initial results and preliminary findings, other aqueous solutions were tested for PBI-T at room temperature and also at 50^{o}C. We observed that applying temperature did not improve lupanine recovery and increasing the concentration of HCL from 0.1 M (Figure 5) to 1 M increased the recovery from 5.37 % to 42.38 %. Although not reaching ideal recovery percentages, this result is promising, and in the future, it could be tested in consecutive recovery steps to obtain higher recovery percentages.

Since EtOH also presented promising results with 73.40 % recovery for PBI-T , other recovery solvents (butanol, isopropanol and MTBE) were also tested for PBI-T. The results showed that butanol and isopropanol could recover 98.43% 72.46 % of lupanine respectively, contrary to MTBE that resulted in a low recovery percentage of only around 10 %.

These results demonstrated that PBI-T could be used to bind and recover Lupanine, without the need to perform a pH conditioning (PBI-TA and PBI-TB) and that several solvents could be chosen to recover lupanine, for higher recovery percentages THF would be the best choice, but a solvent as ethanol would be more economic and environmentally friendly, although THF has a lower bowling point and it's easier to evaporate decreasing evaporation time (as well as methanol). It's important to refer that unfortunately although each experiment had a duplicate, when the experiments were repeated the recovery results were not reproducible giving lower lupanine recovery percentages.

3.5. Bitter Orange Preliminary Study

Amine Extraction For quantification of synephrine present in bitter oranges collected in

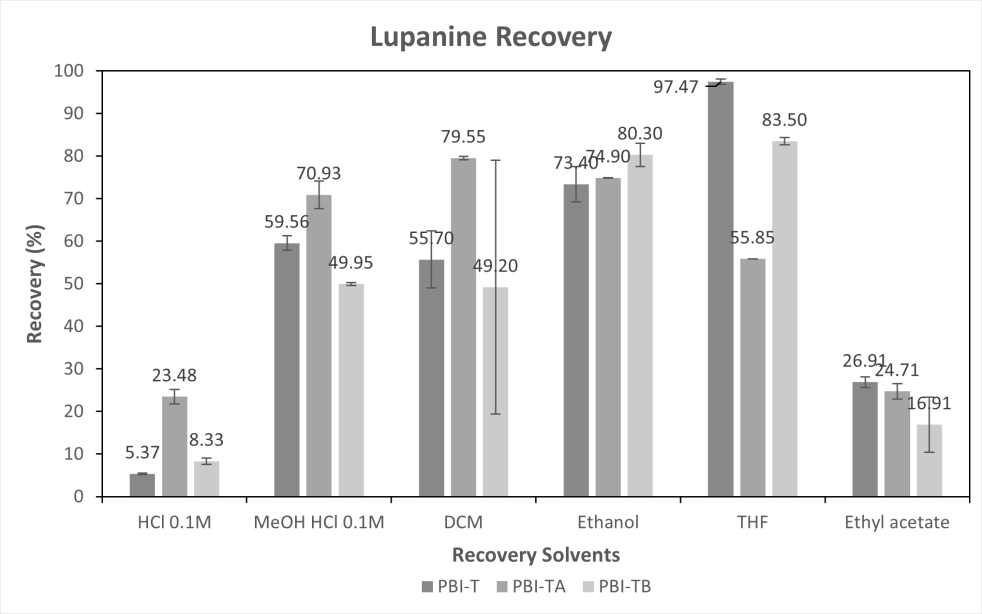

Figure 5: Lupanine recovery for the different PBI polymers using several washing solutions. PBI-T: PBI raw polymer with thermal treatment, PBI-TA: PBI-T with acid treatment, PBI-TB: PBI-T with basic treatment.

# Extraction	Synephrine (ppm)
1	353
2	122
3	18
Total	493

Table 3: Concentration of synephrine in bitter orange.

IST campus, dried bitter oranges were extracted three times with water, the extract was filtered and lyophilised, and then, it was redissolved in methanol and analysed in the HPLC. The peak of synephrine had a retention time of 3.73 min (this peak was identified using an internal standard) corresponding to a total concentration of 493 ppm, over the 3 extractions (Table 3). These results are in accordance with the values obtained in the literature [57].

Flavonoids Extraction : For flavonoid assessment in bitter oranges collected in IST campus, dried bitter oranges were extracted twice with ethanol. The extracts were filtered and lyophilized, redissolved in methanol and analysed in the HPLC. Nnaringin peak had a retention time of 21.57 min and a total concentration of 345 ppm, over the two extractions (Table 4). This result are in accordance with the values obtained in the literature [57]. In this sample, hesperidin was not detected .As naringin concentration in bitter orange increases when the fruit matures, and hesperidin diminishes

#Extraction	Naringin (ppm)
1	291
2	54
Total	345

Table 4: Concentration of naringin in bitter orange

[59], it is natural that we weren't able to detect hesperidin in the extraction samples.

Synephrine and Naringin Preliminary Binding Pellati et. al [57] report synephrine and naringin to be present in mature bitter orange in the range of 500 ppm. There is evidence that the concentration of this compounds are lower in the juice than in the peel [58]. So Based on these values we estimated the concentration of this compounds and prepared a solution at 40 pm and 50 ppm of synephrine and naringin, respectively in water and adjusted the pH of the solution to 2.65 [29], to simulate a synthetic biter orange juice to perform adsorption studies. In these studies, several resins were assessed of different nature.

25 mg of each resin were mixed with 1 mL of the synthetic juice and left overnight at room temperature, under magnetic agitation (100 rpm). Then, the samples were centrifuged and the supernatant was lyophilised and redissolved in methanol for HPLC analysis.

From Figure 6 we observe that, for anion exchange resins (IRA-68 and IRA-458) there was some interference in the quantification of synephrine.

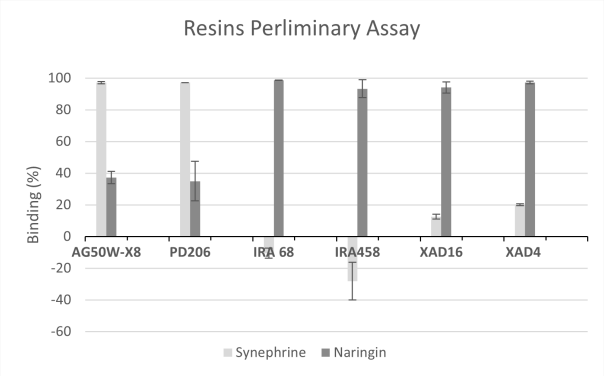

Figure 6: Bitter Orange Binding Resins perliminary assay

This can be due to the lack of resin pre-treatment before the binding. For naringin, these resins presented high binding values around 95 %. This same trend was present by the polymeric adsorbent resins (XAD-16 and XAD-4) with naringin binding around 95 %. For these resins, a small amount of synephrine, around 18 %, was also adsorbed. Polymeric adsorbents interact with organic compounds due to the presence of the aromatic rings, as naringin as more aromatic rings than synephrine it had more affinity to these resins. These resins may be useful if we envisage to isolate the flavonoids for further processing, selling or addition to other food formulations for antioxidant properties enhancement, for example.

The cation resins (AG50W-X8 and Purolite PD206) presented the highest bindings for synephrine around 97 %, showing to be ideal adsorbers for synephrine removal from the juice. However, they also adsorbed around 35 % of naringin. These results are in accordance with the theoretical hypothesis made in the Introduction, where synephrine, having a pKa of 9.76, is protonated in the juice acidic solution, as is present as an ion with a positive charge. Therefore, it was expected that the best resins for the isolation of this compound would be the acid cation exchanger resins.

4. Conclusions

The thermal treatment and pH conditionings used on commercial PBI improved the binding percentage of Lupanine. PBI-T had the highest binding percentage for Lupanine pure solution and the basified effluent. PBI-TB had the highest binding percentage for the pure effluent.

The binding kinetics showed that PBI-T binding follows the pseudo-second-order kinetics with a maximum adsorption capacity of 33 mg of Lupanine per g of PBI-T this value corresponds to the amount of lupanine present in the 1ml effluent added to PBI-T, so in the future, it should be tested if this value of maximum adsorption capacity is the same for higher concentrations of lupanine in the effluent. The Binding Adsorption Isotherm for PBI-T showed that PBI-T follows the Freundlich adsorption model(n = 2.14 and K_f = 2.30).

But further testing showed that although PBI-T was always effective in binding lupanine from every effluent (average binding percentage of 90 %) the recovery results were not reproducible.

The extractions of dried bitter Oranges with water (amine extraction) and ethanol (flavonoids extraction) got a concentration of 493 ppm of synephrine and 345 ppm of naringin, there wasn't detected any amount of hesperidin. A preliminary binding assay was performed with a synthetic mixture of bitter orange juice (40 ppm of synephrine; 50 ppm naringin pH 2.65) mixed with 25 mg of resin (2 acidic resins, 2 basic resins and 2 polymeric adsorbers), from which the acidic resins were the best to bind synephrine while keeping most of the healthy flavonoid (naringin) in solution.

Ideally, after some more optimizations and scale-up tests, it will be possible to remove these alkaloids from their food products and get a source of healthy food products that came from agricultural plantations that are resistant to drought and climate alterations that could help produce food in harsh conditions.

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