Isolation of organic compounds with high added values from agro-industrial solid wastes

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Abstract

Phenols are organic compounds with high antioxidant activity. Occurring mainly in plants, where they act as pigments or even as part of defense mechanisms against insects and herbivores. Given the positive impact on health human, their isolation and purification from agricultural products is of particular interest for the production of nutritional, pharmaceutical and cosmetics supplements. In our study different materials rich in phenolic compounds were used, in order to separate the phenolic content and maximum condensation using physicochemical methods such as solvent extraction, filtration through membranes, adsorption/desorption on resins and vacuum distillation. The materials tested were solid wastes from winery, cocoa residuals, olive leaves, etc. The first step for the treatment was the extraction of phenolic content using water-ethanol solutions which was initially optimized. Then, sequential membrane filtration of the extracts by Ultrafiltration membranes, Nanofiltration and Reverse Osmosis was performed to separate the contained compounds, based on their molecular weight. To remove non-polar compounds, with similar molecular weights with phenols, methods of adsorption/desorption on specific resins were developed, in order final ethanolic solutions rich in phenolic compounds to be obtained. Finally, the ethanol was removed by vacuum evaporation at low temperatures. The purification of olive leaf phenols is illustrated in details in the present work. The final obtained concentrate, was a rich phenolic concentrate contained 98 g/L phenols in gallic acid equivalents. This technique, after modification, can be applied to a variety of phenol-rich byproducts, allowing the operation of phenol separation plant adjustable to local agricultural activities.

Keywords: membrane filtration, adsorption/desorption, phenols isolation, separation and purification

1. Introduction

Olive tree and vineyard cultivations have a long history in the Mediterranean countries, and even today consist an important cultural, economic and environmental aspect of the area. Together with the precious products (extra virgin olive oil and wine) large amounts of agricultural byproducts are co- produced every year, causing significant environmental problems. The problem from the uncontrolled disposal of byproducts to the environment can be reduced by the exploitation and purification of their phenolic content.

Phenolic compounds are important phytochemicals, abundant in nature. They are present in most plants in great variety, and may differ not only from plant to plant, but also with season, maturity and region. Their role may be to act as pigments, or even a defense mechanism for plants [1]. Phenols are characterized as antioxidants, with beneficial health effects for humans [2]; as a result their isolation for the production of high-added value products is of great interest. In our studies, different plant materials or byproducts, rich in phenolic compounds were examined for the purification of their phenolic content, through solid-liquid extraction, membrane filtration and resin adsorption/desorption. In the present work, the study was focused on the extraction of oleuropein from olive leaves which has been named as an excellent phenolic compound with antimicrobial and antioxidant activities [3-6]. Generally, phenols were identified for their antioxidant, antimicrobial, anti-inflammatory, anti-HIV, antiviral, antitumor, hypotensive, hypoglycemic, antiallergenic, cardio-protective, anti-thrombotic, vaso-dilatatory and hepatoprotective activity [1-6].

Solid-liquid extraction can be utilized for the treatment of solid byproducts, rich in phenols, in order to move the targeted compounds from the solid matrix to a solution. Phenolic compound extraction from a solid matrix is commonly achieved through solvent extraction with different techniques [7-15]. This extraction can be further enhanced with the use of ultrasounds or microwaves, increasing its efficiency [11, 12]. The most common solvents used are water, methanol, ethanol, ethyl acetate and other organic solvents, pure or in mixtures water-ethanol [3, 4], or water –methanol [6, 13] or just pure water [3, 14]. Water-ethanol mixtures are preferred in the food industry, as they do not affect the human health. Important parameters of the extraction are the composition of the solvent, temperature, solid/solvent ratio and duration, use of microwaves or ultrasound[3, 11]. Another extraction technique involves supercritical fluids, like supercritical CO_2.

Membrane filtration can then separate phenolic compounds according to their molecular weight. The most important, size exclusion, membrane applications are microfiltration (MF),

ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). The advantages of membrane application include: low energy consumption compared to other separation methods that involve phase change, high selectivity and low temperatures (crucial when handling thermally unstable compounds like phenols). Membrane filtration was already tested and found useful for the isolation and purification of phenols from olive mill wastewaters [16-18], and other agricultural byproducts such as grape marc pomace [19] or other fruit byproducts (bergamot juice, kiwifruit juice, citrus and carrot juices) [20-22]. Membrane filtration of this type of extracts (olive leaves) is not well documented in literature, but in the UF step, removal of suspended solids is expected, while in the NF step the phenolic content of the extract should be retained, as oleuropein has higher molecular weight (541 g/mol) than the MWCO of the membrane used (470 g/mol).

Further treatment of the phenol enriched concentrate can be carried out through resin adsorption/desorption of polar compounds like phenols. In the experiments presented by Li et al. [23], the adsorption/ desorption of olive leaf extracts on synthetic macroporous resins, resulted in 13-fold increase of the total flavonoids concentration while, according to the results of Bayçın, Altıok, Ülkü and Bayraktar [24], silk fibroin was successfully used as adsorbent for the separation of oleuropein. As shown in our previous work [16, 19], the use of resins can separate the polar phenols from the non polar carbohydrates of the plant material. Carbohydrates are present in most plant extracts, hindering the concentration of phenolic compounds. As they have similar molecular weights to phenols, they cannot be separated with membrane filtration, but this is possible with the use of adsorption resins. Finally, after the removal of carbohydrates, the purified phenolic compounds can be further concentrated through vacuum evaporation for increased concentrations of phenolic compounds.

In the present work a detailed experimental investigation was performed for the extraction, isolation and purification of phenols in olive leaves using a combination of solid-liquid extraction, membrane filtration and resin adsorption/desorption, for the production of phenolic concentrates. The final products of the proposed process contain a large percentage of the byproducts' phenolic content, in a small fraction of the initial volume.

2. Materials and Methods

2.1. Olive leaves

The olive leaves used in this study originated from "Koroneiki" olive tree variety, and were collected in December 2014, from the region of Ilia, Greece. The sample was refrigerated at -25 °C until used, for the preservation of its phenolic content. Prior to every experiment, the samples were defrosted and ground.

2.2. Analytical techniques

Phenols (Ph) were measured with the Folin-Ciocalteu method [25], using gallic acid as standard at 760 nm, and carbohydrates (Ch) were measured with L-tryptophan reagent and glucose as standard at 525 nm [26].

2.3. Extraction

The extraction experiments were carried out in a jar test apparatus and more specifically for this purpose the Flocculator "FLOC-6", supplied by RAYPA, was used. Six containers of 600 mL in volume were employed and the extraction took place under mechanical stirring with 100 rpm. After the extraction, the extract was separated from the solid matrix with vacuum filtration through Whatman® glass microfiber filters with 1.6 μm pore diameter.

2.4. Membrane modules

The membranes used were of semi pilot scale in cross flow mode (flowrates up to 100 L/hr). The UF module was ceramic, Zirconia, with 0.24 m^2 filtration area and 100 nm pore size. The NF module was polymeric, with 2.4 m^2 filtration area and 470 g/mol MWCO determined experimentally in the lab through filtration of PEG solutions with different MW. Both membranes were supplied by HAR SpA, Milan, Italy (more information in authors' previous work [27, 28]).

2.5. Resins

The resins used were supplied by Sigma-Aldrich. Three resins were tested, Amberlite XAD4, XAD16N and XAD7HP which have been reported to yield good adsorption results for phenols. Prior to every experiment, the resin was pretreated as follows: firstly it was eluded in acetone for 8h under magnetic stirring and then dried at room temperature. This step ensured the removal of any monomers trapped in the resin matrix and removal of pore blockage. After drying, the exact weight of the resin used could be measured after moisture removal. The resin was then rinsed with 5 mL of ethanol per g of resin and finally rinsed three times with triple distilled water before further use [16, 19].

3. Results and Discussion

3.1 Extraction

The first step for the purification of olive leaf phenols was their extraction. For the optimization of the extraction process three parameters were examined: solvent composition, solid/solvent ratio, and duration. Even though temperature is an important extraction parameter, it was not examined, because of the heat sensitivity of the phenolic compounds. Moreover, in the results presented by Tsakona, Galanakis and Gekas [5], it is reported that the deference in the concentration of phenolic compounds extracted from olive leaves at 25 °C and 40 °C is only 10%.

Water-ethanol mixtures were chosen as solvent, as they have exhibited good extraction results in the literature [4, 5], and they are not harmful to human health or to the environment. In contrast to what it has been reported in literature, the ethanol percentage in the solvent did not significantly alter the extracted phenols, but had a more intense effect in the extraction of carbohydrates. More specifically, the increase of ethanol percentage lowered the amount of carbohydrates extracted, while at 99% ethanol, chlorophyll which is insoluble to water was extracted, as implied by the green color of the extract (Fig. 1). This difference to the results reported in literature may be attributed to the fact that fresh leaves were used in this study, while in the majority of the published papers regarding extraction of oleuropein from olive leaves, dried leaves are used. The drying of leaves is mostly used as preservation technique (although it also has other effects that will be discussed later on) but since the leaves used in this study were refrigerated at -25 °C, drying was not implemented.

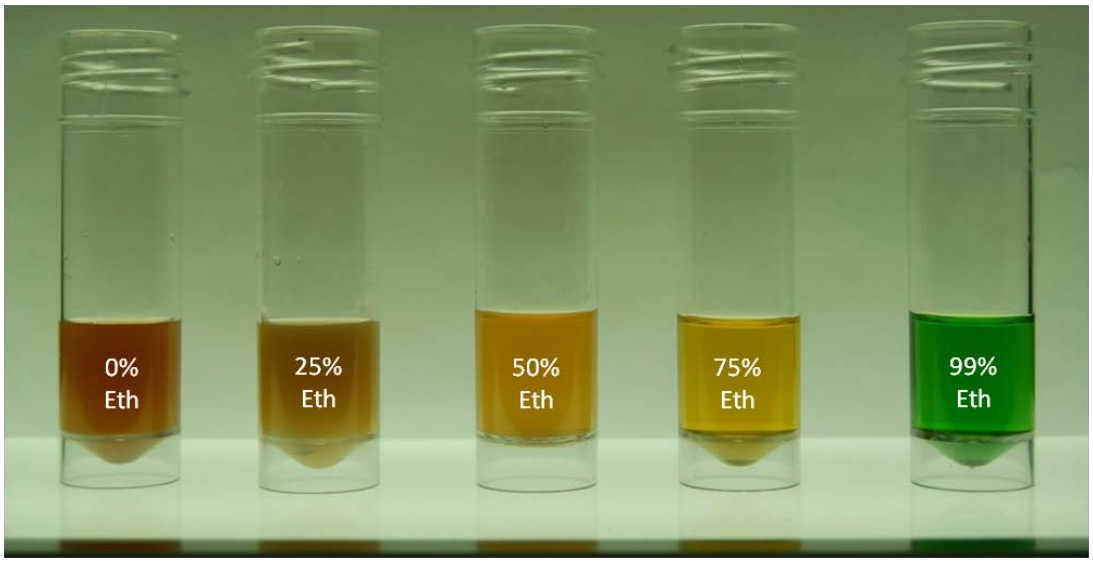


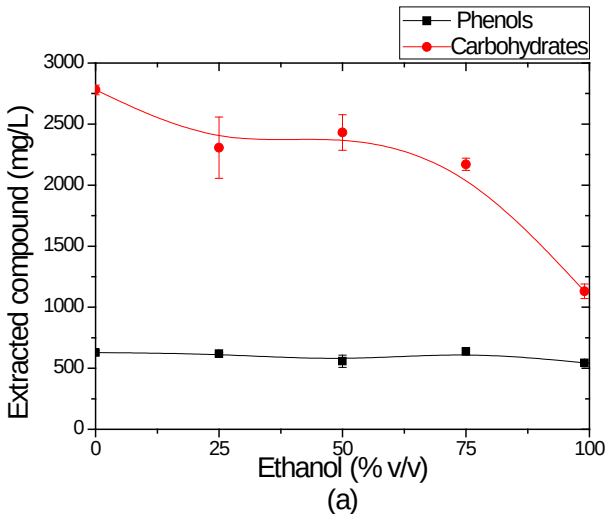
Fig. 1: Olive leaf extracts, with different hydro-ethanolic mixtures.

The results of the extraction process optimization are presented in Fig. 2.a-c.

As the extracted carbohydrates were to be separated in the resin process, the use of ethanol was deemed unnecessary (Fig. 2.a). Moreover, the presence of ethanol would be problematic for the membrane step, prior to which it would have had to be removed. For the rest of the extraction experiments, pure water was used as solvent.

The amount of phenols extracted appeared to be enhanced when the solid/solvent ratio was increased, without reaching a plateau (Fig. 2.b), but the maximum solid/solvent ratio that could be examined was 250g/L, as further increase of the solids would lead to improper submersion of the solid material in the solvent. As a result, 250 g/L was the chosen solid/solvent ratio that was used for the rest of the extraction experiments.

The extraction duration proved to be longer, compared to other materials examined, with the rate of extraction decreasing significantly only after 120 minutes (Fig. 2.c). This may be attributed to the larger particle size of the sample, compared to the other materials examined in our previous work [16, 19], as, because of the low density of the leaves, their grinding proved to be problematic, with the resulting material having a size of around 1cm x 1cm.



(a)

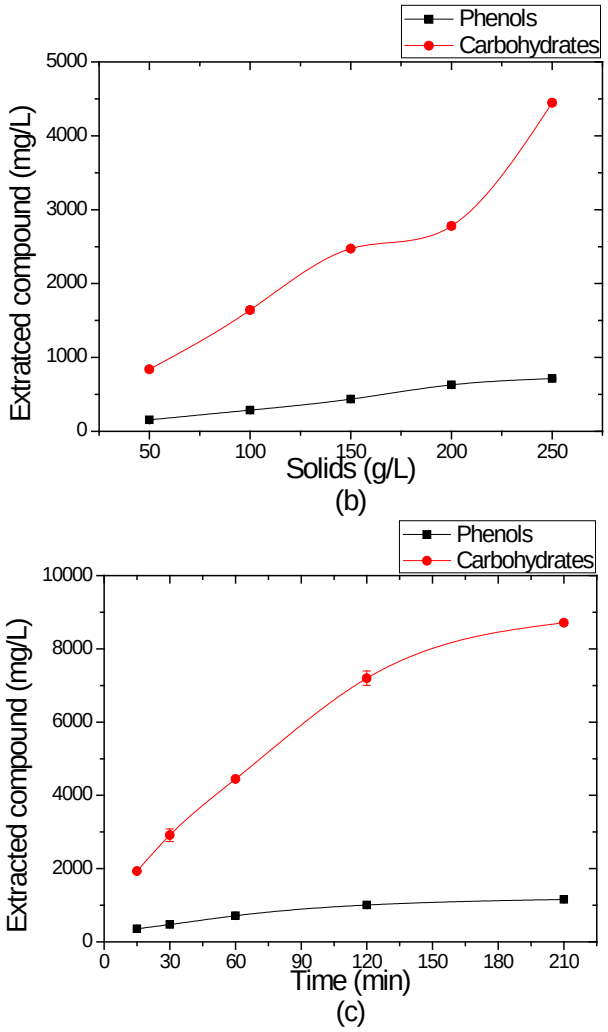


Fig. 2: Optimization of extraction parameters. (a) Variable Ethanol %, constants: 200 g/L solid/solvent, duration 1h, (b) Variable g/L of solid/solvent, constants: 100% water, duration 1h (c) Variable duration, constants: 100% water, 250 g/L solid/solvent.

The optimum extraction process, as identified by the three experimental series described in Figs. 2.a-c, is extraction with 100% water, 250 g of olive leaves per L of solvent, with 120 min duration. The extracted phenols with the optimum conditions were around 400 mg/100 g of olive leaves, compared to 1200 -1700 mg/100 g of olive leaves reported in literature [29]. This difference may be attributed to the initial samples, or sample pretreatment, like insufficient grinding.

After the determination of the optimum extraction conditions, extraction was carried out with a large amount of olive leaves for the production of the extract that would be treated in the pilot scale membrane process. More specifically, 20 kg of olive leaves were extracted, with 80 L of water.

3.2. Membrane filtration

Prior to membrane filtration, the extract was sieved through stainless steel sieves with final pore diameter 0.125 mm. This step was necessary for the removal of the solid matrix and the protection of the membrane modules that are sensitive to high suspended particles concentration. The removal of the suspended solids larger than 0.125 mm resulted in the reduction of the extract volume by 5L.

Fig. 3 illustrates the membrane procedure employed, alongside the volume balances in each step, which consisted of inline UF and NF. The samples obtained in each step are illustrated in Fig. 4.

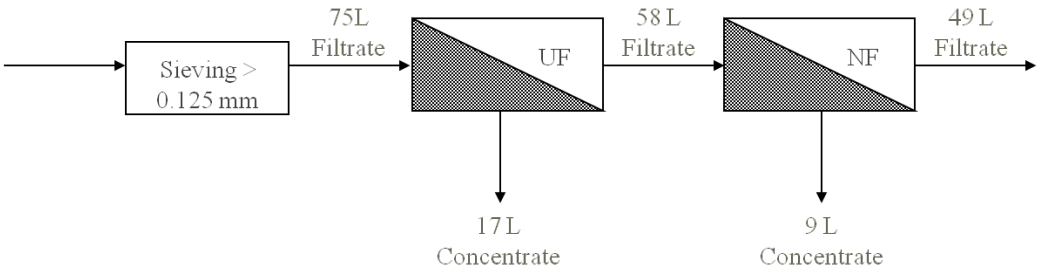


Fig. 3: Membrane process applied for the separation of olive leaf extract phenols.

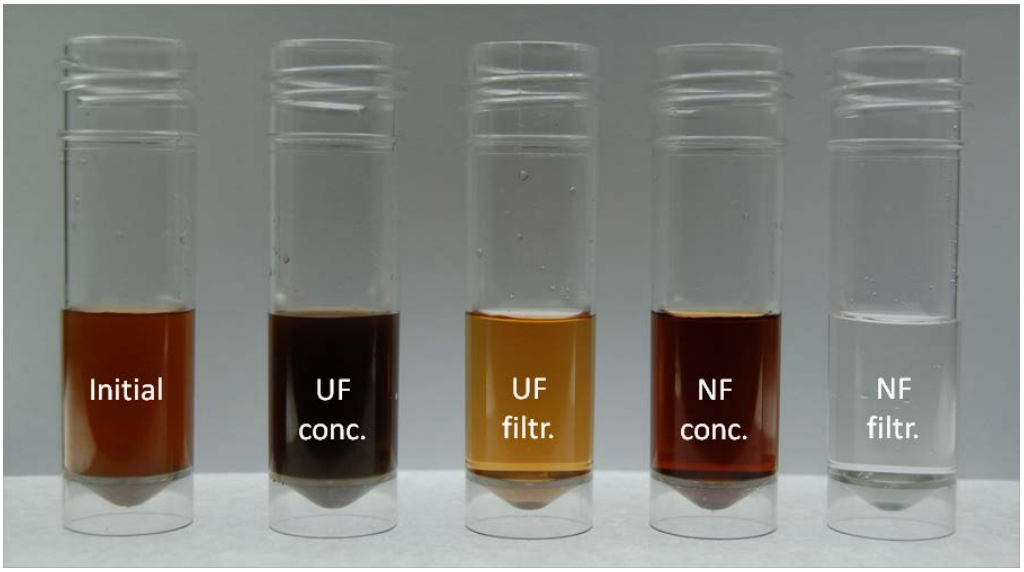


Fig. 4: Fractions of olive leaf extract obtained through membrane filtration.

In the UF step, phenols and carbohydrates appeared to be partly rejected (Table 1). The compounds rejected at this step probably correspond to the ones contained in the smaller solid particles (<0.125 mm) that are fully rejected by the UF membrane with 100 nm pore size. The dissolved compounds contained in the UF filtrate were then rejected by the NF

membrane at a high percentage leading to their concentration. Finally, the NF filtrate is considered mostly free from phenolic compounds and usable for membrane cleaning.

Table 1: Concentration of olive leaf extract total carbohydrates and total phenols in all the steps of membrane separation.

	Initial	**UF conc.**	**UF filtr.**	**NF conc.**	**NF filtr.**
Volume L	75	17	58	9	49
Total Ph mg/L	468 ±15	774 ±3	325 ±7	988 ±25	88 ±1
Total Ch mg/L	2801 ±30	3458 ±27	2140 ±179	5410 ±37	1249 ±24

As expected, flux drop phenomena were observed, with the occurring flux being at acceptable levels (Table 2). In both UF and NF steps, flux drop was mostly reversible after a cleaning cycle.

Table 2: Flux and fouling results of the membrane filtration of olive leaf extract.

		Flux L/(h*m^2)					
	TMP bar	**Water**	**Sample**	**Water after cleaning**	**% Flux drop**	**% Reversible flux drop**	**% Irreversible flux drop**
UF	1	295	237	290	20	98	2
NF	10	35	20	29	33	83	17

The resulting NF concentrate was free of suspended solids and with increased concentration of phenolic compounds.

3.3. Resin Batch experiments

For the further purification of the phenols contained in the NF concentrate, resin adsorption/desorption was used. For the assessment of the resin adsorption efficiency, batch adsorption experiments were carried out, where different amounts of resins were added to a fixed volume of NF concentrate. More specifically, resin was added to 100 mL of NF concentrate and the sample was mechanically stirred at 100 rpm for 1h. Then, the resin was separated from the sample, and the not-adsorbed part was measured.

The results presented in Fig. 5 illustrate the adsorption of phenols and carbohydrates.

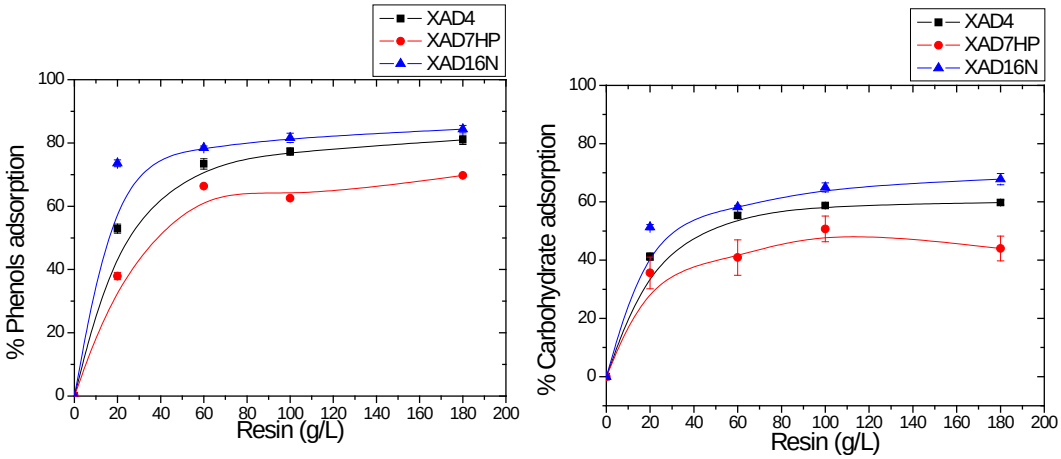


Fig. 5: Adsorption of olive leaf extract phenols and carbohydrates on different resins.

All three resins appeared to uptake the phenols contained in the NF concentrate to an acceptable extend, but, on the other hand, significant adsorption of carbohydrates took place as well. The high adsorption percentage of carbohydrates (along with some results that will be discussed later), indicate the presence of complex compounds, like phenol glycosides, which can be detected as phenols and carbohydrates. The compound that fits this description and is detected in olive leaf extracts at large amounts is oleuropein-glycoside. Here, it must be noted that in many studies, oleuropein-glycoside is simply reported as oleuropein, while the glucose free compound referred to as oleuropein-aglycon. The oleuropein-aglycon molecule consists of a hydroxytyrosol molecule and an elenoic acid molecule, with the glycoside having an extra glucose molecule (Fig. 6). Oleuropein-glycoside has lower antioxidant power than oleuropein-aglycon. In plant tissue, oleuropein is kept in its dormant form, away from enzymes like β-glycosidase, but when the plant structure is damaged, β-glycosidase converts oleuropein-glycoside to oleuropein-aglycon by hydrolyzing the bond with the glucose molecule, as a defense mechanism against herbivores [29]. Several researchers have used microorganism or β-glycosidase to convert oleuropein-glycoside to oleuropein-aglycon, and even produce hydroxytyrosol with pH adjustment or the use of esterase in order to hydrolyze the hydroxytyrosol-elenoic acid bond [30-32]. Alternatively, drying of leaves can increase their oleuropein-aglycon concentration [33]. This kind of pretreatment may lead to better separation results and recovery of higher antioxidant activity and added value compounds.

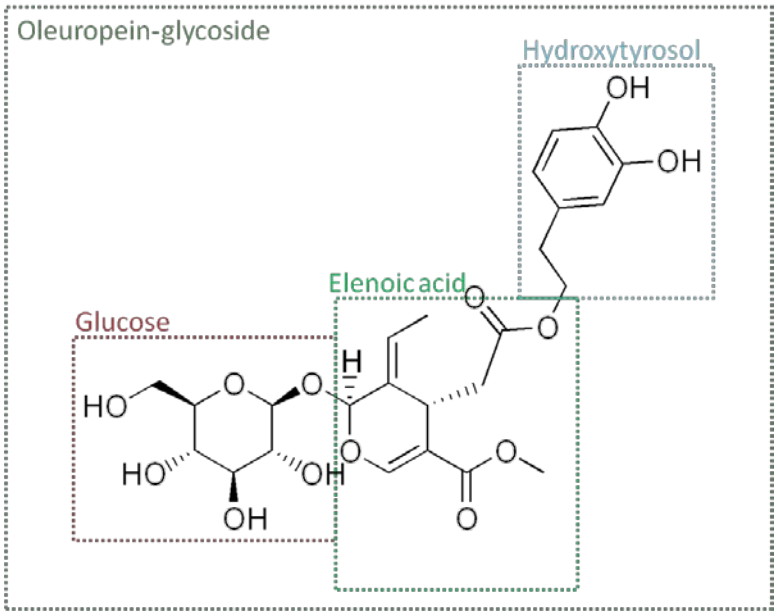


Fig. 6: Oleuropein-glycoside molecule.

For better comparison with other studies, the phenol adsorption results presented in Fig. 5 can be transformed to q_e vs C_e diagrams (Fig. 7), where q_e is the adsorption density (mg of adsorbed solute/g of adsorbate) and C_e the concentration of the not adsorbed solute (mg/L). Here it must be noted, that the experiments were conducted at ambient temperature.

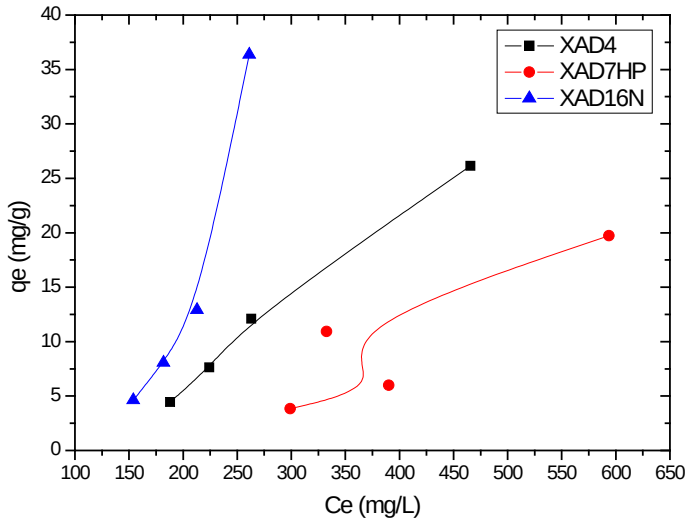


Fig. 7: Adsorption isotherms, occurring from the batch adsorption of olive leaf extract NF concentrate.

As observed in Fig. 7, apart from XAD7HP, the resins exhibited a mostly linear behavior. The irregularity of the XAD7HP results may be attributed to its structural instability that led to fragmentation of its particles during the acetone elution step of its pretreatment. As shown in author's previous work [16, 19] where the resins exhibited similar behavior, the linearity

of the q_e-C_e relation may be attributed to high maximum adsorption density (q_{max}) of the resin, much higher than the q_e examined.

Langmuir and Freundlich models were also and the results are presented in Table 3.

Linear: $$q_e = K_{Lin} C_e \quad (1)$$

Langmuir: $$q_e = \frac{q_{\max} K_{Lang} C_e}{1 + K_{Lang} C_e} \quad (2)$$

Freundlich: $$q_e = K_F C_e^{1/n} \quad (3)$$

Table 3: Comparison of Linear, Langmuir and Freundlich models, for the results of batch adsorption of olive leaf extract NF concentrate phenols.

	Linear		Langmuir			Freundlich		
	R^2	K_{Lin}	R^2	q_{max}	K_{Lang}	R^2	K_F	n
XAD4	0.98875	0.07687	n.d.	n.d.	n.d.	0.92876	$3.0\ 10^{-4}$	0.536
XAD7HP	0.68177	0.0475	n.d.	n.d.	n.d.	0.4936	$8.4\ 10^{-5}$	0.518
XAD16N	0.85923	0.29695	n.d.	n.d.	n.d.	0.97595	$1.6\ 10^{-8}$	0.2598

The linear model best described the XAD4 behavior, while XAD7HP could not be fitted properly because of the irregularity of the results. Even though Freundlich model appears to better describe the behavior of XAD16N resin, the occurring *n* is not in the typical range of 1-5 [34]. XAD16N was chosen as the most appropriate resin for the adsorption of olive leaf phenols.

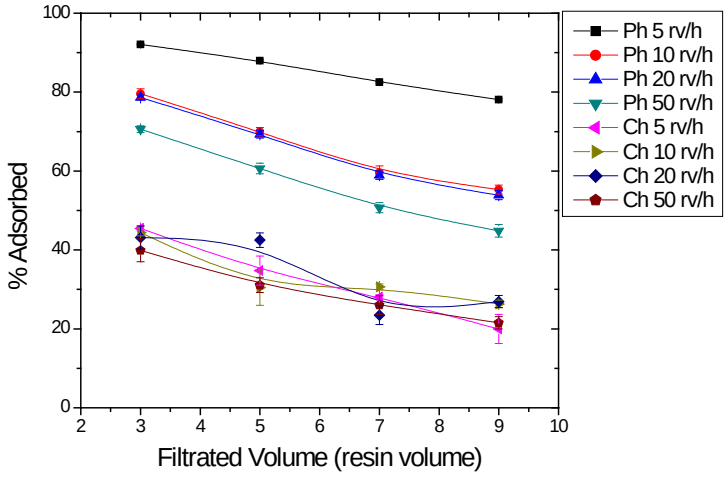
3.4. Resin kinetic experiments

After the selection of the most appropriate resin, columns were packed with a fixed volume of resin and kinetic experiments for the adsorption and desorption of phenols and carbohydrates took place. The results are presented as a function of the filtrated volume, which corresponds to time through the constant filtration rates. The target of the kinetic experiments was the optimization of the filtration rate and the total volume of sample that could be treated prior to resin saturation. The volume is expressed in resin volumes (rv) that correspond to the dry volume of the resin in the column, for easier scale up. In the experiments presented, rv was 5 mL.

The first step was the optimization of the adsorption step. Sample was filtered through the packed column with different filtration rates and the effluent was analyzed. Fig. 8 illustrates the effect of different filtration rates in adsorption (Fig. 8a) and desorption steps with water (Fig. 8b) and ethanol (Fig. 8c). The best adsorption behavior was observed with 5 rv/h filtration rate, while, after the filtration of 6 rv, the adsorption efficiency dropped below 85%.

As mentioned above, the use of different solvents in the desorption step facilitated the separation of phenols from carbohydrates. More specifically, water seems to selectively desorb the adsorbed carbohydrates while ethanol desorbs the targeted phenols [16, 19]. After the adsorption under the conditions proposed above, water was filtrated through the column at 10 rv/h and the occurring results are presented in Fig. 8.b. Desorption percentages were calculated based on the amount of phenols and carbohydrates that were adsorbed in the first step and remained in the column.

With water, around 35% of the adsorbed carbohydrates, were desorbed, with phenolic compound desorption being below 7%. On the other hand, during the filtration of ethanol (Fig. 8.c), the phenols and carbohydrates that remained in the column from the previous steps are desorbed by the same percentage (80%). This comes in contradiction to the results that occurred for the rest of the examined materials in the previous works [16, 19], where ethanol selectively desorbed phenols. This behavior may be explained once more, by the presence of complex compounds, and more importantly oleuropein-glycoside, that are part carbohydrates part phenols.



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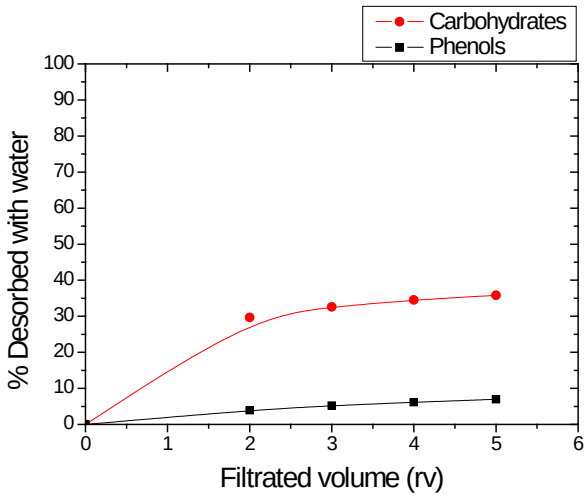

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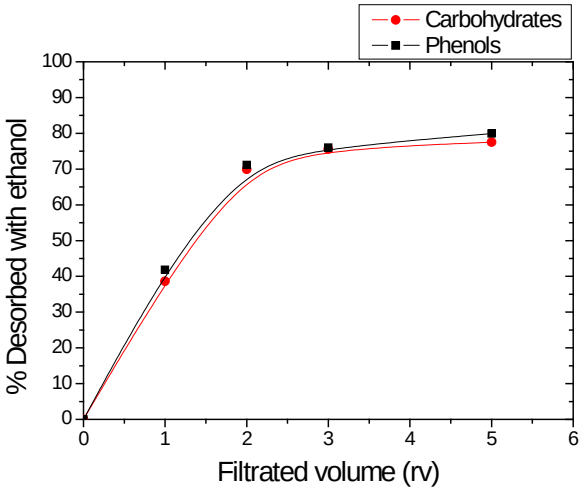

(c)

Fig. 8: Adsorption and desorption of olive leaf extract NF_c phenols and carbohydrates in kinetic experiments (rv: resin volume).

The three steps of the resin process and the overall separation efficiency of phenols from carbohydrates are summarized in Fig. 9. The most important results are, firstly that the final product contains 65% of the initial phenols and only 23% of the initial carbohydrates. Secondly, the volume of the ethanolic solution is only 3 rv, compared to 6 rv of the initial sample and, finally, the solvent was changed from water to ethanol, that can be evaporated at lower temperatures with lower energy consumption. An important problem that occurred was the retention of significant amount of phenols by the resin, even after the filtration of ethanol. This may indicate that a different solvent, or procedure, should be used for the regeneration of the resin prior to another adsorption/desorption cycle. The overall efficiency of the proposed resin process is presented in Fig. 10.

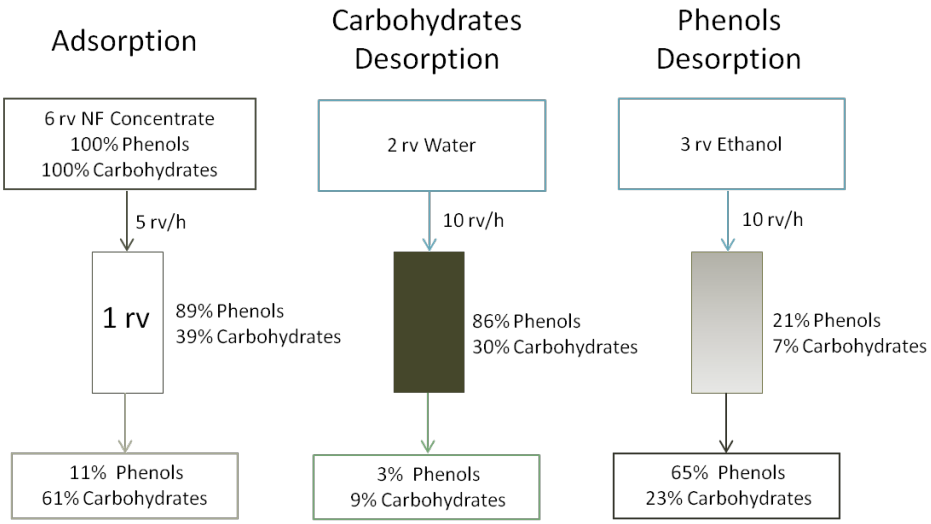

Fig. 9: Proposed resin process for the separation of olive leaf extract NF_c phenols from carbohydrates.

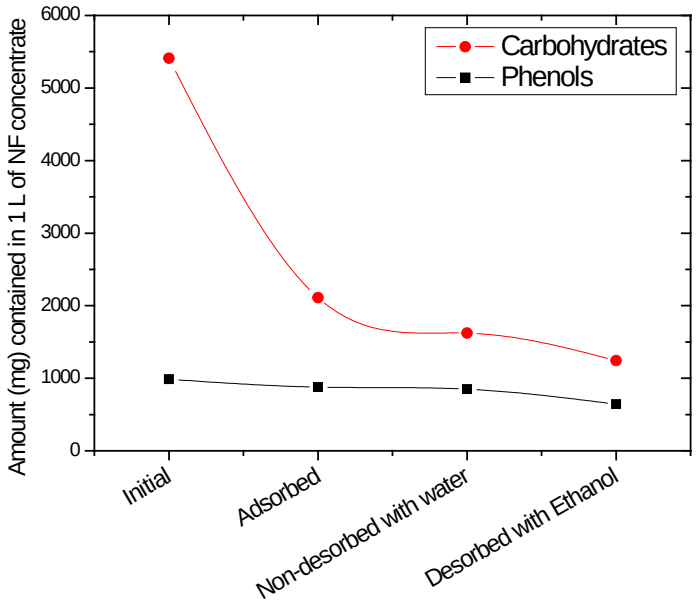

Fig. 10: Overview of the resin process efficiency for the separation of olive leaf extracts NF_c phenols-carbohydrates.

Here it must be noted that temperature is a key parameter in the adsorption/desorption process. As the results presented herein were obtained at room temperature, further optimization of the process can be carried out through temperature control.

3.5. Final concentration

With the removal of carbohydrates from the NF concentrate, further concentration can be achieved through evaporation. For this purpose 1.44 L of NF concentrate were treated with the proposed resin process, leading to the production of 0.72 L of ethanolic effluent that was evaporated under vacuum (0.05 bar, 50 °C). The final concentrate had a volume of 10 mL and is presented in Fig. 11. The concentration of total carbohydrates and total phenol are presented in Table 4.

Fig. 11: Final concentrate of the extracted phenols from olive leaves, after vacuum evaporation of the resin ethanolic effluent.

Table 4: Concentration of total carbohydrates and phenols in the NF_c, resin process product and final concentrate.

	Volume mL	**Total Phenols mg/L**	**Total Carbohydrates mg/L**
NFc	1440	988 ±25	5410 ±37
Desorbed	720	1480 ±1	5260 ±35
Final concentrate	10	97890 ±1230	322333 ±3933

It is important to note that with the resin process, the concentration of phenols was increased, as the ethanolic effluent had significantly lower volume compared to the NF concentrate sample treated. Finally, with the evaporation of ethanol, solvent recycle was achieved and the final product had significantly reduced volume with very high phenolic compound concentration.

4. Conclusions

With the proposed method, significant separation of olive leaf phenols was achieved. Firstly optimization of the extraction conditions was carried out, in terms of solvent ethanol percentage, solid/solvent ratio and duration. Then 20 kg of olive leaves were extracted with 80 L of water, and the extract was treated with membrane filtration. In the UF step, the suspended particles were removed, while NF concentrated the majority of the contained phenolic compounds. Resin adsorption/desorption was used next for the separation of phenols in the NF concentrate from non-polar compounds like carbohydrates. With batch adsorption experiments, XAD16N was proven to have the best adsorption behavior, and was used in resin packed beds to treat a larger amount of NF concentrate. The occurring resin ethanolic product contained 65% of the NF concentrate phenols and 23% of the contained carbohydrates. This separation, although significant, was affected by the presence of

complex phenolic compounds, like oleuropein-glycoside, part phenols and part carbohydrates. Better separation results and possibly higher added value phenolic products could have been obtained with the use of enzymes like β-glycosidase in the NF concentrate, in order to hydrolyze these complex compounds to simpler phenols. Moreover, the use of such enzymes, prior to membrane filtration could lead to the increase of hydroxytyrosol concentration and its partial separation with an extra RO step in the membrane process.

The ethanolic product of the resin process was finally treated with vacuum evaporation, with the finally product containing around 98 g/L phenolic compounds in gallic acid equivalents, compared to 0.5 g/L of the initial extract.

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