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Synthesis of caffeic acid and p-hydroxybenzoic acid molecularly imprinted polymers and their application for the selective extraction of polyphenols from olive mill waste waters

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Abstract

Using caffeic acid and *p*-hydroxybenzoic acid as templates, two molecularly imprinted polymers (MIPs) were prepared that were used for isolation of polyphenols from olive mill waste water samples (OMWWs) without previous pre-treatment. For the preparation of the caffeic acid MIPs 4-vinylpyridine, allylurea, allylaniline and methacrylic acid were tested as functional monomers, ethylene glycol dimethylacrylate (EDMA), pentaerythritol trimethylacrylate (PETRA) and divinylbenzene 80 (DVB80) as cross-linkers and tetrahydrofuran as porogen. For *p*-hydroxybenzoic acid 4-vinylpyridine, allylurea and allylaniline were tested as functional monomers, EDMA and PETRA as cross-linkers and acetonitrile as porogen. The performance of the synthesized polymers was evaluated against seven structurally related compounds by means of polymer-based HPLC. The two polymers that presented the most interesting properties were further evaluated by batch rebinding and from the derived isotherms their capacity and binding strength were determined. Using solid-phase extraction (SPE), their ability to recognize and bind the template molecule from an aqueous solution as well as the pH dependence of the binding strength were explored. After establishing the best SPE protocol, an aqueous model mixture of compounds and a raw OMWWs sample were loaded on the two best polymers. The result of the consecutive use of the two polymers on the same sample was explored. It was concluded that acidic conditions favour the recognition abilities of both polymers and that they can be used for a quick and efficient isolation of the polyphenol fraction directly from raw OMWW.

Keywords: Molecular imprinting; MIPs; Solid-phase extraction; Olive mill waste waters; *p*-Hydroxybenzoic acid; Caffeic acid

1. Introduction

The need for separation of specific compounds from complex mixtures, industrial or biological, has lead to an increase in the synthesis and use of molecularly imprinted polymers (MIPs), which in fact act as biomimetic materials [1]. The synthesis of a MIP is based on the polymerization of a certain monomer, containing functional groups complementary to the one present on the target molecule, thus forming adequately strong covalent or non-covalent bonds. A cross-linker is added to co-polymerize with the monomer and produce a rigid polymer network with the desired physicochemical properties. The polymerization takes place in solution, usually initiated by a free radical initiator. After the polymerization is complete the template is removed under mild extraction conditions (e.g. Soxhlet extraction using a polar solvent) and the cavities left are complementary to the template in terms of size, shape and functionality, thereby serving as recognition sites for the template used.

MIPs have been extensively used for the imprinting of pharmaceuticals, pesticides, carbohydrates, peptides and other molecules of biological interest [2]. Comparatively little attention has been paid on phenolic compounds, which are nonetheless of great interest for both food and pharmaceutical industry, mainly because of their antioxidant properties. At the same time, they are among the main pollutants found in liquid waste of the food industry (e.g. wine production, olive

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oil production, etc.). They are water soluble and when found in increased concentrations present phytotoxical properties [3,4].

Among the wide group of polyphenols, *p*-hydroxybenzoic acid has attracted some attention and has been successfully imprinted [5–8] using 4-vinylpyridine, acrylamide and methacrylic acid. Protocatechuic acid has also been used as template for the synthesis of an acrylamide-based MIP for the separation of structurally related compounds from plant material [9]. As far as caffeic acid is concerned, a MIP using methacrylic acid as monomer has been synthesized but it aimed to the separation and purification of the structurally related chlorogenic acid [10]. Except for the work of Dmitrienko et al. [6], where the recognizing ability of the polymer was evaluated in aqueous solutions, the rest of the cited publications refer to the behaviour of the polymer in an organic solvent, mainly acetonitrile. Even though in all of the above works successful imprinting of phenolic compounds has been proved plausible, the authors feel the need of further testing such polymers under aqueous conditions given the polyphenols' high occurrence in aqueous waste. Their efficient removal from aqueous waste is of great environmental interest and at the same time of economical value since these type of materials can be reused many times without significant loss of properties.

The present paper deals with the synthesis of MIPs using caffeic acid and *p*-hydroxybenzoic acid as template molecules. More emphasis has been put on the synthesis of an effective caffeic acid MIP, since *p*-hydroxybenzoic acid MIPs have been more extensively investigated, as mentioned above. The produced MIPs were evaluated for their efficiency in recognizing the template molecules primarily in an organic solvent by means of polymer-based HPLC and secondly in an aqueous environment by means of SPE. Especially, their recognition ability in an aqueous environment was assessed by using a mixture of seven structurally related compounds. Finally, raw olive mill waste water samples were also applied on the polymers in order to evaluate their efficiency towards such a complex aqueous mixture. Summarizing, the goal was to propose an alternative method to conventional liquid–liquid extraction used so far, for the isolation of polyphenols from olive mill waste waters.

2. Experimental

2.1. Materials

Gallic acid (GA), *p*-hydroxybenzoic acid (*p*-HBA), vanillic acid (VA), caffeic acid (CA) and vanillin (V) were of HPLC grade and were purchased from Fluka (Buchs, Switzerland). Tyrosol and veratric acid were a kind donation of Laboratory of Agroindustrial Chemistry, INP (Tolouse, France). 4-Vinylpyridine (4-VPy), methacrylic acid (MAA), allylurea and allylaniline monomers along with EDMA and DVB80 cross-linkers were provided by Sigma–Aldrich. PETRA cross-linker was purchased from Fluka. Azo-bis-dimethylvaleronitrile (ABDV) initiator was purchased from Wako (Neuss, Germany). Water (HPLC grade), methanol (MeOH; HPLC grade), acetonitrile (MeCN; HPLC grade) and tetrahydrofuran (THF; HPLC grade) were purchased from Merck (Darmstadt, Germany). Triethanolamine (TEA), ethyl acetate (EtOAc), acetic acid and phosphoric acid 85%, all of analytical grade, were provided by Fluka. For the HPLC evaluation of the polymers system, a system comprising of two LC-10ADVP Shimadzu HPLC pumps controlled by a SCL-10A$_{VP}$ Shimadzu pump controller (Kyoto, Japan), a manual Rheodyne injector with a 20 μL loop (Cotati, CA, USA), a column oven and a Shimadzu UV-diode array detection (UV-DAD) system, model SPD-M6A were used. For data collection and peak area calculations the softwareClass-LC10 (Shimadzu) was used. HPLC evaluation was performed at 25 °C at a flow rate of 1 mL/min and the detector was set at 254, 280 and 325 nm. Each compound was injected at least twice. MeCN with 0.05% CH_3COOH was used as elution solvent, in order to slightly reduce the long retention times. Acetone 1 mM in MeCN was used as void marker.

2.2. Synthesis of MIPs

Seven caffeic acid and four *p*-hydroxybenzoic acid imprinted polymers were synthesized. The composition of the pre-polymerization mixtures is described in Table 1. In all cases, the pre-polymerization mixture was dissolved in the porogen, which was THF for the caffeic acid-based MIPs and MeCN for the *p*-hydroxybenzoic acid MIPs, and was placed in a thick-walled glass tube with a narrow neck. The mixture was emerged in an ice-bath and purged with nitrogen gas for 5 min in order to remove oxygen and establish inert supernatant atmosphere. Afterwards, the glass tube was hermetically sealed, placed in a water bath at 40 °C and left for 24 h for the polymerization to proceed. The glass tubes were smashed for the removal of the polymer monolith. The collected polymers were slightly ground and subjected to Soxhlet extraction with MeOH in order

Table 1
Composition of the produced polymers (ABDV 1%, w/w was used as the free radical initiator)

Polymer	Template (T)	Monomer (M)	Cross-linker (C)	Ratio T:M:C
MIP 1	CA	4-VPy	EDMA	1:4:20
NIP 1	–	4-VPy	EDMA	1:4:20
MIP 2	CA	4-VPy	PETRA	1:4:12
NIP 2	–	4-VPy	PETRA	1:4:12
MIP 3	CA	4-VPy	DVB80	1:4:20
NIP 3	–	4-VPy	DVB80	1:4:20
MIP 4	CA	Allylurea	PETRA	1:4:12
NIP 4	–	Allylurea	PETRA	1:4:12
MIP 5	CA	MAA	PETRA	1:4:12
NIP 5	–	MAA	PETRA	1:4:12
MIP 6	CA	MAA	EDMA	1:4:20
NIP 6	–	MAA	EDMA	1:4:20
MIP 7	CA	Allylaniline	EDMA	1:4:20
NIP 7	–	Allylaniline	EDMA	1:4:20
MIP 8	*p*-HBA	4-VPy	EDMA	1:4:20
NIP 8	–	4-VPy	EDMA	1:4:20
MIP 9	*p*-HBA	4-VPy	PETRA	1:4:12
NIP 9	–	4-VPy	PETRA	1:4:12
MIP 10	*p*-HBA	Allylurea	PETRA	1:4:12
NIP 10	–	Allylurea	PETRA	1:4:12
MIP 11	*p*-HBA	Allylaniline	EDMA	1:4:20
NIP 11	–	Allylaniline	EDMA	1:4:20

to remove the template and remaining un-reacted monomers and soluble oligomers. Thereafter, it was ground and sieved to particles of 25–50 μm (for HPLC) and 50–100 μm (for SPE) in size, followed by repeated sedimentation in methanol in order to remove fine particles (<25 μm). For the preparation of non-molecularly imprinted polymers (NIPs) the same procedures were applied, except for the addition of template in the synthesis step.

2.3. HPLC analysis

The synthesized polymers were evaluated by means of HPLC. The produced ground and sieved polymer particles were packed in an HPLC column (30 mm × 4.6 mm I.D.) which was subsequently connected to the main HPLC unit. Columns were equilibrated with the eluent solvent for 1 h prior to the first injection and for 15 min between injections. As a measure of the polymers' efficiency, the retention factors were calculated using the equation:

$$k = \frac{t_R - t_O}{t_O} \quad (1)$$

where t_R is the retention time of each analyte and t_o is the retention time of the void marker. For better clarification of the imprinting effect, the imprinting factor for each analyte is presented, which was calculated using the equation:

$$\text{I.F.} = \frac{k_{MIP}}{k_{NIP}} \quad (2)$$

2.4. Batch rebinding

The imprinted polymers that exhibited the best recognition ability as assessed by HPLC, were subjected to batch rebinding experiments. Thus, 5 mg of imprinted and non-imprinted polymer was weighed in HPLC glass vials and incubated for 24 h with a 1 mL solution of template in MeCN with concentrations ranging from 0.1 to 20 mM. The supernatant was removed and analyzed by HPLC in order to measure the concentration of the analyte that was not bound by the polymers. Each experimental point is the average of three repetitions. The derived binding isotherm data were least-squares fitted by a Freundlich isotherm.

2.5. SPE analysis

In order to establish the optimum conditions under which the templates can be recognized by the corresponding MIPs, a standard solution of each template in water was initially prepared and used in SPE mode. Thus, 100 mg of polymer, MIP or NIP, was packed between polyethylene frits in 3 mL polypropylene tubes. Samples were loaded at different pH values (3.5, 5.5 and 7) while for the elution, mixtures of ethyl acetate, methanol and acetic acid were tested, with the concentrations of methanol and acetic acid ranging from 1 to 90% and from 1 to 10%, respectively. Ethyl acetate was incorporated in the elution mixture as it is widely used in liquid–liquid extractions of OMWW samples and is considered to be a selective solvent for low molecular mass polyphenols [11]. Analogous procedure was followed for the *p*-hydroxybenzoic acid polymer but the sample pH values used were 2, 3.5, 5.5 and 7.

Once establishing the most effective protocol for both MIPs, a reference mixture consisting of gallic acid, protocatechuic acid, tyrosol, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid and veratric acid in water was applied on the cartridges. The concentration of each analyte in the mixture was 0.3 mg/mL. After each step, the eluate was collected and analyzed using the above described HPLC system. The column used for the analysis was a C-18 Nucleosil, 250 mm × 4.6 mm I.D., 5 μm (Macherey-Nagel). Phosphate buffer at pH 2 (A) and MeCN + 10% MeOH + 10% phosphate buffer pH 2 (B) was used as a mobile phase, the flow rate used was 0.75 mL/min and the

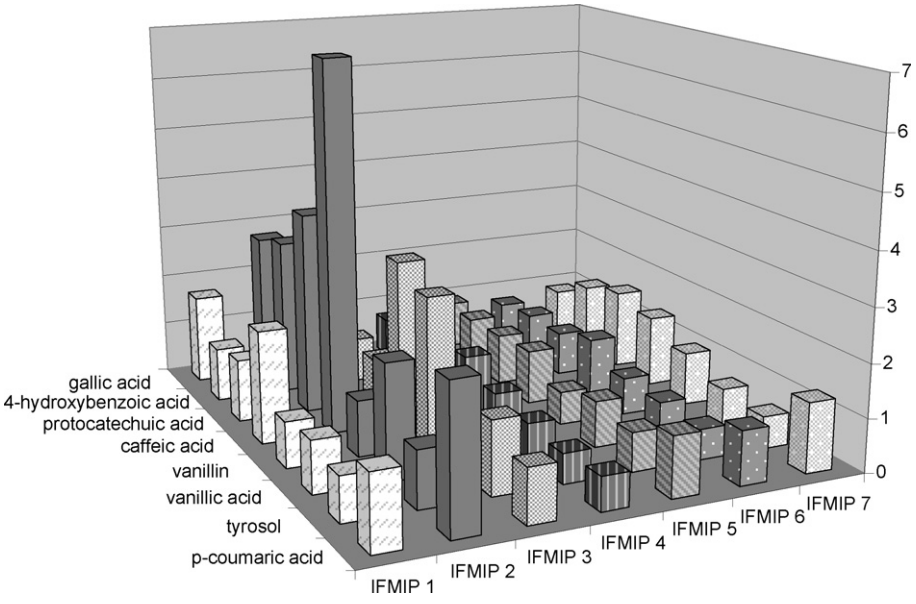

Fig. 1. Imprinting factors of the selected polyphenols on the polymers prepared using caffeic acid as a template.

analysis of the polyphenol mixture was conducted at 35 °C under the following gradient program: 0–5 min 98% (A), 5.01–10 min 95% (A), 10.01–15 min 90% (A), 15.01–25 min 85% (A), 25.01–30 min 80% (A), 30.01–40 min 65% (A), 40.01–50 min 50% (A), 50–55 min 20% (A), 55–60 min 98% (A). Detection was done at 254, 280 and 325 nm.

Both the established protocols and the developed HPLC method, were also used for the application of OMWW on the polymers.

2.6. Liquid–liquid extraction

For comparison purposes, a liquid–liquid extraction of the polyphenol fraction of OMWW was performed, in order to assess the clean-up potency of the prepared polymers. The adopted method, slightly modified, is a well established one [11–13]. Specifically, 5 mL of sample was acidified to pH 2 and centrifuged to remove the solids. The supernatant was collected and extracted 3 times with 20 mL hexane in order to remove the lipid fraction and then 3 times with 35 mL ethyl acetate. The ethyl acetate layers were collected and combined, dried over Na_2SO_4 and evaporated to dryness. The yellowish residue was re-dissolved in methanol and analyzed with the above HPLC method.

3. Results and discussion

3.1. HPLC analysis results

The recognition ability of the produced MIPs was examined by HPLC and the results are presented in graph form in Figs. 1 and 2 as a function of the imprinting factor. As can be seen from the figures, successful imprinting occurred mostly in the case of MIP 2 and MIP 8. The difference between the IF for the templates and the other tested compounds (Fig. 3) reveals the selectivity of these polymers towards their corresponding templates. The rest of the prepared polymers seem to be largely non-selective, since they retain the templates and their structurally related compounds almost equally.

3.2. Effect of functional monomer and cross-linker

The monomers used for the imprinting of caffeic acid were 4-vinylpyridine, methacrylic acid, allylaniline and allylurea and the same were used for the imprinting of *p*-hydroxybenzoic acid except for methacrylic acid. The main aim for the preparation of these MIPs was their use directly on OMWW samples for an efficient isolation of the polyphenols that they contain, avoiding the voluminous use of solvents and multiple extraction steps that liquid extraction and other techniques require. For this reason, commercial monomers were used instead of novel synthesized specific monomers for these acidic templates, like those developed by Sellergren and coworkers [14]. The above monomers were selected because of their ability to form strong electrostatic bonds with the templates.

Among the monomers used, 4-VPy exhibited the best recognition ability both for CA and *p*-HBA. The pK_a of caffeic acid is ∼4.9 which is close to that of MAA (pK_a 4.7) and 4-VPy (pK_a 5.4). On the other hand, allylaniline has a slightly lower pK_a value (pK_a 4.2), yet it shows better recognition than MAA. It is argued that except for the electrostatic interactions and the hydrogen bonds, that apply in varying strength for all the monomers used, additional π–π interactions between these two monomers and CA account for the better recognition of the produced polymers. Both allylurea- and allylaniline-based polymers were additionally tested in a basic environment, with the addition of TEA in the elution solvent, but no considerable increase of the IF occurred and thus, these polymers were not further examined. As far as *p*-HBA is concerned, it was also best imprinted using 4-VPy [5,6,8]. Its pK_a value of 4.6 is closer to

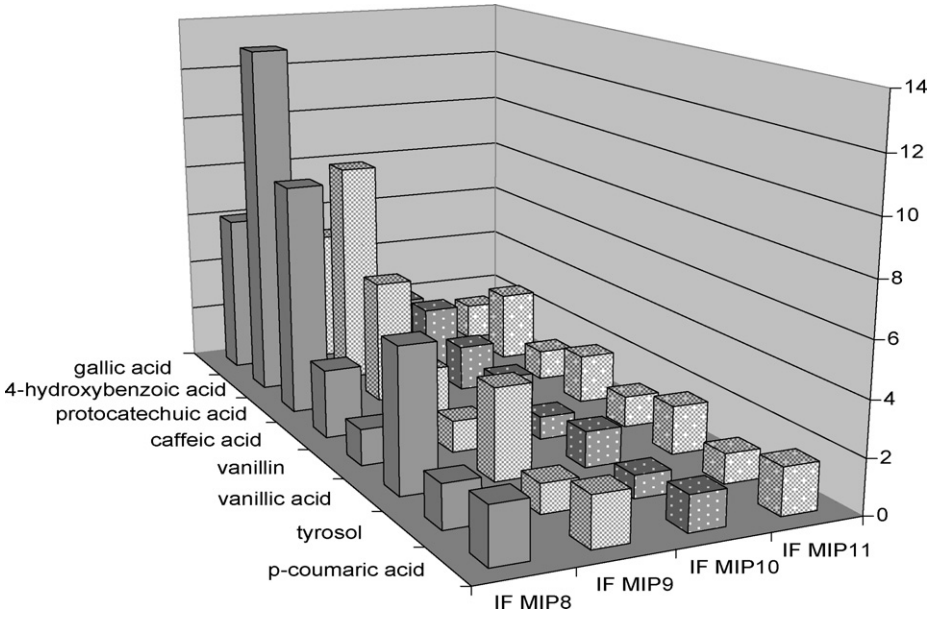

Fig. 2. Imprinting factors of the selected polyphenols on the polymers prepared using *p*-hydroxybenzoic acid as a template.

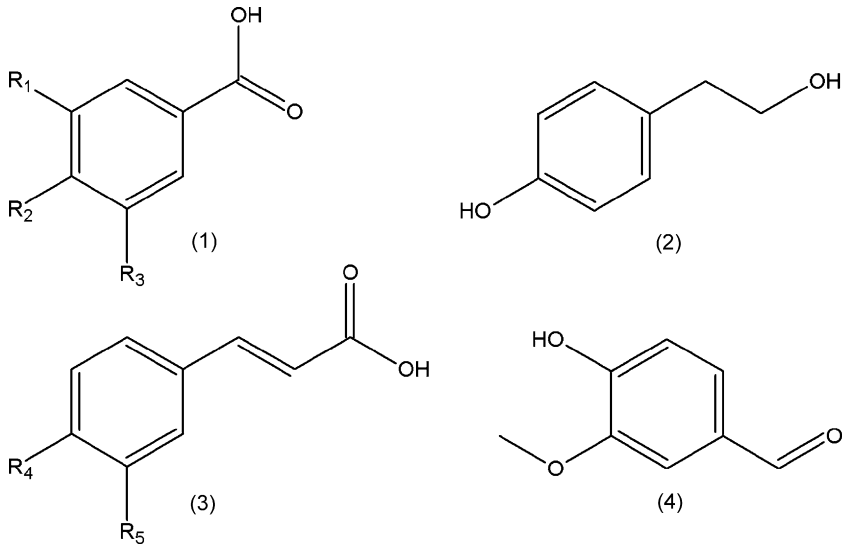

Fig. 3. Structures of the studied templates: (1) $R_1 = R_3 = H$, $R_2 = OH$ *p*-hydroxybenzoic acid; $R_1 = R_2 = OH$, $R_3 = H$ protocatechuic acid; $R_1 = R_2 = R_3 = OH$ gallic acid; $R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$ vanillic acid; (2) tyrosol; (3) $R_4 = OH$, $R_5 = H$ *p*-coumaric acid; $R_4 = R_5 = OH$ caffeic acid; (4) vanillin.

that of allylaniline than to that of 4-VPy, yet it forms a stronger complex with 4-VPy and the explanation for this probably lies upon the stronger basic character of 4-VPy.

The retention of the rest of the compounds examined on MIP 2 agrees with the number of hydroxyl groups present in the molecule. Gallic acid and protocatechuic acid bear hydroxyl groups in the same positions as caffeic acid and probably for this reason they can bind to the sites created for caffeic acid, yet more weakly since their size is a lot smaller. The existence of the methoxy groups in the molecules of vanillin and vanillic acid reduces their potency to form strong hydrogen bonds and the same applies for tyrosol which, even though similar in size with caffeic acid, does not bear a carboxyl group and cannot interact strongly with the basic 4-VPy.

Similar observations apply for MIP 8. The order of retention is gallic acid > protocatechuic acid > *p*-hydroxybenzoic acid > caffeic acid > vanillic acid > *p*-coumaric acid > tyrosol > vanillin, which is consistent with the number of hydroxyl groups in the molecule, the size of the molecule and the presence of methoxy or carboxyl groups.

In both of the above polymers, even though the retention of some compounds is higher than that of the template, the imprinting factor of the templates is higher and therefore successful imprinting has occurred. The order of retention at the respective NIPs is in both cases: gallic acid > protocatechuic acid > caffeic acid > *p*-hydroxybenzoic acid > *p*-coumaric acid > vanillic acid > tyrosol > vanillin. Since in the case of the NIPs there are no specific sites present, the interactions are mainly of ionic nature and thus, non-specific. Hence, the above order can be explained as it coincides with the number of hydroxyl groups present in the molecule. Considering these, the higher retention of gallic acid and protocatechuic acid on the synthesized MIPs could be attributed to non-specific ionic interactions.

As far as the cross-linkers are concerned the more flexible, highly hydrophilic PETRA performs the best in caffeic acid imprinting but the more rigid EDMA seems to be the cross-linker of choice in the case of *p*-hydroxybenzoic acid. This could be attributed to the fact that caffeic acid has a flexible long C-chain similar to PETRA, while *p*-hydroxybenzoic acid is a rigid molecule and is presumably better imprinted using a rigid cross-linker such as EDMA. DVB80 was also used as a cross-linker in the imprinting of CA. It is known to enhance the rigidity of the polymer chains, and probably for this reason it appears to slightly improve the recognition ability of the produced MIP. Yet it lacks oxygen groups and therefore its only possible contribution, apart from the rigidity of the network, is the additional π–π interactions with the template, which obviously did not contribute enough to the recognition ability of the produced polymer.

3.3. Batch rebinding

Batch rebinding experiments were conducted for both polymers in MeCN. The resulting isotherms are displayed in Figs. 4 and 5 along with the fitting of the experimental points to the Freundlich isotherm:

$$q = aC^m$$

where q (μmol g^{-1}) is the amount of adsorbed analyte per unit of polymer mass and C (mmol L^{-1}) is the concentration of the analyte in solution at equilibrium. The a parameter of the Freundlich equation reflects the distribution of binding sites of different binding strength that are present in the polymer and from this parameter the capacity (N) and the average affinity (K_a) can be calculated. The parameter m is the heterogeneity index, with values from zero to one, by one indicating homogeneity of the sites [15,16].

The Freundlich isotherm was chosen over the Langmuir or bi-Langmuir isotherms for the fitting of the experimental data as the experimental points fall on a straight line when plotted in a log q vs. log C format. The results of the mathematical treatment of the obtained data reveal some interesting facts (Table 2). In both polymer sets the observed differences in binding performance

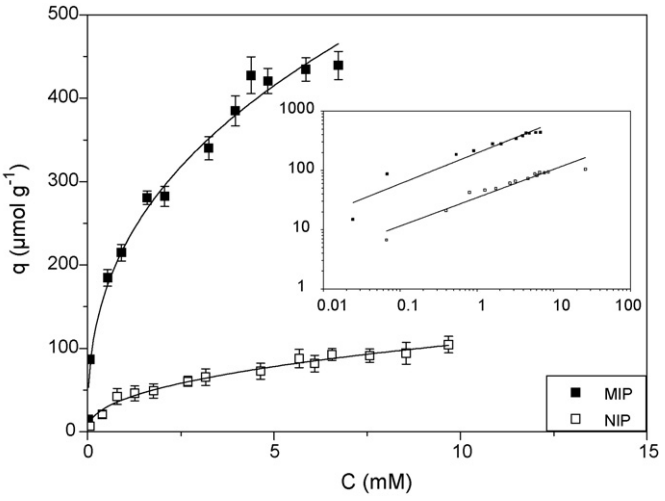

Fig. 4. Batch rebinding adsorption isotherm of caffeic acid in MeCN on MIP and NIP 2 and fitting to the Freundlich isotherm (solid curves).

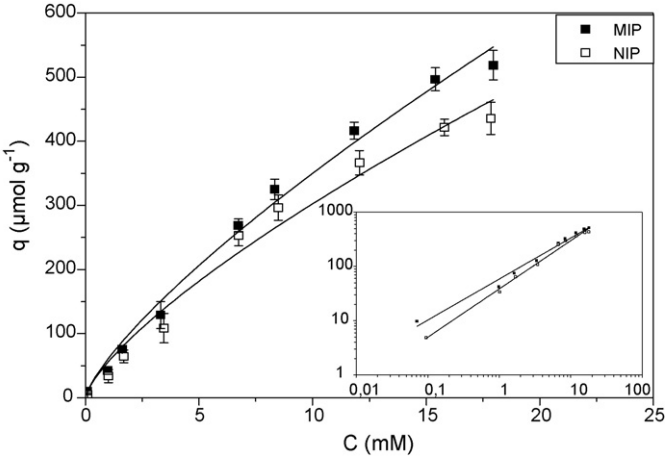

Fig. 5. Batch rebinding adsorption isotherm of *p*-hydroxybenzoic acid in MeCN on MIP and NIP 8 and fitting to the Freundlich isotherm (solid curves).

can be attributed, mainly, to the difference in the number of binding sites (N) as in both cases the affinity constants (K) are essentially the same. Thus, both MIPs studied possess almost 6 times the number of sites of the corresponding NIPs. This is not unexpected since the templates at hand have no significant geometric characteristics leading to the generation of sites of limited shape recognition and, thus, limiting the binding effect essentially to the electrostatic attraction between an acid and a base. Such sites, however, seem to be able to achieve significant discrimination between the templates as shown by the results of the chromatographic evaluation.

Table 2
Fitting parameters of the batch rebinding data to the Freundlich isotherm and calculated average association constants and average number of binding sites

	a	m	N (μmol g^{-1})	K (M^{-1})
MIP 2	196.01	0.518	12.01	972
NIP 2	34.56	0.520	2.09	968
MIP 8	58.70	0.764	1.28	417
NIP 8	38.52	0.891	0.24	322

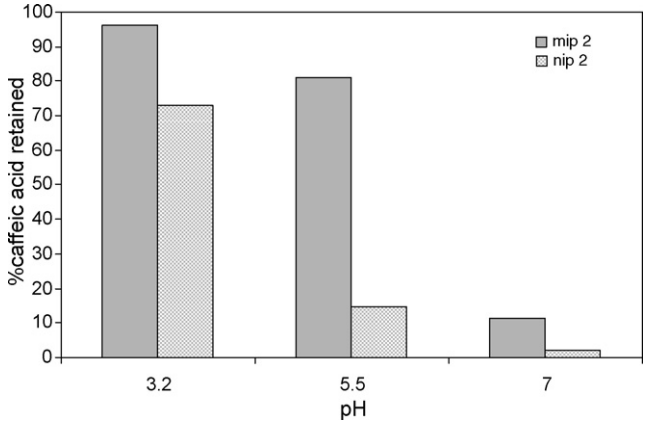

Fig. 6. Effect of pH on the percentage of caffeic acid retained on the imprinted and non-imprinted polymer.

3.4. SPE testing and optimization

3.4.1. MIP 2 prepared for caffeic acid

After establishing the efficiency of the polymer to rebind the template in acetonitrile, its applicability in a water sample was evaluated. For this reason, aqueous caffeic acid samples were prepared. In all cases, the cartridge was conditioned with 2 mL water, followed by the application of 1 mL of sample solution and a first washing step with 1 mL water, to bring forward the hydrophobic properties of the MIP [9]. The initial screening showed that a 1 mL wash with MeCN was crucial in order for the selectivity to be revealed, as washing the columns with MeCN caused the elution of caffeic acid only from the NIP column. In the aqueous working environment, the template and the polymer interact largely due to hydrophobic interactions and the addition of an organic washing step is needed so as to bring forward the polymer's specificity [17]. Testing revealed that pH played an important role in the performance of the MIP under aqueous conditions (Fig. 6).

At first the pH of the solution was not altered, as the dilution of caffeic acid leads to a solution of pH 3.5. At this pH the selectivity of the MIP is not pronounced. When the pH of the solution was increased to 5.5 the difference became obvious. The retention of caffeic acid on the MIP after the washing steps, increased to 80%, while only 15% was retained on the NIP and the rest was eluted during the intermediate steps.

At the acidic pH of the caffeic acid solution, the latter is mostly in its acid form while 4-VPy is highly protonated. Therefore it can be argued that the electrostatic interactions between the polymer and the template are the main reason for the increased binding observed on both polymers, thus, masking the selectivity of the imprinted polymer, which is attributed to the size and shape of the template. Increasing the pH of the sample closer to the pK_a of 4-VPy, both the polymer and the template are close to their isoelectric points which is the ideal situation for the recognition process. Thus, the imprinted polymer that possesses specific binding sites can retain the template effectively as opposed to the non-imprinted which retains the template mainly due to electrostatic interactions, whose weak nature becomes obvious during the elution steps [18]. Increas-

Table 3
The recovery percentages of caffeic acid from MIP 2 using different elution mixtures

Elution systems tested	% of recovered caffeic acid
4 mL 98% EtOAc + 1% MeOH + 1% CH_3COOH	41.5
4 mL 50% EtOAc + 45% MeOH + 5% CH_3COOH	80.83
4 mL 35% EtOAc + 60% MeOH + 5% CH_3COOH	81.71
4 mL 10% EtOAc + 85% MeOH + 5% CH_3COOH	70.88
4 mL 90% MeOH + 10% CH_3COOH	77.05

ing pH to 7, 4-VPy remains neutral but the carboxylic group of caffeic acid is now completely deprotonated. Thus, caffeic acid has lost its ability to form hydrogen bonds at the binding sites and accordingly both retention and selectivity are dramatically decreased [19].

Upon establishing the optimum pH conditions for the MIP, it was attempted to increase the amount of water and acetonitrile used during the washing steps in order to achieve the best possible clean-up. It was found that by increasing the amount of water up to 3 mL caused an increase in the quantity of caffeic acid eluted equal to 3.2% of the amount washed out by 1 mL of water. Additionally, the increase of MeCN volume up to 4 mL did not cause any further elution of caffeic acid. Therefore, it was decided that 2 mL of water and 3 mL of MeCN to be used at the washing steps.

Further testing regarding the elution system resulted in a solution consisting of 35% ethyl acetate, 60% methanol and 5% acetic acid as being the optimum elution solvent mixture. In Table 3 are presented the release percentages achieved with some of the elution mixtures tested.

The final established protocol consisted of an initial conditioning of the column with 2 mL water, application of 1 mL of sample at pH 5.5, washing with 2 mL water and 3 mL of MeCN, elution with 4 mL 35% ethyl acetate + 60% MeOH + 5% CH_3COOH and subsequent washing of the column with 2 mL water pH 7, to restore the charge of the column, and with 2 mL MeOH.

3.4.2. MIP 8 prepared for p-hydroxybenzoic acid

For the *p*-hydroxybenzoic acid MIP the same procedure was followed, that is, an aqueous sample was applied on the polymer and the influence of the pH conditions on its performance was examined. The results are presented in Fig. 7.

Even though the functional monomer used for both polymers is the same, it interacts differently with each template. It appears that *p*-hydroxybenzoic acid is bound mostly in its neutral form, as noticed also by Dimitrienko et al. [6], with electrostatic interactions playing a smaller role in the recognition procedure, since by increasing the pH, the binding decreases. In view of the fact that at pH 3.5 the MIP's selectivity is more pronounced, this value was selected for further testing.

In view of the above, it can be concluded that for both polymers the pH of the solution is the factor that reveals the specific binding capacity of the polymers and enhances their recognition ability which is a result of the efficiency of imprinting. Consid-

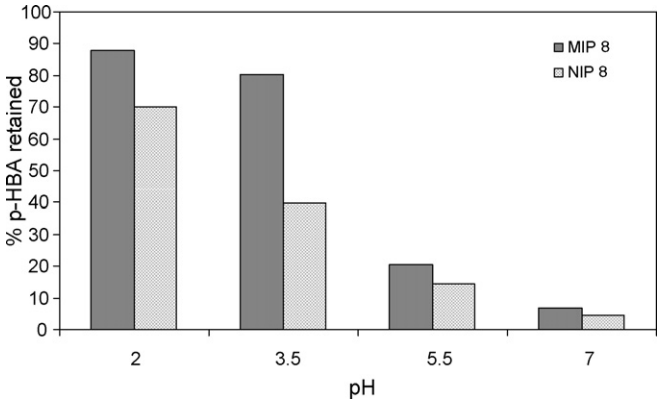

Fig. 7. Effect of pH on the percentage of *p*-hydroxybenzoic acid retained on the imprinted and non-imprinted polymer.

ering that the template–monomer complexes can be of, both, molecular and electrostatic nature, selecting the optimum pH value is essential not only for achieving high capacity, but also for bringing forward the selectivity of the polymer [20,21] in aqueous conditions.

The SPE protocol already adopted for MIP 2 was employed for MIP 8 as well. The initial screening revealed that washing with 2 mL H_2O did not affect the binding capacity of the polymer, but at the second washing step, the addition of more than 1 mL of MeCN caused increased elution of the bound template. The elution mixture and quantity was found to be suitable and therefore was not altered. Thus, it was decided to follow the above mentioned protocol of caffeic acid MIP for the *p*-hydroxybenzoic acid MIP as well, altering only the amount of MeCN during the washing steps by using only 1 mL.

3.5. SPE separation of a mixture of structurally related compounds

After establishing the optimum protocol for the application of caffeic acid on the MIP, a mixture of structurally related compounds was used in order to evaluate the selectivity of the polymer for caffeic acid. The compounds selected were among those commonly found in OMWW. From the compounds used *p*-coumaric acid bears the best structural resemblance, as its only difference is the luck of a hydroxyl-group at the *para* position. Nonetheless, all the compounds bear carboxyl and multiple hydroxyl groups being for this reason prone to forming hydrogen bonds with the polymer matrix. Thus, strong competition for both specific and non-specific binding sites is expected. The adjustment of the loading pH of the sample should enhance the selectivity towards the template, according to the findings discussed above. A slight modification of the protocol established was also attempted, by using hexane and hexane/THF mixtures instead of MeCN as a second elution step. These solvents were selected with the view of using the MIP with OMWW, for the removal of active non-polar compounds. The results showed that even though these solvents did not affect the retention ability of the polymer, they did not enhance its selectivity either and were therefore abandoned.

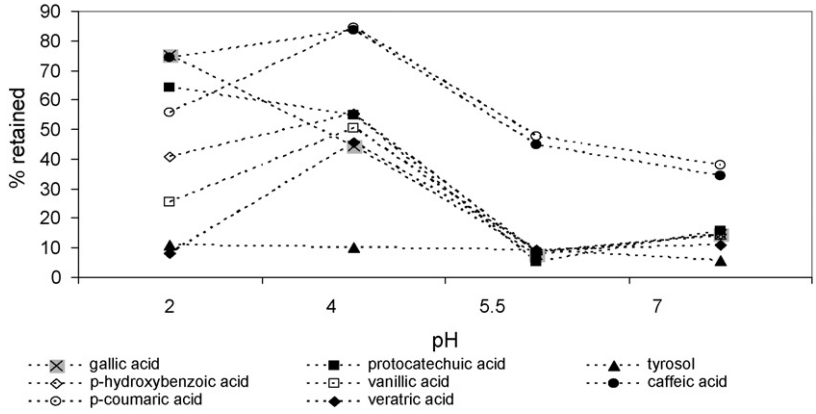

Fig. 8. Effect of pH on the percentage of each polyphenol retained on MIP 2.

The effect of pH on the selectivity of the polymer was re-evaluated for the mixture of the compounds to ensure that its recognition ability for the template at pH 5.5 is not affected by the presence of structurally related compounds. The results for the retention on MIP 2 are presented in Fig. 8.

At the acidic pH 2, 4-VPy is protonated while polyphenols are mostly undissociated. The percentage of the retention of the compounds follows the number of hydroxyl groups in their molecule. Both, gallic and protocatechuic acids are smaller in size compared to caffeic acid and possess hydroxyl groups at positions 3 and 4 as does caffeic acid, therefore they possibly occupy some of the specific binding sites and are for this reason well retained. *p*-Hydroxybenzoic acid and vanillic acid have only one contact point, either through their carboxyl group or sole hydroxyl group, while veratric acid can interact solely through its carboxyl group. Increasing pH to 4, the degree of protonation of the polymer is reduced while an increasing percentage of the compounds approach their isoelectric point. At this stage both hydrogen bonding and electrostatic interactions account for the binding, which is mainly non-specific, and thus retention is increased. Even though the retention at this pH is high, it is not considered as a suitable working pH, since the final solution contains considerable amounts of all of the compounds. At pH 5.5 the polymer matrix has reached its pK_a and therefore has an overall neutral charge, but the polyphenols are negatively charged due to the dissociation of the carboxyl group. Therefore, they attach to the surface through electrostatic interactions and for this reason are easily eluted during the washing steps. On the contrary, caffeic acid is bound to the specific cavities and is retained only to be eluted during the final elution steps. Unfortunately, the same applies for *p*-coumaric acid. The retention ability of the MIP for caffeic acid from the mixture is reduced compared to retention from a solution of caffeic acid alone, but this can be attributed to the structural resemblance of the compounds employed, their competition for binding to the active sites, and the interactions between them. Increasing the pH further has a negative effect on both retention and recognition, as already discussed above. Since at pH 5.5 the recognition of caffeic acid is pronounced, this pH value was selected as the working pH. Still, should the objective be the removal of a polyphenol fraction from an aqueous sample, lower pH values could be used, since the polymer is less specific and presents increased capacity.

The major part of the polyphenols loaded onto MIP 2 is washed off during the washing with water. For this reason it was decided to collect these eluates and load them onto MIP 8, in order to achieve a higher recovery from the initial mixture. Taking into account that at pH 3.5 the polymer recognizes better the template, the collected eluates were brought to this pH prior to loading them on the MIP column. The recovery rates ranged from 9 to 51%, with the highest being that of the template. Most of the other compounds were eluted during washing with MeCN, with the exception of tyrosol which was eluted almost completely with the aqueous wash, presenting the same behaviour as in the case of MIP 2. The retention order of the compounds is associated with their potency to form strong hydrogen bonds and therefore follows the order of the number of hydroxyl and carboxyl groups. Gallic acid and protocatechuic acid are the stronger retained, followed by *p*-coumaric and caffeic acid. Vanillic acid, veratric acid and tyrosol are the less retained. Nonetheless, the polymer's selectivity for the template is lessened in the aqueous environment and even though it maintains a high capacity, it retains gallic and protocatechuic acid in almost equal amounts as *p*-hydroxybenzoic acid.

3.6. Application on real OMWW sample

The two polymers were used consecutively for the removal and isolation of polyphenols from a real OMWW sample. Adequate quantity of OMWW was brought to pH 2 and centrifuged in order to free polyphenols bound to sugars and remove the suspended solids. The sample was then brought again to pH 5.5 in order to be applied onto MIP 2. The SPE protocol followed was the one established before. The eluate of the sample and the 2 mL of the aqueous wash were collected, brought to pH 3.5 and subsequently, were applied onto MIP 8. The final collected fractions from both polymers were analyzed by HPLC and the chromatograms are presented below (Fig. 9).

As can be seen from the chromatograms, MIP 2 can achieve a very significant pre-concentration of caffeic acid as indicated by chromatogram b while the sample as a whole is significantly cleaner. As far as MIP 8 is concerned, it is evident from chro-

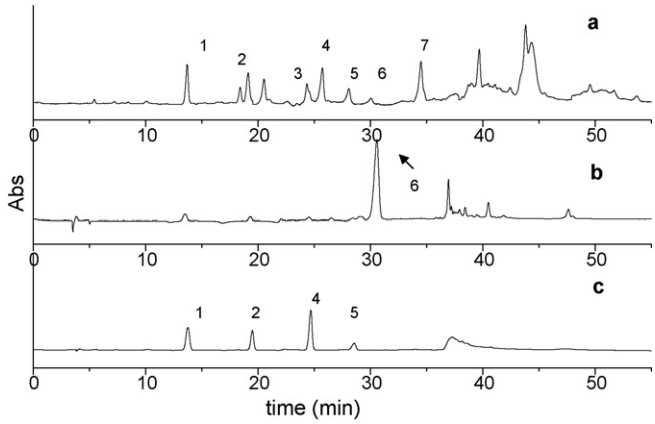

Fig. 9. Chromatograms of (a) olive mill waste water sample after liquid–liquid extraction (280 nm), (b) final extract obtained from MIP 2 (325 nm), (c) final extract obtained from MIP8 (280 nm). The peaks identified are (1) gallic acid, (2) protocatechuic acid, (3) tyrosol, (4) *p*-hydroxybenzoic acid, (5) vanillic acid, (6) caffeic acid, (7) *p*-coumaric acid.

matogram c that the sample is very well cleaned up, and the peak of *p*-HBA is dominant. Still, as already shown for the reference mixture, the selectivity is not as pronounced due to the aquatic environment and therefore gallic acid, protocatechuic acid and vanillic acid are co-retained considerably. Hydroxytyrosol and tyrosol were the only compounds that were not contained in the final extracts. Due to their structure they cannot be retained on the polymer and are quickly eluted during the washing steps with water.

4. Conclusion

In this work, the technique of molecular imprinting was used successfully for the preparation of polymers capable of recognizing caffeic acid and *p*-hydroxybenzoic acid from an aqueous mixture of structurally related compounds. The polymer prepared for caffeic acid was more hydrophilic, due to the existence of PETRA as a cross-linking agent. The polymer's specificity was pronounced at a slightly acidic pH, close to the pK_a value of the monomer and the template. On the other hand, the polymer prepared for *p*-hydroxybenzoic acid had EDMA as a cross-linking agent and even though it demonstrated good recognition ability in MeCN, it was not as selective in aqueous environment. Still, by properly adjusting the pH, the two polymers can be used sequentially for an efficient removal and recovery of the polyphenol fraction of a complex environmental sample such as olive mill waste water.

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