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Scale-up of a sorption process working with molecularly imprinted adsorbents for enrichment of winemaking residues and improvement of bioactivity

Catarina P. Gomes [a,c], Cristina N. Duarte [a,c,e], Cláudia D. Martins [a,c], Joana S. Amaral [a,c], Getúlio Igrejas [b,c], Maria João V. Pereira [b,c], Mário Rui P.F.N. Costa [d], Rolando C.S. Dias [a,c,*]

[a] *Centro de Investigação e Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia 5300-253, Bragança, Portugal*
[b] *Centro de Investigação em Digitalização e Robótica Inteligente (CeDRI), Instituto Politécnico de Bragança, Campus de Santa Apolónia 5300-253, Bragança, Portugal*
[c] *Laboratório Associado para a Sustentabilidade e Tecnologia em Regiões de Montanha (SusTEC), Instituto Politécnico de Bragança, Campus de Santa Apolónia 5300-253 Bragança, Portugal*
[d] *LSRE-LCM, Faculdade de Engenharia, Departamento de Engenharia Química, Universidade do Porto 4200-465, Porto, Portugal*
[e] *Grupo de Investigação em Polifenóis (GIP-USAL), Faculdade de Farmácia, Universidade de Salamanca E-37007, Salamanca, Espanha*

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ABSTRACT

This work presents the scale-up of a sorption process for the fractionation and enrichment of bioactive compounds in winemaking residues using molecularly imprinted adsorbents. The process works with hydroalcoholic solvents and the improvement of the bioactivity of the produced fractions, comparatively to the raw extracts, is demonstrated. The proposed approach was experimentally validated through the designing and running of a pilot size sorption prototype for the automation of the fractionation method. The synthesis of the molecularly imprinted adsorbents was made at the gram-scale and spent diatomaceous earth, used for wine filtration, was considered as a possible source of bioactive compounds in winemaking residues.

The different fractions produced were evaluated for their antioxidant activity through three different assays, namely radical scavenging activity, reducing power and inhibition of lipid peroxidation. The results obtained show the improvement of the bioactivity of most of the fractions comparatively to the original diatomaceous earth extract. The most enriched fraction is estimated to have a total phenolic content c.a. 3.8 times higher than the original extract. The radical scavenging activity, the reducing power and the inhibition of lipid peroxidation for this fraction were measured to be 6.4, 4.2 and 4.5 times higher, respectively, than the initial diatomaceous earth extract.

This work provides new insights on biomass valorisation and circular bioeconomy by combining in the same research materials development, process design and application to real extracts with proved improvement of the bioactivity of purified products.

1. Introduction

Nowadays, the potential reuse and valorization of residues/by-products from agri-food industries has gained prominence as it contributes to the bio-circular economy (Jimenez-Lopez et al., 2020).

The winemaking industry is one of the sectors that generates a significant number of by-products, with grape pomace, which includes mostly grape skins and seeds, being the major and most known. Nevertheless, other undervalued residues like wine lees (sediment consisting of yeast, grape solids, and other substances) and diatomaceous earth (used as a filtration agent) are also produced. These different winemaking residues have been reported to contain relevant amounts of added-value bioactive compounds, mainly polyphenols, with high market potential to be used by food, chemical, nutraceutical, cosmetic and pharmaceutic industries (Kalli et al., Dec. 2018, Duarte et al., Mar. 2023). These compounds are well-known for their potent antioxidant properties with several showing additional activities such as antimicrobial and anti-inflammatory. Their bioactivity may vary with the molecules configuration and the position and number of hydroxyl groups (Michalak, 2006), but mainly stems from their ability to

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* Corresponding author
E-mail address: rdias@ipb.pt (R.C.S. Dias).

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scavenge free radicals and reactive oxygen/nitrogen species (ROS/RNS), reduce oxidized intermediates, induce metal chelation, and inhibiting enzymes (Efenberger-Szmechtyk et al., Jan. 2021). By showing these beneficial properties, they have been positively associated to the prevention of certain diseases, including cancer, diabetes, cardiovascular issues, and degenerative disorders (Beya et al., Feb. 2021, Ashwin et al., Dec. 2021)

Within polyphenols, flavonoids are particularly significant and are further categorized into various classes based on their chemical structure (Ashwin et al., Dec. 2021). Extracts from winemaking residues, especially grape pomace (GP) and diatomaceous earth (DE), have been shown to be rich sources of these polyphenolic compounds, offering various health benefits, as aforementioned. Moreover, numerous studies have further investigated the use of the natural extracts obtained from these residues to replace synthetic additives in various industries such as pharmaceutical, cosmetic, and food (Beya et al., Feb. 2021), (Bucić-Kojić et al., 2020, Silva et al., Oct. 2018, Soto et al., Aug. 2015, Maluf et al., Jul. 2018). To this aim, El-Zainy et al. (El-Zainy et al., Jan. 2016) extended the shelf-life of raw beef sausages by adding grape seed polyphenols, offering a healthier alternative to synthetic additives. Ferreira et al. (Ferreira and Santos, Jan. 2022) explored replacing synthetic antioxidants with grape pomace extracts in cosmetics, and Maluf et al. (Maluf et al., Jul. 2018) demonstrated the efficacy and safety of grape pomace as an antioxidant in cosmetic formulations. Recently the potential cosmeceutical activity of extracts obtained from diatomaceous earth has been described by Duarte et al. (2023).

These studies underscore the potential of substituting synthetic additives with natural extracts from agricultural waste, with the dual goal of promoting consumer preference for natural products and aligning with the principles of a bio-circular economy.

Overall, the interesting properties exhibited by polyphenols have ignited a growing interest in their application across the food, pharmaceutical, and cosmetic industries, thus contributing to the rise of their market value. According to the Grand View Research in 2022, the global market size for polyphenols was estimated at 1.6 billion US dollars, with an expected compound annual growth rate of 7.4 % between 2022 and 2030 (Grand View Research 2023). The market value of phenolic compounds experiences a substantial increase with the purity of the product. To achieve this, among various techniques, sorption and desorption processes are employed, making use of efficient and selective adsorbents. These processes are utilized to enrich, concentrate, or even purify phenolic compounds present in agricultural waste (Pradal et al., May 2018). An extensive overview of various adsorbents that find practical applications in the valorization of agro-industrial residues, particularly in the concentration of polyphenols has been reviewed by (Pérez-Larrán et al., Oct. 2018). Indeed, sorption/desorption processes are an attractive possibility for complex extract handling because of the inherent lower energy consumption, cyclability, preservation of temperature-sensitive compounds, operational flexibility, amenability to intensification, and industrial scalability. The successful use of sorption and desorption processes related to the valorization of winemaking residues has also been reported but is dependent on the availability of suitable adsorbents (Drevelegka and Goula, Mar. 2020, Heravi et al., Jul. 2022). One possibility for working with such kinds of biomass is the consideration of Molecularly Imprinted Polymer (MIP) sorbents, which are synthesized using the molecular imprinting technique. Often, MIPs make use of synthetic monomers with the capability to grant to the final adsorbent the capability to recognize and bind to specific molecular structures. This selectivity arises from the distinct cavities integrated into the polymeric network of the material, which are formed during the polymerization process. Our research group has already demonstrated the potential application of MIP adsorbents in continuous processes for valorizing polyphenolic compounds from agricultural residues (Bzainia et al., 2023, Gomes et al., Nov. 2017, Gomes et al., 2019, Gomes et al., 2020, Bzainia et al., 2022, Gomes et al., Mar. 2023, Gomes et al., Jul. 2021, Bzainia et al., 2023). Specifically, MIPs containing pyridyl moieties have been proposed because of their remarkable efficacy in adsorbing phenolic contaminants, outperforming other materials like styrene-DVB copolymers (Varshney and Mishra, 2023). The strong binding between the hydroxyl groups and pyridyl groups makes them highly effective for retaining phenolic compounds (Bzainia et al., 2023, Gomes et al., Nov. 2017, Gomes et al., 2019, Gomes et al., 2020, Bzainia et al., 2022, Gomes et al., Mar. 2023, Gomes et al., Jul. 2021, Bzainia et al., 2023).

The objective of the present work is to scale-up the synthesis of a pyridyl-based molecularly imprinted polymer (using quercetin as template) considering an inverse suspension polymerization towards its use as an adsorbent at a pre-industrial scale, and also the designing and built-up of a sorption/desorption process prototype to work with the packed MIP particles. This approach will ultimately lead to the production of enriched polyphenol fractions from an initial hydroethanolic extract, obtained with winemaking residues as a potential source to be valorized, namely the diatomaceous earth used to filtrate the wine, which is a less explored residue of this huge industry.

2. Materials and methods

2.1. Winemaking by-products

The winemaking by-products were provided by Caves Campelo S.A. (Portugal) during the harvest and production season in September 2022. The samples of diatomaceous earth were arranged on separated trays and dried in an oven (Scientific, Series 9000, Scientific Engineering, Johannesburg, South Africa) at 40°C for 3 days, and then ground into powder in a mill (IKA, A11 basic) to pass a sieve of 20 mesh. The samples were stored at 20 °C inside a container and protected from light.

2.2. Extraction method

Phenolic compounds were extracted using ethanol/water 80:20 (v/v). Fifteen grams of diatomaceous earth was extracted with 100 mL of solvent by sonication (SONO SWISS, sw1) during 5 min followed by stirring (Lbx instruments, S03 series) for 1 hour, protected from light at room temperature. The samples were centrifuged (Eppendorf, Centrifuge 5810 R) at 7871 × *g* for 5 min, and the pellet was re-extracted. After collecting the supernatants, they were gathered and the ethanol (Fisher Scientific, Loughborough, UK) was evaporated under vacuum in a rotary evaporator (Julabo, TW12, Julabo Labortechnik, Seelbach, Germany) at 40°C. The solution obtained for the diatomaceous earth extract was freeze dried at -20 °C and submitted to lyophilisation. The same extraction procedure was follow considering a Red Grape Pomace (GP) as polyphenols source.

2.3. Characterization of phenolic compounds by HPLC-DAD

Samples were analysed using a high-performance liquid chromatography (HPLC) system, KNAUER, comprised of a binary pump (P6.1L) with a degasser, an autosampler (6.1L), a column thermostat (CT2.1) operating at 45°C, and a diode array detector (6.1L). For chromatographic analysis, an Ascentis C18 (Supelco) packed column with 5 μm particle size and dimensions of 25 cm × 4.6 mm was employed. The HPLC analyses were performed using the following solvent-gradient method: starting with 100 % solvent A, which consisted of H_2O (pH=3)/acetonitrile (ACN) in a ratio 90/10 (v/v), and ending with 100 % solvent B, composed of H_2O (pH=3)/ACN in a ratio of 10/90 (v/v). The pH of water was adjusted with acetic acid. The gradient from solvent A to solvent B took 45 min, with a flow rate of 1 mL/min. The ClarityChrom software was used to control the system and process the obtained data.

2.4. Molecularly imprinted adsorbent

2.4.1. Reagents

Quercetin (hydrate, purity 95 %) was supplied by Acros Organics and was used as template in the synthesis of molecularly imprinted polymers. The functional monomer 4-vinylpyridine (4VP, 95 % purity) was provided by Alfa Aesar. The crosslinker ethylene glycol dimethacrylate (EGDMA, 98 % purity) and the initiator 2,2′-azobis(2-methylpropionitrile) (AIBN, 98 % purity) were supplied from Sigma-Aldrich. The surfactant Span 80 (sorbitan oleate) and the *n*-heptane were provided from Sigma-Aldrich. The solvent dimethylformamide (DMF, 99 % extra pure) was purchased from Acros Organics, acetonitrile (ACN, 99.8 % HPLC grade), ethanol (99.8 % purity, analytic grade), acetic acid glacial (AcOH, $\geq$ 99.7 % analytical reagent grade) and methanol (99.8 % purity, HPLC grade) were provided from Fisher Chemical. The used water was ultrapure grade and supplied locally.

2.4.2. Synthesis of Quercetin-molecularly imprinted sorbent at gram-scale

The quercetin-molecularly imprinted sorbent was obtained by inverse suspension polymerization following a similar procedure as described previously (Gomes et al., Nov. 2017, Gomes et al., 2019). *n*-Heptane was chosen for the continuous phase in the inverse suspension polymerization, with span 80 as stabilizer. Firstly, Span 80 was dispersed in *n*-heptane under vigorous stirring at room temperature. In parallel, the chosen template, quercetin, was mixed with the selected functional monomer (4VP) and the solvent ACN/DMF in a ratio 85/15 (v/v) until the formation of a homogeneous solution. During this step, the interaction between the template and the functional monomer (T-FM) was promoted for 30 minutes in an ultrasound bath. Subsequently, the remaining reagents, including the crosslinker (EGDMA) and the initiator (AIBN) were dissolved in the solution and added to the continuous phase. The final solution was transferred to a Parr 5100 pressurized glass reactor with maximum capacity of 1 L. The reactor was pressurized with argon at 2 bar and the temperature set-point was 60 °C. A total polymerization volume of 600 mL was considered. The reactor is equipped with a magnetic drive internal stirrer including double turbine type six-blade impellers at 45° angle. The polymerization was carried out with a stirring speed of 400 rpm. Other details on the synthesis of related 4-vinylpyridine adsorbents can be found in (Gomes et al., Nov. 2017, Gomes et al., 2019, Gomes et al., 2020, Bzainia et al., 2022, Gomes et al., Mar. 2023, Gomes et al., Jul. 2021, Bzainia et al., 2023, Gomes et al., 2024, Almeida et al., 2024).

2.5. Designing and Running of a Pilot Size Sorption/Desorption Prototype

Sorption/desorption experiments were conducted using a continuous process prototype designed to perform the efficient recover of the high value products from winemaking residues. Initially, the experiments involved packing the synthesized adsorbent particles (via a slurry process) into an empty column (approximately 17 g of adsorbent in a column with dimensions L×D =250 mm × 20 mm). Subsequently, the packed column was placed in the continuous process prototype. The column was first cleaned and conditioned using the same solvent employed during the loading process. Subsequently, the chosen winemaking residue extract was percolated through the column, followed by the necessary steps for recovery and cleaning.

The prototype was designed to simulate sorption/desorption processes at an industrial scale. It included tanks for storing the hydroalcoholic extracts obtained from the winemaking residues such as diatomaceous earth and grape pomace extracts. These extracts were added to the tank to initiate the sorption process. From the feeding tank the extracts were filtered through a material with a sufficiently low pore size (e.g., in this case 1 μm) to remove particles that could obstruct the adsorption columns. After this, the stream was pumped into the core of separation processes, which consisted of a column packed with the molecularly imprinted sorbent. Four-way valves were employed to pipe the extract and the desorption solvents to the column during the sorption/desorption cycles. The temperature of the sorption/desorption columns was controlled within a typical operating range of 15-25 °C for sorption and 45-60°C for desorption the processes. At the outlet, another four-way valve drives the eluent through a UV-vis sensor, whose response was considered to monitor the process. The prototype also includes a binary pump to produce the change of the solvent composition along the desorption process. Two reservoirs, each containing solvents with predefined reference compositions, were used to provide the necessary supply for this gradient pump. All parameters, including flow rate, temperature, valves positions, experiments execution and UV data acquisition were controlled using LabVIEW software. A schematic diagram of the continuous sorption/desorption prototype developed and used for these experiments is presented in Fig. 1.

2.6. Total phenolic content

The total phenolic content of the hydro-ethanolic extracts prepared from diatomaceous earth and of the corresponding obtained fractions was determined by spectrophotometry in a microplate reader (Epoch 2, Biotek), using the Folin-Ciocalteu reagent. Different concentrations (0.005; 0.01; 0.05; 0.1; 0.25 and 0.5 mg/mL) of an ethanolic solution of gallic acid, used as standard, were used to obtain the calibration curve. For the analysis, the extracts and corresponding fractions were evaporated under vacuum (Stuart RE 300 rotary evaporator), freeze-dried and lyophilized. Then, the lyophilizates were redissolved in ethanol 80:20 to known concentrations, using serial dilutions (1:2) if necessary. In a test tube, 250 μL of extract from each sample or standard were mixed with 1.25 mL of Folin-Ciocalteu reagent (1:10 v/v in water) and 1 mL of sodium carbonate (75 g/L). The tubes were vortexed for 15 sec and placed to rest in the dark for 30 minutes at 40°C for colour development. At the same time, a negative control using ethanol was prepared. After, the tubes were centrifuged at 12000 rpm for 2 minutes and the solutions from each test tube were then transferred to a 96 wells microplate with the help of a micropipette. Finally, the absorbance was measured at 765 nm in a microplate reader (Epoch 2, BioTek Instruments, USA). The results were expressed in mg of gallic acid equivalents per gram of extract (mg GAE/g extract). Similarly, an analogous assessment was conducted for the total phenolic content of the GP extract.

2.7. Antioxidant activity

Stock solutions with known concentration were prepared from the lyophilized extracts (DE extract, GP extract and the eluted fractions) by re-dissolution in ethanol/water (80:20, v/v). After serial dilution (1:10) of the stock solutions, the antioxidant activity was measured following three different assays, namely DPPH radical scavenging activity, reducing power and inhibition of lipid peroxidation in porcine brain tissue. Briefly, DPPH radical-scavenging activity was evaluated as previously described (Duarte et al., Mar. 2023) using an Epoch 2 microplate reader (BioTek Instruments, USA) and calculated as a percentage of DPPH discoloration using the formula:

$$EC_{50} = \frac{A_{DPPH} - A_S}{A_{DPPH}} \times 100 \qquad (1)$$

where A_S is the absorbance of sample solution, and A_{DPPH} is the absorbance of the DPPH solution at 515 nm. Reducing power evaluated the capacity to convert Fe^{3+} into Fe^{2+}, by measuring the absorbance at 690 nm as described by Silva et al. (Silva et al., Feb. 2020). The lipid peroxidation inhibition was evaluated based in the formation of thiobarbituric acid reactive species (TBARS) in a suspension of lipid-rich porcine brain as described by Spréa et al. (Spréa et al., 2020). The absorbance was measured at 532 nm. The results of the in vitro assays were expressed as EC_{50} values, corresponding to the sample concentration providing 50 % of antioxidant activity in the DPPH and TBARS

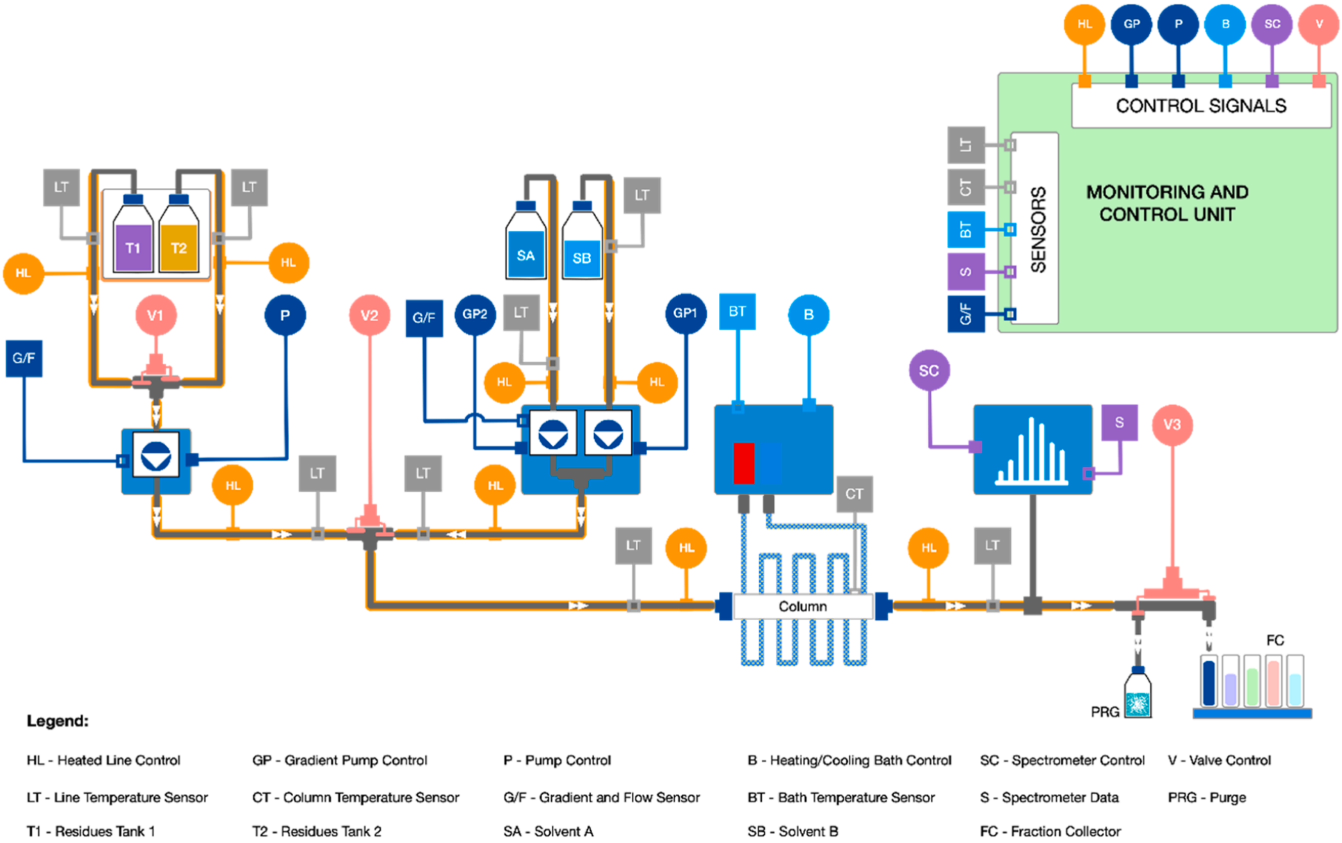

Fig. 1. Schematic diagram of the pilot size sorption/desorption prototype used in this work.

assays, or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

3. Results and discussion

3.1. Rationale of MIP synthesis

When the synthesis of molecularly imprinted adsorbents is considered, it is meaningful a preliminary study on the interactions between the chosen template molecule and the functional monomer, as well as on the working conditions of the final material. Pyridine-based polymers are extensively used in various fields, serving as advanced materials with a broad range of applications encompassing wastewater and industrial effluent treatment, enzyme and protein adsorption, electronics biomedicine, and catalysis, among others (Varshney and Mishra, 2023, Xiao and Lin, Sep. 2020, Neolaka et al., Feb. 2020, Atif et al., Nov. 2019). Considering that adsorbents containing pyridyl moieties have shown a strong affinity for phenolic compounds present in diverse plant extracts, they hold promising features for the valorisation of this class of bioactive compounds (Bzainia et al., 2023, Gomes et al., Nov. 2017, Gomes et al., 2019, Gomes et al., 2020, Bzainia et al., 2022, Gomes et al., Mar. 2023, Gomes et al., Jul. 2021, Bzainia et al., 2023). Particularly, the design of polymer networks capable of adsorbing a specific compound in a selective way and that can be applied in continuous processes is highly advantageous. In previous works (Gomes et al., Nov. 2017, Gomes et al., 2019, Gomes et al., 2020), we successfully demonstrated the strong interaction between quercetin (the template molecule) and 4-vinylpyridine (4VP, the functional monomer) resulting in materials with molecular imprinting effect and improved selectivity. Therefore, these synthesis conditions were used in the present work to scale-up the production and apply this type of adsorbents at a pre-industrial scale.

Quercetin is one of the flavonoids more abundant in plant matrices, it is composed by three aromatic rings, five hydroxyl groups and a carbonyl function. The predicted interaction of quercetin with 4VP is presented in Fig. 2, where the strong hydrogen bonds formed between the pyridyl group of 4VP and the hydroxyl groups of quercetin are highlighted.

Considering that quercetin is relatively affordable and this aglycone and its derivatives are abundant in most plant matrices, including winemaking residues (as observed in this study), this molecule was selected as a template for the synthesis of the MIP adsorbent. Furthermore, quercetin can also be regarded as a kind of surrogate molecule for other flavonols, a pratice often employed in the pharmaceutical industry

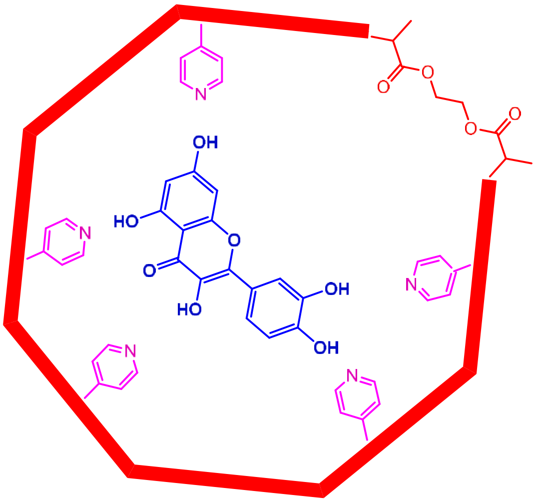

Fig. 2. Plausible interactions between the functional monomer (4VP) and the template molecule (quercetin).

with other chemicals. Our approach considers the structural similarities between quercetin and other target polyphenols for the introduction of a broader degree of specificity for flavonols into the designed adsorbents.

Nonetheless, the molecular imprinting effect is not only dependent on the interactions between template and functional monomer. Other parameters such as the solvent(s) used and system composition considered in the copolymerization also affect the molecular imprinting efficiency. For example, the use of protic solvents, such as water, can disrupt the hydrogen bonds between the functional monomer and the template. In contrast, aprotic solvents like acetonitrile (ACN) are commonly used to enhance hydrogen bond interactions.

Recently, our research group was devoted at finding improved synthesis conditions to valorise the bioactive compounds present in plant matrices. Different experiments, considering diverse templates and functional monomers were carried out (Bzainia et al., 2023, Gomes et al., Nov. 2017, Gomes et al., 2019, Gomes et al., 2020, Bzainia et al., 2022, Gomes et al., Mar. 2023, Gomes et al., Jul. 2021, Bzainia et al., 2023) using extracts from different raw biomass such as onion skin, winemaking residues, olive leaves and almond hull/shell/skin. Often, the materials were synthesised at small scale (e.g. final quantity of produced material around 200 mg). The performance of the materials was studied in solid phase extraction cartridges and in small columns with dimensions between L(mm)×D(mm)=10×4.6 and L(mm)×D(mm)=100×4.6. In this work we proceeded to the scale-up of the synthesis of the molecularly imprinted adsorbent with subsequent application in a pilot size sorption/desorption prototype.

When considering the final application of such kinds of materials as adsorbents into continuous process, the morphology of the particles becomes a critical parameter. Too small particles can lead to high pressures, rendering them unsuitable for use in continuous processes. Inverse suspension polymerization is a technique enabling the production of particles with a desirable morphology for efficient packing and utilization in continuous processes. However, it is important to note that the final morphology is also dependent on the specific synthesis conditions. Table 1 summarizes the recipe employed to produce the material considered as adsorbent in this work. With these polymerization conditions, particles with size suitable for use as adsorbent in continuous processes working at high pressure were successfully obtained.

The polymerization was performed using the inverse suspension polymerization technique with the AIBN initiator and the EGDMA crosslinker, at 60 °C for 24 h.

The parameters used in Table 1 to describe the initial state of the polymerization systemare defined as follows:

- Y_M - mass fraction of functional monomer and crosslinker in the solution (%);
- Y_I - mole fraction of initiator relative to the FM and the crosslinker (%);
- Y_{CL} - mole fraction of the crosslinker in the mixture FMs + crosslinker (%);
- $Y_{FM/T}$ - mole ratio of the FM to the template molecule;
- $Y_{C/D}$ - mass ratio between the oil phase and the monomer phase in inverse suspension polymerization.

3.2. FTIR and SEM characterization

FTIR analysis provides key information on the formation of the molecularly imprinted sorbent, specifically regarding the incorporation of the functional monomer into the polymeric network. Fig. 3 presents the FTIR spectra obtained for the MIP synthesized and used in this work.

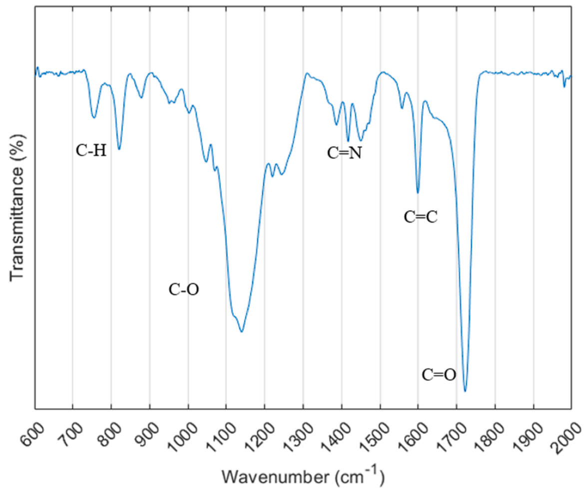

Fig. 3. FTIR spectra collected for the molecularly imprinted material synthesized with the functional monomer 4VP, the crosslinker EGDMA and the template molecule quercetin.

Taking into consideration the 4VP monomer, characteristic peaks at 1600 cm^{-1} corresponding to C=C pyridyl, 1418 cm^{-1} (C=N), 820 cm^{-1} and 755 cm^{-1} corresponding to the C-H bending in the pyridine ring, these assignments provide evidence of the successful incorporation of 4VP into the synthesized material. The peaks at 1720 cm^{-1} and 1130 cm^{-1} are correspondent to C=O and C-O, respectively, characteristic of EGDMA (crosslinker). The observation of these peaks also confirms the incorporation of the crosslinker in the final material.

Fig. 4 shows the SEM micrographs for the synthesized MIP sorbent. Particles agglomerates with sizes in the rough range 5 to 100 μm were formed. It is worth to mention that the agglomeration observed is due to the selected inverse suspension polymerization technique involving the 4VP functional monomer, the EGDMA crosslinker and the ACN/DMF solvent in the dispersed phase. These aggregates connect particles with diameters <1 μm (precipitated at early stages of the polymerization) in final particle assemblies that are suitable for application as packing materials for continuous sorption/desorption processes, as explored in this research. The too low size of polymer particles hindering their application due to the possible backpressure problem is circumvented through this synthesis approach. Actually, in addition to the reactants concentrations and solvent composition, affecting particle agglomeration, other factors such as the thermodynamics of phase separation and stirring conditions also change the size and morphology of the final particles. The synthesis method above described for the MIP particles attempts the addressing of these issues and look for the feasibility of the extension to the sorbent production at the gram-scale.

3.3. Processing of the diatomaceous earth extract with the developed MIP sorbent

Recently, Duarte et al. (Duarte et al., Mar. 2023) reported for the first time the phenolic composition of an hydroethanolic extract obtained from diatomaceous earth used for wine filtration. The study found that the diatomaceous earth extract is a promising and unexplored source of phenolic compounds, notably flavonols and anthocyanins. As expected, the extract in the current research also exhibited a complex mixture of these phenolic compounds. Fig. 5 illustrates both the 3D HPLC-DAD

Table 1
Polymerization conditions used to synthesize the molecularly imprinted adsorbent used in this work.

Template	Functional Monomer	Solvent (v/v)	Y_M	Y_I	Y_{CL}	$Y_{FM/T}$	$Y_{C/D}$	Yield (%)
Quercetin	4VP	ACN/DMF (85/15)	30.43	3.97	51.48	15.69	3.62	~100

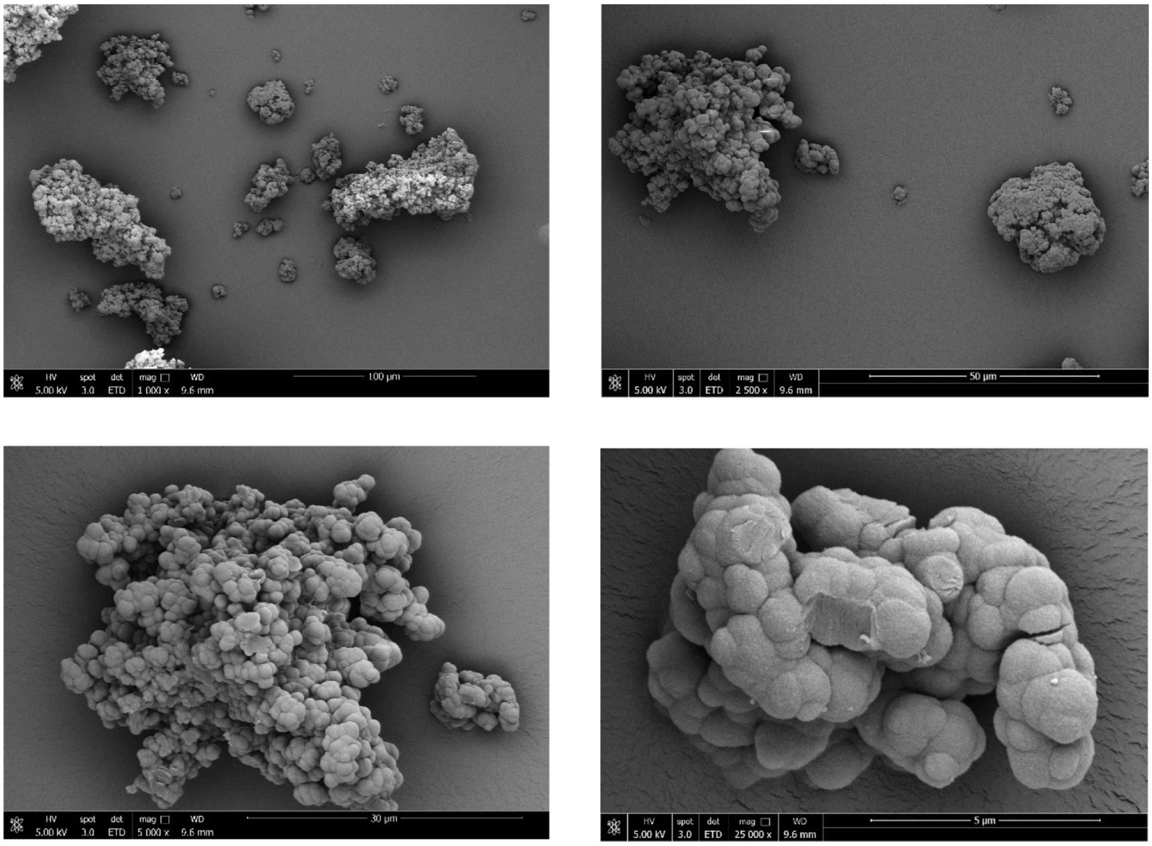

Fig. 4. SEM images for the molecularly imprinted adsorbent synthesized in this work through an inverse-suspension approach.

chromatogram and the corresponding 2D HPLC-DAD chromatogram measured at λ=280 nm for the DE extract used in this study. Tentative identification of the contained compounds was accomplished by comparison with the HPLC-DAD analysis for representative polyphenol standards. Considering the retention time and UV-vis spectrum, the peak eluting at 16.6 min was identified to be quercetin (peak 6 in Fig. 5(b)). Moreover, based on data from previous works by our research group involving HPLC-DAD and LC-DAD-MS/MS analyses for the identification of compounds present in winemaking residues (Duarte et al., Mar. 2023, Bzainia et al., 2022, Gomes et al., 2024), some additional peaks in Fig. 5 were also identified. The outcomes for this tentative identification analysis are summarized in Table 2. Note that peak 5 in Fig. 5 is not associated with a polyphenol molecule but rather with another compound used during the vinification process (Duarte et al., Mar. 2023, Bzainia et al., 2022, Gomes et al., 2024). Interestingly, the flavonol aglycones quercertin, myricetin and kaempferol as well as some correspondent glycosylated molecules are identified in the diatomaceous earth extract, indicating that a relevant amount of such bioactive compounds are lose during the wine filtration process.

The extract prepared from the DE residue was used with the prototype illustrated in Fig. 1 to highlight the efficacy of the synthesized molecularly imprinted adsorbent with the fractionation of the target classes of molecules. For that purpose, initially, the material was loaded with diatomaceous earth extract by circulating approximately 890 ml of the extract at T=25 °C with a flow rate of 2 mL/min, for 24 h, considering a recycling mechanism. The solvent considered was a mixture of ethanol and water in an 80/20 (v/v) ratio and extract concentration of 3.75 mg/mL. The adsorption process was monitored by collecting fractions at specific intervals, which were subsequently analyzed in a UV-vis spectrophotometer. Fig. 6 shows the UV spectra from the collected fractions during the loading process and the normalized breakthrough curve measured at λ=280 nm. Since the prototype was used with a recycling mechanism, it is crucial to note that the breakthrough curve may be influenced by the competitive adsorption phenomenon, which involves various compounds present in the DE extract and the availability of binding sites.

The driving force for a possible separation of different polyphenols in plant extracts by sorption/desorption is quantified by the particular isotherms describing the change of the adsorbed amounts with concentration for a specific sorbent and working solvent. These features were recently demonstrated with standard polyphenols and using an adsorbent similar to that here considered, with practical application to the purification of olive leaf extracts (Almeida et al., 2024). However, when addressing real plant extracts, the determination of the isotherms quantifying these concentration effects for all compounds is not practical due to the plethora of chemicals there contained. Therefore, for productivity improvement and simplicity of the sorption/desorption process, working with an as higher as possible extract concentration is advantageous. The upper limiting value is defined by the global solubility of the extract in the selected solvent if precipitation of less soluble compounds is not desired. A key feature of the 4-vinylpyridine sorbents here explored, namely in comparison with common commercial alternatives, is the possibility to work with hydroalcoholic mixtures with high alcoholic content and having also a high retention capability, as demonstrated before (Gomes et al., Nov. 2017, Gomes et al., 2019, Gomes et al., 2020, Bzainia et al., 2022, Gomes et al., Mar. 2023, Gomes et al., Jul. 2021, Bzainia et al., 2023, Gomes et al., 2024, Almeida et al., 2024). Thus, in previous works, the running of adsorption processes with different winemaking residues extracts concentrations as higher as 1 mg/mL in EtOH/water 80/20, 1.1 mg/mL in EtOH/water 54/46 and 3.75 mg/mL in EtOH/water 80/20 were demonstrated to be possible (Gomes et al., 2024). The same is observed in the present work with the consideration of an extract concentration of 3.75 mg/mL in EtOH/water 80/20, as highlighted in Fig. 6.

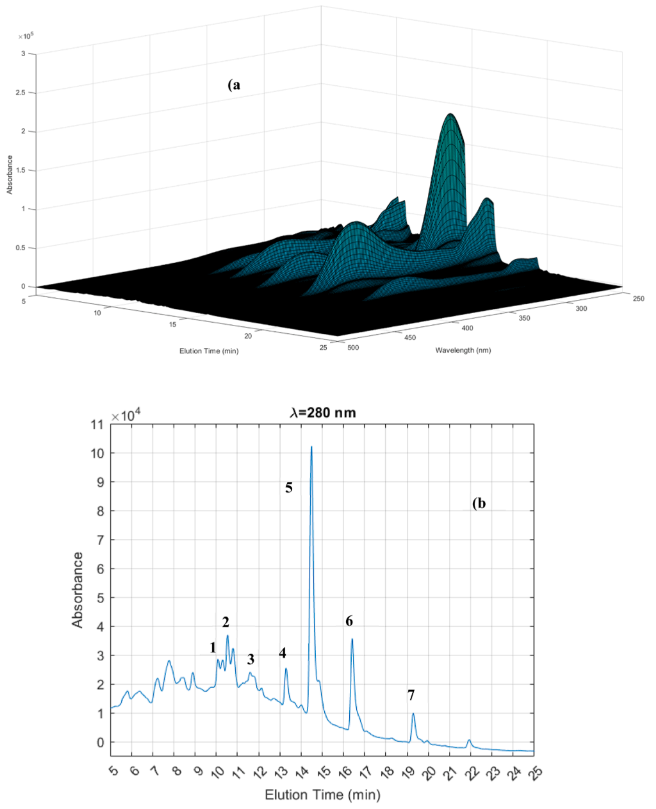

Fig. 5. a) 3D HPLC-DAD chromatogram and correspondent b) 2D chromatogram measured at λ=280 nm for the diatomaceous earth extract processed with the developed molecularly imprinted polymer particles.

Table 2
Tentative identification for relevant phenolic compounds in the raw diatomaceous earth extract (see Fig. 5).

Peak	Elution Time (min)	Tentative Identification
1	10.2	Myricetin-O-hexoside
2	10.7	Quercetin-O-hexuronoside
3	11.7	Malvidin-3-O-acetylglucoside
4	13.4	Myricetin
6	16.6	Quercetin
7	19.4	Kaempferol

After the extract loading step in the MIP particles, the fractionation (elution) process was carried out at T=45 °C. The subsequent stage involved the use of eight distinct elution solvents, which included pure water, mixtures of ethanol/water, pure ethanol, pure methanol (MeOH) and MeOH/AcOH (90/10 v/v). Table 3 presents the solvents used and the corresponding compositions for each elution fraction. By changing the eluting solvent, it became possible to obtain fractions with a simplified composition compared to the initial DE extract. Fig. 7 shows the HPLC-DAD 2D chromatogram of the diatomaceous earth extract in ethanol/water 80/20, used with the load of the MIP particles, and the corresponding fractions collected after elution with the different solvents described in Table 3. Measurements at 280, 360 and 550 nm are presented to emphasize the fractionation and consequent purification/concentration of different families of flavonoids (such as flavonols and anthocyanins).

A more comprehensive analysis of selected fractions collected during this desorption process is illustrated in Fig. 8, featuring HPLC-DAD-3D chromatograms. These results underscore the efficacy of the synthesized MIP adsorbent for fractionate and concentrate compounds based on their distinct binding strengths. One significant outcome, also observed in Fig. 7, is the removal of the non-polyphenolic compound (peak 5) when increasing the percentage of organic solvents (ethanol and methanol). Indeed, fractions F8 and F9 do not contain this peak.

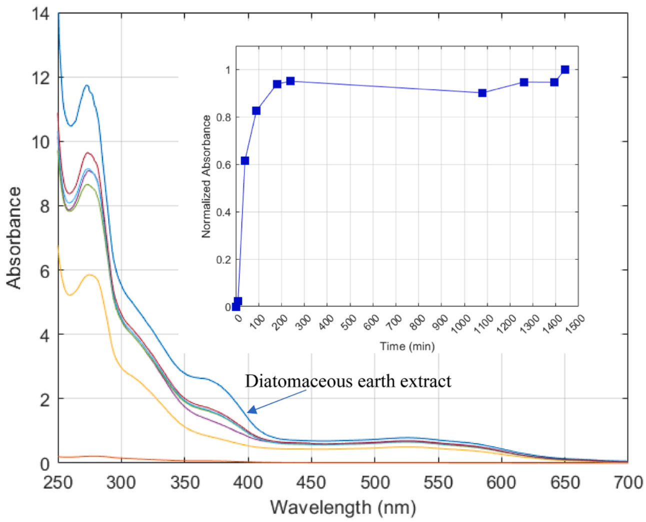

Fig. 6. UV-vis spectra for the samples collected during the saturation process of the molecularly imprinted adsorbent with a diatomaceous earth extract in ethanol/water 80/20 (v/v) at concentration of 3.75 mg/mL. Column outlet was sampled at different time instants for quantification of the dynamics of adsorption. The inset in the plot shows the normalized breakthrough curve measured at λ=280 nm.

Table 3
Solvent and their compositions used in each elution fraction.

Fractions	Solvent	Composition (v/v)
F1	Water	100
F2	Water	100
F3	Ethanol/water	20/80
F4	Ethanol/water	40/60
F5	Ethanol/water	60/40
F6	Ethanol/water	80/20
F7	Ethanol	100
F8	MeOH	100
F9	MeOH/AcOH	90/10

Another important result in Fig.s 7 and 8 is the production of fractions (F7, F8, and F9) that are highly enriched with flavonols, specifically quercetin (peak 6) and kaempferol (peak 7). The fractionation of one identified anthocyanin (peak 3) from the flavonols group, specifically quercetin-O-hexuronoside, quercetin, and kaempferol, is also achievable in some fractions such as F3, F4, and F5.

Based on the 2D HPLC-DAD chromatograms illustrated in Fig. 7, was performed the estimation of the enrichment factors (EF) for the fractions generated during the sorption/desorption process. Fig. 9 displays these outcomes considering the flavonols as reference compounds and the enrichment achieved for such kind of polyphenols in some fractions is evidenced (e.g. EF>2 for F7 and F8). It is worth to mention that the enrichment factor for these fractions has the potential for further enhancement by implementing an alternative solvent gradient during the elution step. This approach would entail using a specific solvent composition until all the compounds are fully extracted, potentially leading to higher levels of enrichment.

3.4. *Total phenolic content (TPC) of the purified fractions*

Numerous studies have focused on the phenolic composition of grape pomace and other residues from the winemaking production, either by determining their overall content in phenolic compounds or by

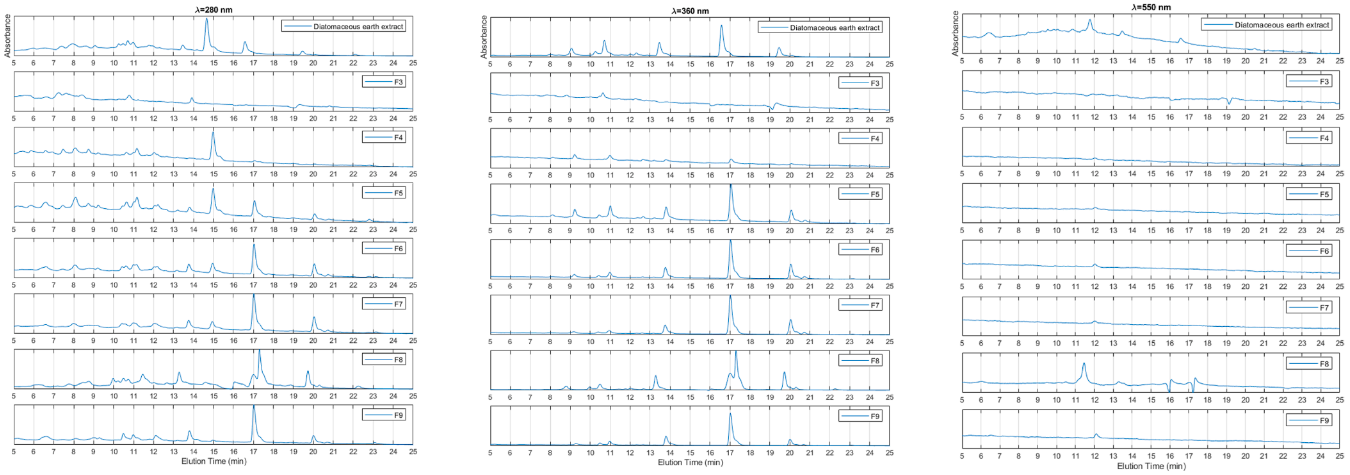

Fig. 7. 2D HPLC-DAD chromatograms for the initial DE extract and for different fractions collected during the desorption process of the compounds retained in the molecularly imprinted adsorbent. Previous loading step was performed with a DE extract in ethanol/water 80/20 (v/v) at a concentration of 3.75 mg/mL. Measurements at 280, 360 and 550 nm are here presented to highlight the fractionation of different families of flavonoids (flavonols and anthocyanins).

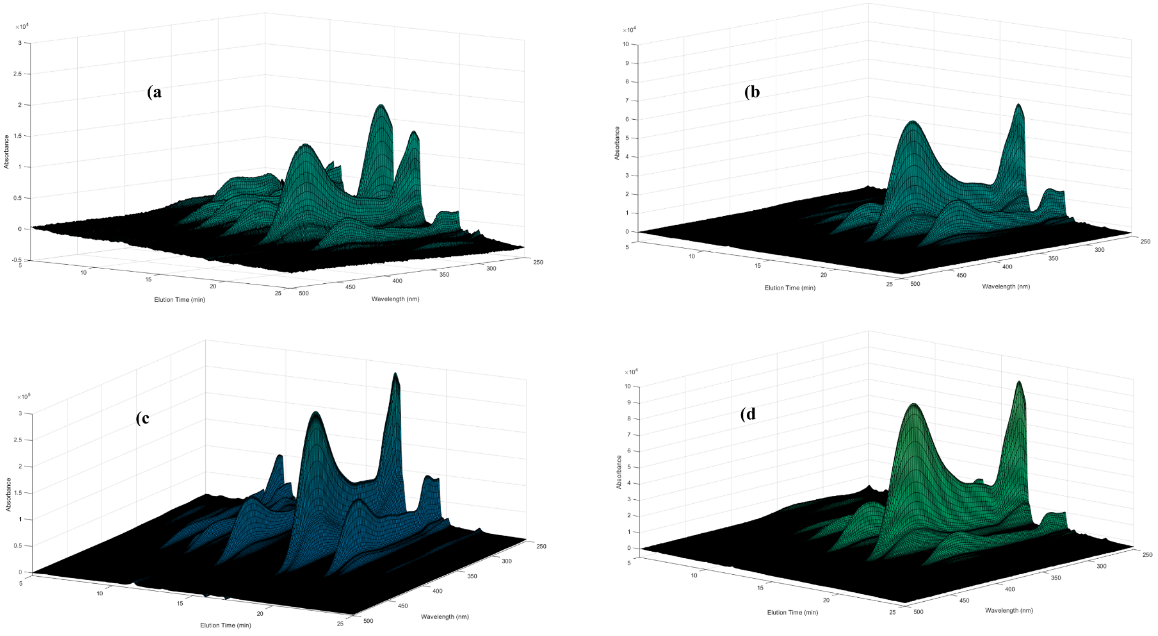

Fig. 8. 3D HPLC-DAD chromatograms for different fractions collected during the desorption of the molecularly imprinted adsorbent. Previous loading step was performed with a DE extract in ethanol/water 80/20 (v/v) at a concentration of 3.75 mg/mL. a) fraction recovered in ethanol/water 60/40 (F5) b) fraction recovered in ethanol (F7) c) fraction recovered in methanol (F8) d) fraction recovered in MeOH/AcOH 90/10 (F9).

identifying and quantifying specific individual compounds (Peixoto et al., Jul. 2018, Matos et al., Sep. 2019, Gil-Sánchez et al., Aug. 2017). However, when it comes to the utilization of diatomaceous earth extracts, limited scientific literature is available (Duarte et al., Mar. 2023). Although chromatographic analysis provides precise information on individual compounds, the determination of total phenolic content (TPC) by the Folin-Ciocalteu reaction has great potential to be used as a screening assay by the winemaking industry. By estimating the TPC in the initial diatomaceous earth extracts and fractions, this simple assay would easily allow to select the most promising purified products. The TPC determined for the initial DE extract and for selected fractions obtained with the MIP particles are shown in Fig. 10.

The TPC value determined for the initial DE extract (75.58 ± 1.0 mg GAE/g of extract) is lower than that previously reported by Duarte et al. (Duarte et al., Mar. 2023) (126.92 ± 0.9 mg EAG/g extract). In this case, it is noteworthy to highlight that, following the sorption/desorption experiments with the developed MIP, except for fraction F3, all obtained fractions exhibited significantly higher TPC values as compared to the initial extract, demonstrating the effectiveness of the molecularly imprinted adsorbent in producing enriched fractions with higher concentrations of total phenolic compounds (see Fig. 10). Furthermore, drawing an analogy with the TPC of fractions obtained from the DE extract and a raw GP extract, known for its notable TPC values (Duarte et al., Mar. 2023) and their direct application in some industries (El-Zainy et al., Jan. 2016, Ferreira and Santos, Jan. 2022, Wasilewski et al., Apr. 2022), it becomes apparent that the TPC values of the fractions from the DE extract consistently exceed those from the GP extract. This highlights the significance of the valorisation achieved with the proposed sorption/desorption process.

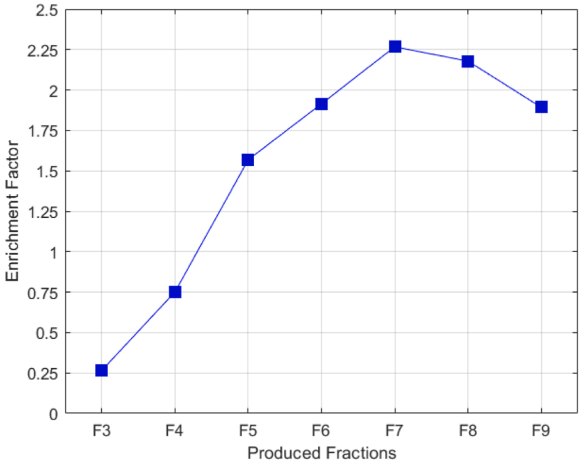

Fig. 9. Enrichment factors calculated from the HPLC-DAD chromatogram areas of the fractions obtained following the sorption/desorption processes with the diatomaceous earth extract. These calculations were performed considering the flavonol compounds as reference.

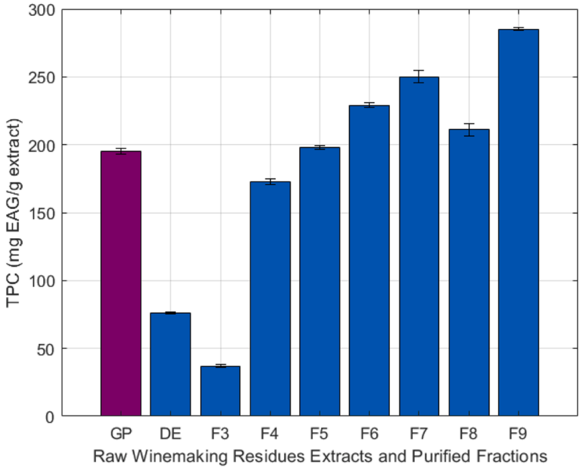

Fig. 10. Total phenolic content (TPC, expressed in mg GAE/g extract) for a GP extract, a DE extract and for the fractions purified through the processing of the DE extract by sorption/desorption with the MIP particles.

3.5. *Antioxidant activity of the purified fractions*

Numerous pathologies, degenerative processes, and health conditions, including diabetes, arterial hypertension, aging, and cancer are intricately linked to oxidative stress. Oxidative stress induces significant cellular damage due to the elevated production of reactive oxygen species (ROS) that target vital macromolecules such as proteins, DNA and lipids. The antioxidant potency of phenolic compounds is related to their reducing properties, as they function as hydrogen or electron donors. This characteristic predicts their potential as free-radical scavengers, essentially acting as antioxidants. Additionally, phenolic compounds exhibit the capacity for metal chelation, particularly with iron and copper ions, effectively suppressing the formation of free radicals catalysed by these metals. Numerous methods have been devoted to assess antioxidant properties, with commonly used assays, including DPPH scavenging activity, reducing power, and TBARS inhibition. The results obtained by applying these assays for the DE extract, a GP extract and for selected fractions obtained after sorption/desorption processes are shown in Table 4 and Fig. 11.

Table 4
Antioxidant activity of DE extract and of selected fractions obtained after the sorption /desorption process with the molecularly imprinted particles.

Extract/ Fractions	Antioxidant activity EC_{50} values (mg/mL)		
	DPPH scavenging activity	Reducing Power	TBARS inhibition
GP extract	0.142±0.002	0.134±0.006	0.008±0.0002
DE extract	0.373±0.008	0.250±0.004	0.036±0.0007
F3	0.310±0.010	0.693±0.015	0.418±0.0050
F4	0.286±0.009	0.164±0.004	0.017±0.0002
F5	0.212±0.016	0.120±0.002	0.014±0.0008
F6	0.302±0.014	0.110±0.002	0.006±0.0009
F7	0.077±0.006	0.060±0.004	0.009±0.0005
F8	0.108±0.008	0.060±0.008	0.017±0.0014
F9	0.058±0.009	0.060±0.003	0.008±0.0004

EC_{50} values correspond to the sample concentration achieving 50 % of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values: 0.0536 mg/mL (DDPH), 0.0451 mg/mL (reducing power), and 0.00251 mg/mL (TBARS)

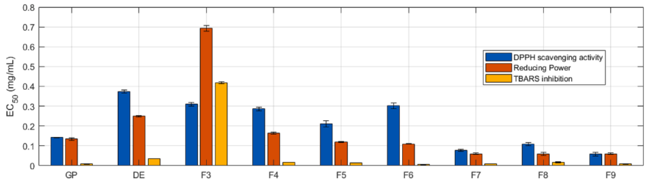

Fig. 11. Antioxidant activity for a GP extract, a DE extract and fractions obtained by sorption/desorption of the DE extract with the molecularly imprinted particles.

The EC_{50} values for the DE extract in various assays were determined as follows: 0.373 ± 0.008 mg/mL in the DPPH assay, 0.250 ± 0.004 mg/mL in the reducing power assay, and 0.036 ± 0.0007 mg/mL in TBARS inhibition. It is important to note that the DE extract has not been extensively studied in terms of its phenolic composition or antioxidant activity, making direct comparisons with reported results challenging. However, when considering the findings published by Cristina et al. (Duarte et al., Mar. 2023) for the same extract, obtained under identical conditions to ours, we observe lower EC_{50} values, specifically 0.17 ± 0.01 mg/mL in the DPPH assay.

In evaluating the outcomes of the sorption/desorption processes using the MIP particles as adsorbent, it is crucial to emphasize the noteworthy findings. It was observed that all fractions, except for F3, exhibited superior results, indicated by lower EC_{50} values, signifying heightened antioxidant potency compared to the initial DE extract (see Table 4 and Fig. 11).

A plausible interpretation of these findings is rooted in Fig.s 9 and 10, depicting the estimated enrichment factors for the fractions obtained after treatment with the MIP sorbent. From that data, a distinct trend emerges as the organic solvent content increases, because there is a noticeable enhancement in the presence of flavonols across nearly all fractions. These results demonstrate the effectiveness of the molecularly imprinted sorbent for the concentration and enrichment of flavonoids, namely flavonols. The enriched fractions exhibit remarkably attractive EC_{50} values, indicating their potential utility across various industries.

Particularly, for fraction F9 was measured a total phenolic content c. a. 3.8 times higher than the original extract (see Fig. 10). Moreover, the DPPH scavenging activity, the reducing power and the TBARS inhibition of this fraction are estimated to be 6.4, 4.2 and 4.5 times higher, respectively, than the initial diatomaceous earth extract (see Table 4). Notably, as presented in Table 4 and Fig. 11, many fractions purified exhibited superior results as compared to a raw grape pomace extract, which is a product currently employed in some industries as a natural antioxidant.

It is worth noting that flavonoids have both antioxidant properties and radical scavenging activity, but the extent of their activity can vary depending on their specific chemical structure and the conditions of the assay. Generally, it is difficult to make a broad statement about which group shows more radical scavenging activity because it can depend on factors such as the specific compound within each class and the type of free radicals being targeted (Pietta, Jul. 2000). Flavonols, such as quercetin and kaempferol, are known for their strong antioxidant properties due to the presence of multiple hydroxyl groups on their structure and they are effective scavengers of various types of free radicals (Pietta, Jul. 2000).

4. Conclusions

This paper focuses on the valorisation of phenolic compounds found in winemaking by-products, specifically in spent diatomaceous earth, used in industry for wine filtration. The study involved the synthesis at the gram-scale of a custom-designed adsorbent through the inverse suspension polymerization technique. Special attention was given to particular polymerization conditions, trying to avoid solvents weakening the interaction between pyridyl groups of the functional monomer (4-vinylpyridine) and the template molecule (quercetin). The selected conditions were specifically designed to produce a material with high performance, making it well-suited for utilization in a large-scale continuous process (e.g. avoiding high backpressure through control of the size of the molecularly imprinted polymer particles). The synthesized adsorbent was subsequently used to work in a designed pilot size sorption/desorption prototype running fractionation processes based on hydroalcoholic solvents.

The different fractions produced with the sorption/desorption prototype were evaluated for their total phenolic content and antioxidant activity with three different assays (radical scavenging activity, reducing power and inhibition of lipid peroxidation). Fractions with a higher TPC than the initial extract were obtained, emphasizing the successful enrichment of phenolic compounds. Interestingly, the rise in phenolic content correlated with increased antioxidant activity of these fractions. Indeed, the results obtained show the improvement of the bioactivity of most of the fractions comparatively to the original diatomaceous earth extract. For the most enriched fraction was measured a total phenolic content c.a. 3.8 times higher than the original extract. The DPPH scavenging activity, the reducing power and the TBARS inhibition of this fraction were estimated to be 6.4, 4.2 and 4.5 times higher, respectively, than the initial diatomaceous earth extract. Notably, many fractions purified exhibited superior results as compared to a raw grape pomace extract, which is a product currently employed in some industries as a natural antioxidant.

This work combines in the same research materials development, process design and application to real extracts with demonstration of improvement of the bioactivity of purified products. The proof of concept here presented can be extended to the purification of other kinds of winemaking residues or to the valorisation of other sorts of biomass with contribution for sustainability and circular bioeconomy.

CRediT authorship contribution statement

Catarina P. Gomes: Writing – original draft, Investigation, Data curation. **Cristina N. Duarte:** Writing – original draft, Investigation, Data curation. **Cláudia D. Martins:** Writing – original draft, Investigation, Data curation. **Joana S. Amaral:** Writing – review & editing, Supervision, Conceptualization. **Getúlio Igrejas:** Writing – review & editing, Supervision, Conceptualization. **Maria João V. Pereira:** Writing – review & editing, Supervision, Conceptualization. **Mário Rui P.F.N. Costa:** Writing – review & editing, Conceptualization. **Rolando C.S. Dias:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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