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Synthesis of water-compatible imprinted polymers of *in situ* produced fructosazine and 2,5-deoxyfructosazine

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ABSTRACT

Fructosazine and 2,5-deoxyfructosazine are two natural chemicals with various applications as flavoring agents in food and tobacco industry; the 2,5-deoxyfructosazine has also anti-diabetic and anti-inflammatory activities. In order to quantify these compounds in natural samples such as plant or food, we have developed a selective technique based on a water-compatible molecularly imprinted polymer (MIP). MIPs are prepared with a covalent approach from 2,5-deoxyfructosazine as template formed *in situ* by the self-condensation of glucosamine with vinylphenyl boronic acid, taken as catalyst and covalent monomer during the pre-complexation step. Acrylamide and polyethylene glycol diacrylate are used as supplementary non-covalent functional monomer and cross-linker, respectively. For the first time, a highly cross-linked but highly polar imprinted polymer of fructosazine and deoxyfructosazine is obtained as a solid material and not a gel. Amount of monomers is optimized to obtain high selectivity for both molecules. Results show that the MIPs prepared have a significant imprinting effect with a resulting imprinting factor of 3 for both templates. Molecularly imprinted solid-phase extraction is then performed and could be used in routine analysis to extract 2,5-deoxyfructosazine and fructosazine from soy sauce.

1. Introduction

The 2,5-*bis*(D-*arabino*-tetrahydroxybutyl)pyrazine (Fructosazine, FZ **1**) and the 2-(D-*arabino*-tetrahydroxybutyl)-5-(D-erythro-2,3,4-trihydroxybutyl)pyrazine (2,5-deoxyfructosazine, DFZ **2**) are natural chemicals which belong to the non-volatile (polyhydroyalkyl)pyrazines (Fig. 1). FZ and DFZ are widely used as flavoring agents and possess various applications such as flavors in food and tobacco industry [1–3]. They have been identified in soy sauce [4], caramel [5] and peanuts where they are involved in roasted food's color [6]. DFZ has been found to exhibit activity against diabetes [7–9] but also against immunological anti-inflammatory diseases [10]. DFZ has been prepared by several synthetic methods including the reaction of glucose [11] and fructose [12] with various ammonium salts in weak acidic medium (pH 5.3∼6.0) [13], the conversion of cellobiose and inulin [14], or by the self-reaction of glucosamine under neutral pH and phosphate buffers [10], or in hot methanolic alkaline solution [15]. More recently, Rohovec et al. reported a clean conversion of D-glucosamine hydrochloride to 2,5-deoxyfructosazine and fructosazine in the presence of phenylboronate or boronate [16]. Another approach is to extract them from plants (tobacco) or foods for products' characterization, quality control or food analysis. Few methods have been developed to extract those products by using soxhlet extraction and solid phase extraction on C_{18} cartridges [17] or using supersonic extraction [18]. However, these extractions performed only from tobacco samples are time consuming and cannot be applied in routine analysis.

In the last two decades, molecular imprinted polymers (MIP), pioneered by Wulff and Sarhan [19] and then expanded by Mosbach and Arshady [20], have emerged as powerful sorbent for selective solid-phase extraction of single compounds or compound classes from complex matrices [21–25]. MIPs are cross-linked polymers able to bind one target compound with high selectivity; for their synthesis, a complex is formed between a template molecule and one or more functional monomers by covalent or non-covalent interactions together with an appropriate porogen solvent and cross-linking monomer. After polymerization (generally through a radical process), the template molecule is removed leading to a polymer containing specific cavities or imprints for the analyte. The choice of monomer that is likely to form strong interactions with the template such as H-donors or acceptors, and non-polar groups is a critical parameter in the development of a MIP for solid phase extraction. Concerning sugars, several glucose imprinted polymers [26,27] are already developed but usually as hydrogels. Because of a larger application range in drug, food or cosmetic analysis, the synthesis of water-compatible polymers able to uptake compounds selectively from aqueous media is an actual tendency. It enables to

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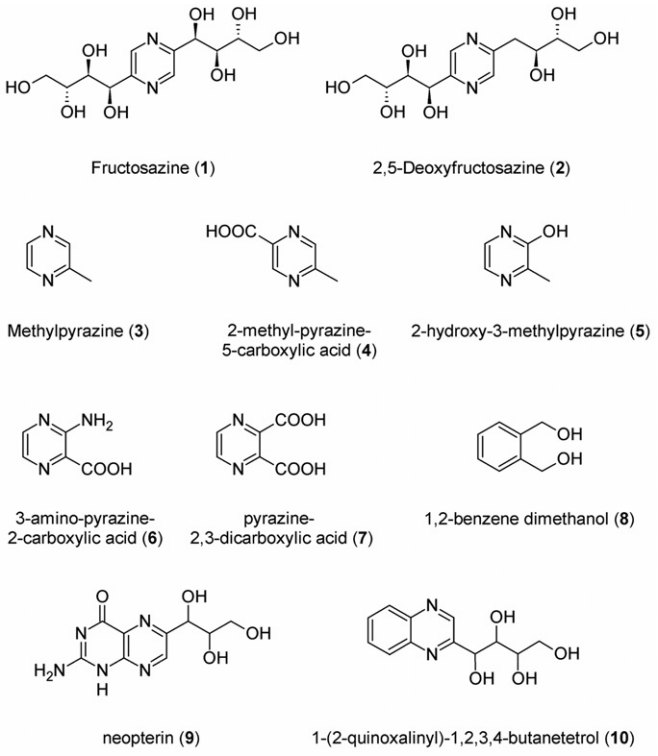

Fig. 1. Structures of *in situ* synthesized templates: fructosazine (1) and 2,5-deoxyfructosazine (2) and analogs used for selectivity studies (3–10).

increase the determination and extraction capabilities of highly polar compounds in aqueous sample [28–34]; however, the low water compatibility of MIP is still an issue, basically due to the lack of general strategies to imprint water-soluble compound as well as the poor recognitions of MIPs in water.

Thus, our first goal was to prepare a water-compatible polymer for FZ (**1**) and DFZ (**2**) extraction from food using water as porogen solvent; the templates (FZ and DFZ) are formed *in situ* from condensation of glucosamine with vinylphenyl boronic acid (VPBA) monomer during the polymer synthesis. In order to evaluate the capability of the extraction process, MIP is packed into a cartridge and a molecularly imprinted solid-phase extraction (MISPE) is performed to extract FZ and DFZ from soy sauce. This can be considered as an eco-friendly approach as both the polymerization and extraction are carried out in water.

2. Experimental

2.1. Chemical and reagents

Glucosamine hydrochloride and acrylamide (AA) were purchased respectively from Alfa Aesar (Schiltigheim, France) and Acros-Fisher Scientific (Illkirch, France). Polyethylene glycol diacrylate (PEGDA), vinylphenyl boronic acid (VPBA) and sodium persulfate were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). All chemicals and solvents were analytical or HPLC grade and were used without further purification. Ultra-pure water was provided by a UHQ system (Elga, High Wycombe, UK).

2.2. Instrumentations

Polymerizations were carried out in a UV irradiation system Bio-Link Crosslinker BLX-E365, Vilber Lourmat, France at the wavelength of 365 nm and with 5×8 W UV-tubes. Determinations of the exact mass of the templates were conducted in HRMS with a Bruker MaXis UHR-Qq-TOF spectrophotometer (Bremen, Germany) in positive electrospray ionization (ESI) mode, with the drying gas flow at 7 L min^{-1}, the drying gas temperature is set at 200 °C, the nebulizer pressure at 0.6 bar, the capillary voltage at 4500 V. Molecularly imprinted polymers are deposited onto silicon slides, and coated with 5 nm of gold using a thermal evaporator (Denton DV-502 A, Denton Vacuum, Moorestown, NJ, USA). Scanning electron micrographs (SEM) were obtained at 2 kV with Hitachi S4500 equipped with a Field Emission Gun (Hitachi Co, Tokyo, Japan). For BET surface measurements, a 150 mg quantity of polymers was degassed for 10 day at 60 °C under reduced pressure (< 7 μm Hg) to remove adsorbed gases and moisture. Surface area was performed at 77 K by Brunauer–Emmett–Teller (BET) on an ASAP 2020 surface area and porosity analyzer (Micrometrics Instrument Corporation, Créteil, France).

2.3. HPLC analysis

The instrumentation used to perform this work included a conventional LaChrom Elite HPLC system from VWR, Hitachi (Fontenay sous Bois, France) equipped with a L-2130 pump, an L-2200 autosampler, a Jetstream column oven and an L-2455 diode-array detector. Analyses were performed at 30 °C with a porous graphitic carbon (PGC) Hypercarb 30 mm × 4.6 mm column with diameter particules of 5 μm (ThermoHypersil, France). The mobile phase consisted of water as elution solvent A and acetonitrile as elution solvent B. The elution gradient was 100% of solvent A from 0 to 5 min then linear gradient to 70% of A at 10 min and going to 100% of B from 13 min to 14 min and remaining this composition stable for 10 min. Flow rate was set at 0.5 mL min^{-1}. Quantification is performed by UV detection at 275 nm. Analyses in semi-preparative HPLC were carried out with a LaChrom HPLC system from VWR, Hitachi (Fontenay sous Bois, France), equipped with a L-7100 pump and a L-2455 diode-array detector. An aliquot of 1 mL of the processed samples was injected into a porous graphitic carbon column 250 mm × 10 mm with diameter particles of 7 μm from Hypercarb (Hypersil). The mobile phase consisted in a volumic mixture of 87% of water and 13% of acetonitrile. Analyses were performed isocratically at 30 °C at a flow rate of 2 mL min^{-1} and chromatograms were recorded at 275 nm.

2.4. In situ synthesis of boronate-FZ (**11**) and boronate-DFZ (**12**)

The synthesis of boronate esters of FZ (**11**) and DFZ (**12**) was realized as described by Rohovec et al. [16]; in the optimized procedure, VPBA (0.5 mmol, 1 eq) was added to a solution of sodium hydroxide (2 eq) in water (3 mL). The solution was stirred at room temperature during 2 h until a clear solution was obtained. Then, D-Glucosamine hydrochloride (0.5 mmol, 1 eq) was added portionwise during 5 min. After 3 h of stirring, the light yellow solution was evaporated to dryness under vacuum at a temperature below 40 °C. The resulting yellow solid was composed of more than 95% of (**12**) and less than 5% of (**11**) and directly used as covalent template-monomers for the MIP. To confirm the structure of the products obtained by self-condensation of glucosamine under those conditions, the yellow solid was acidified (10% HCl) and the boronic acid was extracted with diethyl ether (twice 40 mL). The obtained aqueous phase containing FZ (**1**) and DFZ (**2**) was evaporated under reduced pressure and the resulting compounds were purified by semi-preparative HPLC and identified by HRMS: HRMS (ESI, *m*/*z*) for FZ (**1**): $[M+H]^+$ = calcd for $C_{12}H_{21}N_2O_8$, 321.1297; found, 321.1292; $[M+Na]^+$ = calcd for $C_{12}H_{20}N_2O_8Na$, 343.1117; found, 343.1112;

HRMS (ESI, *m/z*) for DFZ **(2)**: $[M+H]^+$ =calcd for $C_{12}H_{21}N_2O_7$, 305.1349; found, 305.1343; $[M+Na]^+$ =calcd for $C_{12}H_{20}N_2O_7Na$, 327.1168; found, 327.1163.

2.5. MIP synthesis

As stated above, the mixture of the two boronate esters (**11**) and (**12**) was directly diluted with ultrapure water into a glass vial. AA (4 eq) and PEGDA (20 eq) were added and mixed together. After deoxygenating the solution with bubbling nitrogen for 10 min, the initiator, sodium persulfate (0.15 eq) was added and the mixture was irradiated for 24 h under UV light (λ=365 nm, Bio-Link Crosslinker BLX-E365, Vilber Lourmat, France) at 20 °C. The obtained bulk polymers were ground and sieved through a 63 μm sieve and the smallest particles of polymers were removed by sedimentation in methanol. In order to remove the templates, polymers were washed in a soxhlet apparatus with ultrapure water during 10 h to remove FZ- and then with water/acetic acid (90/10, v/v) during 8 h to remove DFZ, then dried under reduced pressure. The efficiency of this procedure was checked by analyzing the washing fractions of polymers by HPLC, determining the elimination of the template. Non-imprinted polymers (NIP) were prepared in the same manner except that boronate esters (**11**) and (**12**) were replaced by VPBA for the NIPa, meanwhile the boronate esters and VPBA were omitted for NIPb.

2.6. Batch and cartridge experiments

To assess the kinetic of rebinding of polymers towards DFZ and FZ, 10 mg of polymer (MIPs or NIPs) was placed in 1.5 mL polypropylene microtubes and incubated at room temperature in solutions of DFZ and FZ (0.5 mL, 0.3 mg L^{-1}) in ammonium acetate buffer 10 mM pH 11. After centrifugation for 5 min at 12,000 rpm to sediment particles, the supernatant was taken and analyzed by HPLC. For cartridge experiments, cartridges were packed with polymers (MIP, NIPa or NIPb, 50 mg) and conditioned with methanol (2 mL) then with ultrapure water (1 mL) followed by ammonium acetate buffer (1 mL, 10 mmol L^{-1}, pH 11). The same buffer solution spiked with DFZ and FZ (1 mL, 1.7 mg L^{-1}) was percolated through the cartridges. The washing step was performed by percolating ethanol (1 mL). FZ was then eluted with ultrapure water (1 mL) meanwhile the DFZ could be eluted with a mixture of ethanol and ammonium acetate (10 mM, pH 11) (80/20, 1 mL). In order to evaluate capacities of polymers, several MISPE were performed by percolating different amounts of DFZ and FZ (1 mL). For each concentration, amounts of binding molecules on MIP and NIPs were recovered.

2.7. Extraction from soy sauce

Commercially soy sauce was diluted 500 times with ammonium acetate buffer (10 mmol L^{-1} pH 11) in order to obtain 1.7 mg L^{-1}-DFZ solution and 0.7 mg L^{-1}-FZ solution. Then, this solution (1 mL) was percolated on polymers cartridges to perform MISPE as previously described.

3. Results and discussion

3.1. Design of MIPs

3.1.1. Choice polymerization solvent and cross-linker

The choice of porogen solvent for polymerization can affect the specific recognition [35], the affinity of imprinted sites [36] as well as the structure of the polymers [37]. In the case of extraction from aqueous solvents, polymerization solvents of MIP are often organic solvents in order to not interfere with polar interactions between template and monomers like hydrogen bonds or sensitive covalent bonds [38]. However, in our case, water provides stable ester bonds [39] and high solubility of glucosamine. Since classical cross-linkers were not suitable for polymerization in water, polyethylene glycol diacrylate (PEGDA) was selected as a cross-linker because of its satisfactory solubility in water [40,41] and the possibility to make bulk polymerization even in water. For the same reason, sodium persulfate was preferred to the more usual 2,2′-azobisisobutyronitrile (AIBN) as polymerization initiator.

3.1.2. Choice of functional monomers

Imprinting of saccharides has been performed by many groups using non-covalent [42], metal-coordinated [43] and covalent approaches (especially through arylboronic acids pioneered by Wulff [19] in the 1970 s). In fact, it is well known that boronic acid can form at alkaline pH covalent bonds with 1,2- or 1,3-*cis*-diols in both organic or aqueous phases [44], the formed cyclic boronate ester being cleaved at pH values lower than the pKa value usually 8. Since numerous hydroxyl groups are attached to the carbohydrate backbone, the formation of polymerizable boronic esters for MIP design is a good way to avoid the competition between the water hydroxyl groups and the saccharides ones. Various saccharide imprinted polymers with different forms such as grafted electrode, bulk, film have been described with boronic acid as functional covalent monomer [45–47]. Thus, to obtain a selective MIP for FZ (**1**) and DFZ (**2**) and because the D-glucosamine can lead to (**1**) and (**2**) by a self-condensation with phenylboronic acid followed by acidic cleavage of the boronate ester, we decided to apply the reported conditions [16] with the 4-vinylphenylboronic acid (VPBA) used as both catalyst and monomer (Fig. 2). However, to increase the low solubility of VPBA in water, two equivalent of sodium hydroxide were requested. Moreover, based on literature [16], we can assume that 1,2-bidentate and tridendate type of coordination in the borate ester exist leading to free hydroxyl groups in bidentate form. Thus, in order to increase the selectivity on the polymeric matrix, a non-covalent neutral monomer, acrylamide (AA) was used for potential complexation through hydrogen bonding of the free hydroxyl groups and the nitrogens of the pyrazine cycle. If methacrylic acid is the most commonly employed functional monomer for non-covalent imprinting, we preferred acrylamide as it is more soluble in water; 2150 g L^{-1} compared to 89 g L^{-1} for acrylic acid and forms stronger hydrogen-bonds in polar protic solvent than acrylic acid. Moreover, to perform non-covalent interactions, an excess of non-covalent functional monomers is usually used [48,49]. The commonly used ratio of non-covalent monomer was chosen (4 eq of AA).

3.2. Optimized synthesis of imprinted polymers

Depending on the concentration of VPBA (0.5, 1, 2, 4 eq) employed for the *in situ* synthesis of templates or for the polymer formation, four MIPs (P-0.5, P-1, P-2, P-4) were prepared from glucosamine (1 eq), VPBA (various amount), PEGDA (20 eq), AA (4 eq) and sodium persulfate (0.15 eq) at basic pH in 3 mL of water containing 2 eq of sodium hydroxide. Five NIPs were also synthesized, NIPa-0.5, NIPa-1, NIPa-2, NIPa-4 includes the same amount of VPBA than MIP but with no glucosamine (so no possible formation of boronate esters of FZ (**11**) and DFZ (**12**)) and NIPb with no glucosamine and no VPBA. Corresponding polymers were packed in cartridges to perform solid phase

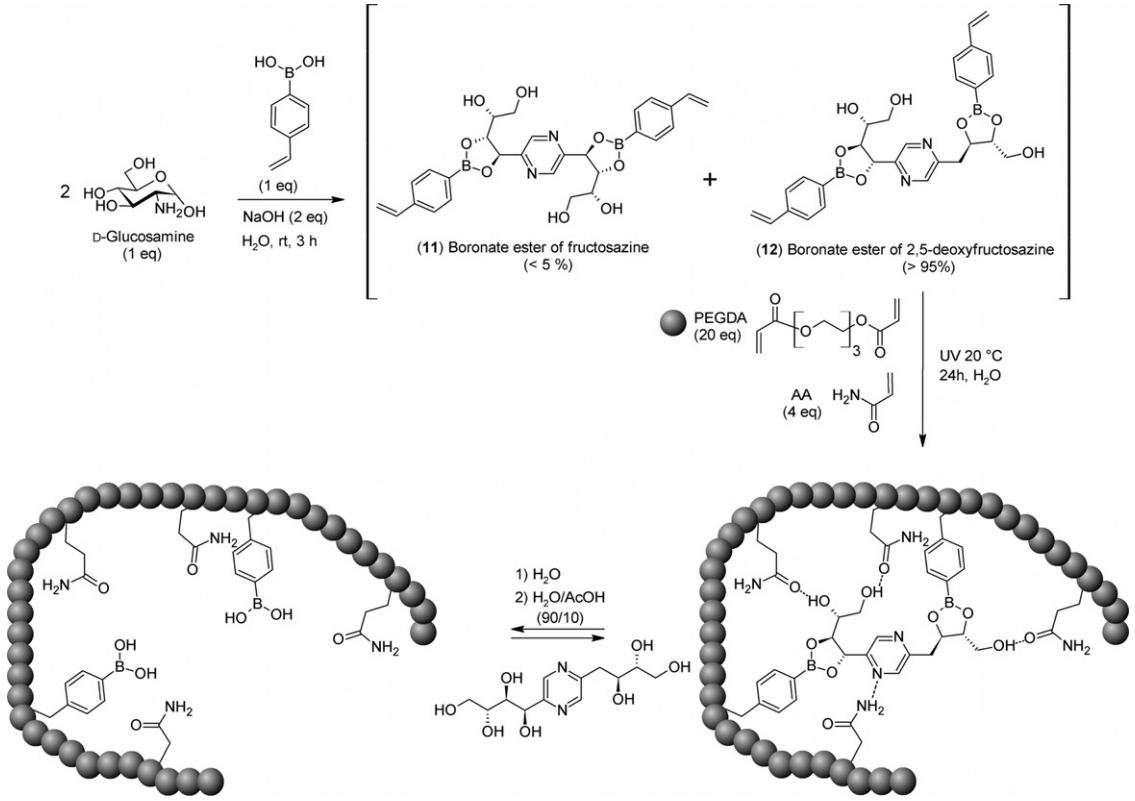

Fig. 2. Schematic representation of our imprinting strategy of DFZ.

extraction in order to select the best MIPs for extraction of FZ and DFZ.

3.2.1. *Influence of VPBA*

We have first studied the effect of different concentrations of VPBA on the ratio of boronic esters of FZ (**11**) and DFZ (**12**), respectively. By HRMS, it can be deduced that from 0.5 eq to 1 eq of VPBA, the major product formed is the boronate ester of DFZ (**12**) with less than 5% of boronate ester of FZ (**11**); with 2 eq of VPBA, the ratio (**11**) to (**12**) is 35/65, meanwhile with 4 eq of VPBA, both compounds are equally formed. The influence of VPBA on the selectivity of polymers was also assessed with the commonly used non-covalent monomers and cross-linker proportions (4 eq of acrylamide and 20 eq of PEGDA). Selectivity of the MIP does not seem to be correlated to the (**11**) to (**12**) ratio during the synthesis. With 0.5 eq, NIPs and P-0.5 are not selective of either FZ (**1**) or DFZ (**2**) since the number of specific cavities formed is not sufficient with rebinding below 15% and 5%, respectively. With 1 eq of VPBA, higher selectivity of P-1 (1 eq of VPBA) for FZ (**1**) ($>$ 90% binding) is observed and satisfactory selectivity for DFZ (**2**) is obtained ($>$ 55% binding). For higher VPBA's amount, while selectivity for FZ (**1**) is maintained, the one for DFZ (**2**) increases. However, non specific binding on NIPs increases in the same time and imprinting factors drop under 1.4 and moreover NIPa rebinds more FZ and DFZ for 4 eq of VPBA. Thus, the selectivity of polymer with 1 eq of VPBA is the most efficient for the extraction of both FZ (**1**) and DFZ (**2**) with a binding of 97% and 56%, respectively and an imprinting factor of 3 for both DFZ and FZ.

3.2.2. *Structures of imprinted polymers*

The surface morphologies of imprinted polymer P-1 were studied by using SEM. As shown on Fig. 3a, non homogeneous particles which with size lower than 63 μm were obtained. The surface of the P-1 with a cauliflower form exhibits high density of macropores (Fig. 3b). This macroporous structure is related to the protic and polar porogen solvent used for polymerization (water). Indeed, polar and protic solvents generally lead to macroporous structure and lower capacity than apolar solvents [50] but higher selectivity in our case.

Similar results of SEM were obtained for NIPb (Fig. 3**c**) and the values of specific surface area by BET measurements were also analogous *i.e.* 11 $m^2\ g^{-1}$ and 9.5 $m^2\ g^{-1}$ for P-1 and NIPb, respectively. This can be explained by the comparable surface polarity of these polymers. Indeed, P-1 contains VPBA inside cavities meanwhile NIPb does not contain VPBA which results in low surface polarity for both polymers and consequently the polymers have the same morphology.

On the contrary, a non-porous structure was obtained for NIPa-1 as we can see in SEM microphotograph (Fig. 3d) which is confirmed by the value of specific surface area (0.8 $m^2\ g^{-1}$). Small particles are joined together into conglomerates. The differences between P-1 and NIPa-1 morphology can be explained by the differences in VPBA's distribution in the polymer. We can presume that due to the presence of VPBA on the surface of NIPa-1, a higher surface polarity is expected and as a result a less porous polymer is obtained in water.

3.3. *Kinetics of binding*

The binding of FZ (**1**) on the imprinted polymers P-1, NIPa-1 and NIPb was assessed by kinetics studies (Fig. 4**a**). Then, kinetic parameters were studied and the adsorption on polymer was found to follow a pseudo-second-order kinetic (Fig. 4b), according to Eq. (1) [51] since t/Q_t *versus* t is a linear relation and $\ln(Q_m/Q_m - Q_t)$ *versus* t is not linear so the kinetic does not follow a

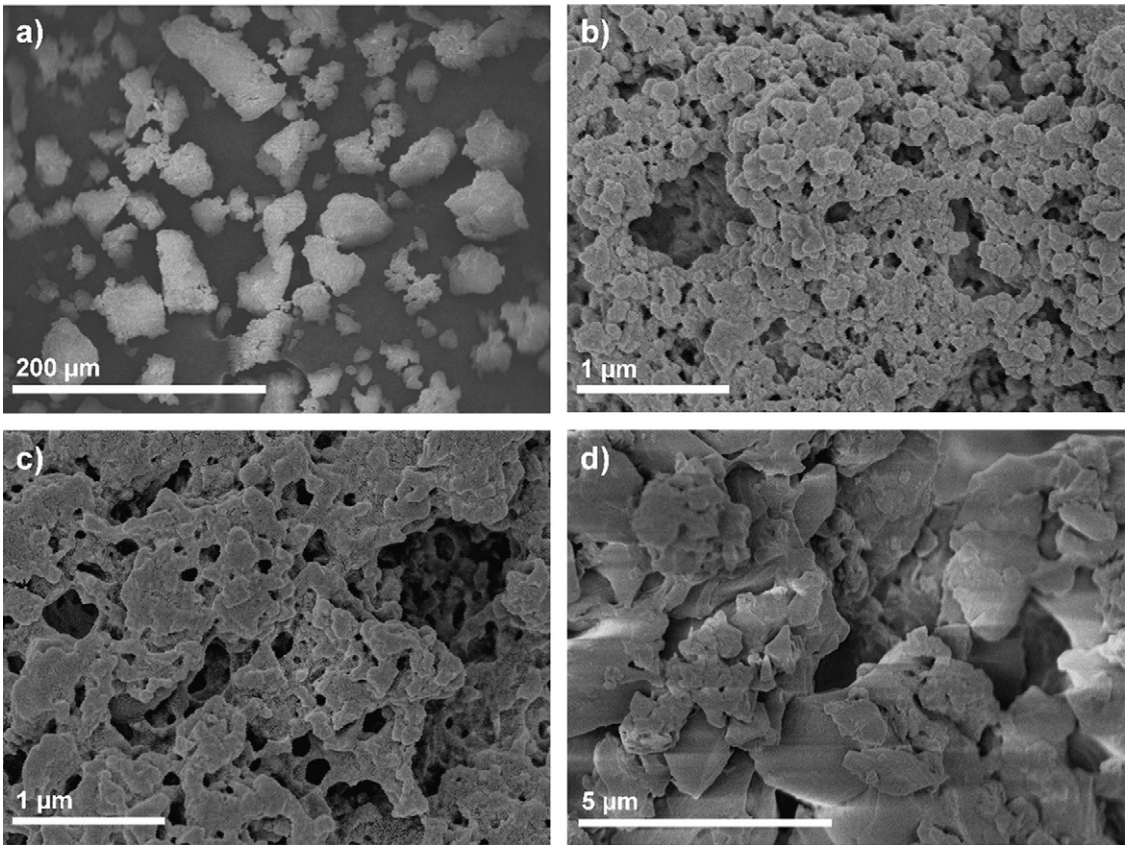

Fig. 3. SEM image of polymers: (a) particles of P-1, macroporous structures of a particle of (b) P-1, (c) NIPb and (d) NIPa-1.

pseudo-first-order kinetic.[52].

$$\frac{dQ_t}{dt} = k_2(Q_m - Q_t)^2 \text{ which can be integrated to } \frac{t}{Q_t} = \frac{1}{k_2 Q_m^2} + \frac{t}{Q_t} \quad (1)$$

where Q_t (mg g^{-1}) is the amount of template adsorbed per gram of polymers at t time, Q_m (mg g^{-1}) is the maximum adsorption on the polymer when the equilibrium state is reached for a given concentration in the solution, k_2 (mg^{-1} g min^{-1}) is the pseudo-second-order kinetic constant and t (min) is the time.

Thus, kinetic parameters for P-1 and the NIPs were determined (Table 1). The half-adsorption time $t_{1/2}$ is the time taken for the polymer to absorb half of the maximum quantity ($Q_m/2$). As we can observe, maximum of adsorption Q_m is higher on P-1 and is reached almost immediately ($t_{1/2}$=0.1 min). Furthermore, Q_m is very low for NIPb and adsorption kinetic is very slow ($t_{1/2}$=45 min) in comparison with NIPa-1 and P-1, which confirms the important role of VPBA on binding. The significant difference for Q_m and $t_{1/2}$ between P-1 and NIPa-1 confirms the different behavior and thus the imprinting effect. The difference in the morphology observed by SEM and BET measurements partially explains the difference in Q_m values.

Consequently, the fast kinetic of adsorption on P-1 makes this method suitable for SPE and routine analysis. Similar results were obtained for DFZ (data not shown).

3.4. Evaluation of the adsorption capacity of polymers

Langmuir model was used to evaluate capacity and affinity of polymer P-1 for the template. Langmuir equation (Eq. (2)) describes equilibrium adsorption on binding sites of polymers.

$$q_e = Q_0 \frac{kC_e}{1 + kC_e} \quad (2)$$

where q_e (mg g^{-1}) is the amount of template adsorbed per gram of polymers, C_e is the equilibrium concentration of the template (mg L^{-1}), Q_0 is the maximum adsorption on the polymer (mg g^{-1}) and k (mL mg^{-1}) is the Langmuir constant related to the affinity of binding sites of polymers and to the measure of the energy of adsorption.

For several concentrations of templates (FZ and DFZ, respectively), evolution of the amount of the binding molecules on the cartridges is reported hereafter (Fig. 5).

The adsorption isotherms of P-1 increase more quickly that the NIPs' ones, which reveals that the MIP contains specific binding sites for both FZ and DFZ (higher k value in Eq. (2)). Moreover, maximum adsorption of DFZ and FZ on P-1 is obtained with 30 mg g^{-1} and with 2 mg g^{-1} of MIP (Table 2), respectively. These results can be compared to those obtained for the kinetic tests in the previous paragraph. For kinetic tests the maximal absorbed quantity of FZ was 1.3 µg g^{-1}. This value is much lower than the overall saturation quantity which is evaluated at 2.07 mg g^{-1}. This confirms that the kinetic values were obtained in a steady state of adsorption (out of the polymer saturation). The values given for NIPs have to be moderated by the kinetic results. Indeed, since the half-adsorption times are significant for NIPs (especially NIPb) the equilibrium state cannot be reached in cartridge experiment. As a consequence the results of the Langmuir isotherm cannot be taken into account at face value but are given to show tendencies.

The higher capacity value of the P-1 for DFZ can be explained by the higher amount of binding sites for DFZ. Indeed, HRMS studies suggested that DFZ is formed in bigger amount during

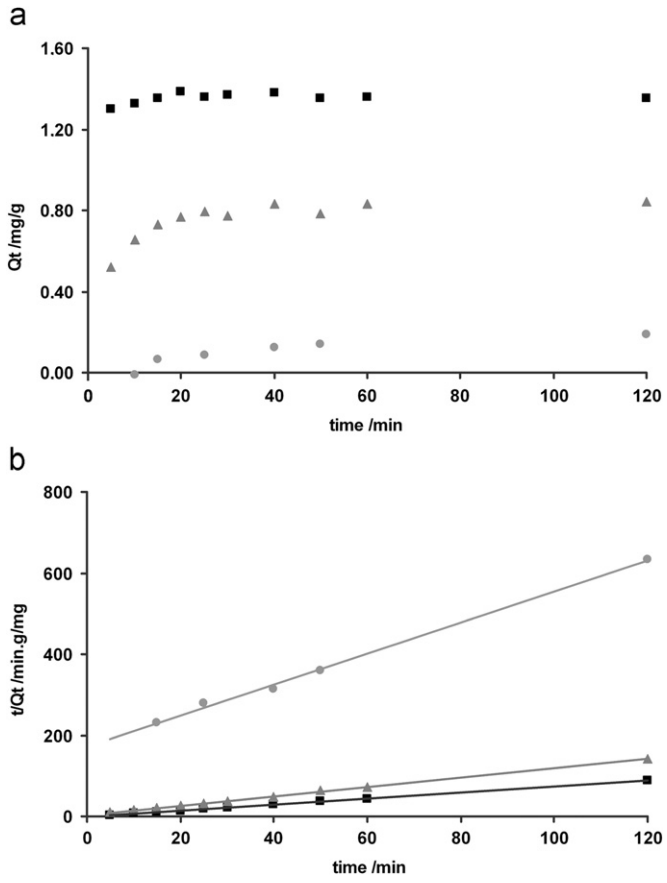

Fig. 4. Kinetics of rebinding of FZ on P-1 (squares), NIPa-1 (triangles) and NIPb (circles) (a) and pseudo-second-order kinetic proved by the linearity of t/Qt *versus* t graph (b).

Table 1
Kinetic parameters for polymers P-1, NIPa-1 and NIPb.

	Q_m (10^{-3} mg g^{-1})	k_2 (mg^{-1} g min^{-1})	$t_{1/2}$ (min)
P-1	1.3553	7408.3	0.1
NIPa-1	0.8655	401.5	2.9
NIPb	0.2612	85.2	45.0

polymerization of P-1. As a result, the higher the amount of DFZ, the higher the capacity value of the polymer P-1. However, the affinity of this polymer P-1, evaluated by Langmuir constants values (k, Table 2) is better for FZ than for DFZ which is probably due to the presence of one more possible interaction with the hydroxyl group. Moreover, the higher polarity of FZ ($\log P_{(FZ)} = -5.7$) compared to DFZ ($\log P_{(DFZ)} = -5.0$), calculated with Marvin Software 4.1.11, leads to stronger interaction with the polar polymer which is acting as a normal phase support. These polar interactions are unusual for a hard highly cross-linked polymer and are usually observed for hydrogels. These results confirm the higher selectivity of imprinted polymer P-1 for FZ.

3.5. Selectivity in cartridge experiments

As discussed above, the imprinted polymer P-1 showed highest selectivity for FZ with an extraction yield of 93% and satisfactory selectivity for DFZ (extraction yield of 41%). FZ is more retained on P-1 due to stronger interactions with boronic acids and the polar polymer. Since P-1 is acting as a normal phase support, elution of the more polar compound (FZ) is performed with the more polar solvent (100% water) whereas less polar

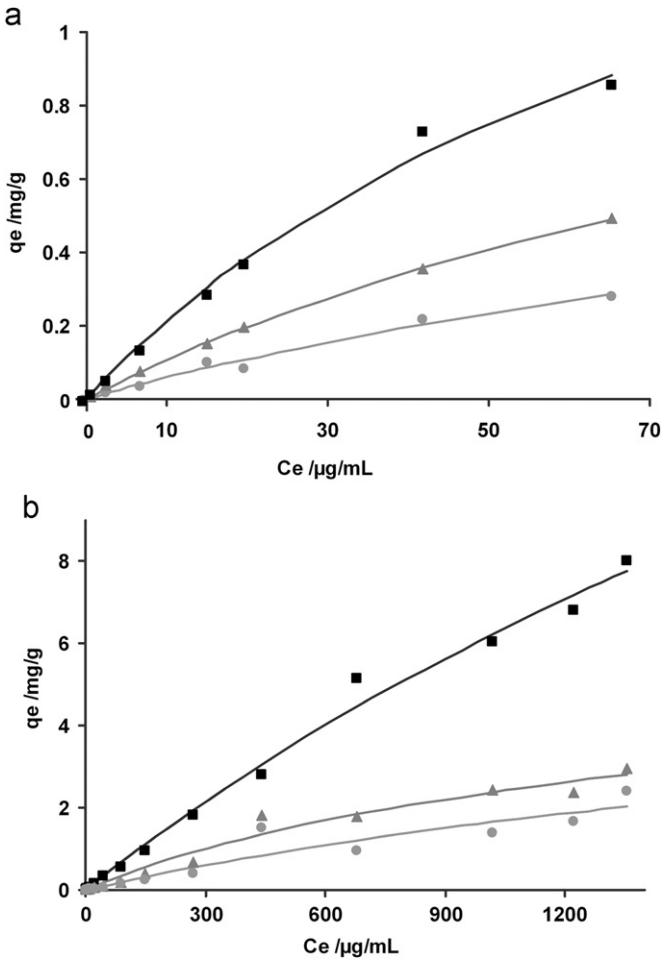

Fig. 5. Adsorption isotherms of polymers evaluated on P-1 (squares), NIPa-1 (triangles) and NIPb (circles) by the Langmuir model for FZ (a) and DFZ (b).

Table 2
Capacity and Langmuir constants of polymers for FZ and DFZ in comparison with the amount of binding molecules on polymers and the percentage of templates synthesized during polymerization.

	Polymers	% *in situ* synthesized	Capacity (Q_0, [mg g^{-1}])	% binding	Langmuir constant (k, 10^{-3} [mL mg^{-1}])
DFZ	NIPb		6.23	8	0.35
	NIPa-1		5.71	20	0.70
	P-1	> 95	30.15	56	0.26
FZ	NIPb		0.97	15	6.38
	NIPa-1		1.43	30	8.00
	P-1	< 5	2.07	97	11.38

solvent is used for the washing step (100% ethanol) or elution of DFZ (ethanol/ammonium acetate buffer pH 11, 80/20). Selectivity studies were performed on imprinted polymers P-1 for various molecules in non-competitive MISPE experiments. Very close structural analytes were chosen as well as analytes which can come from possible degradation of DFZ and FZ and represent thus possible interfering molecules of the real samples. All MISPE experiments for selectivity tests were performed with 2 µg mL^{-1} of various structurally related analytes ((**1**)–(**10**), Fig. 1) loaded on cartridges of 50 mg of imprinted polymer P-1 (Fig. 6). Imprinting factors (IFs) were calculated as the ratio of bindings on P-1 *versus* NIPa-1.

MISPE experiments showed that all analytes which can only develop non-covalent interactions with functional monomers like hydrogen bonds with acrylamide, π-π or ionic interactions with

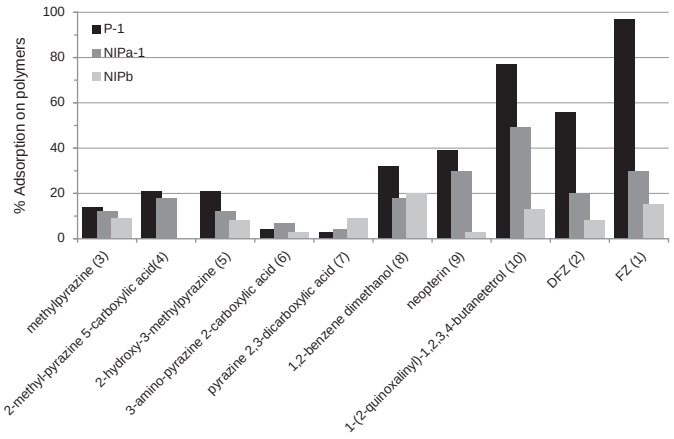

Fig. 6. Selectivity of MIP P-1 in cartridge experiments (50 mg). All compounds were loaded at the concentration of 2 μg mL^{-1}. For conditions see Section 2.6.

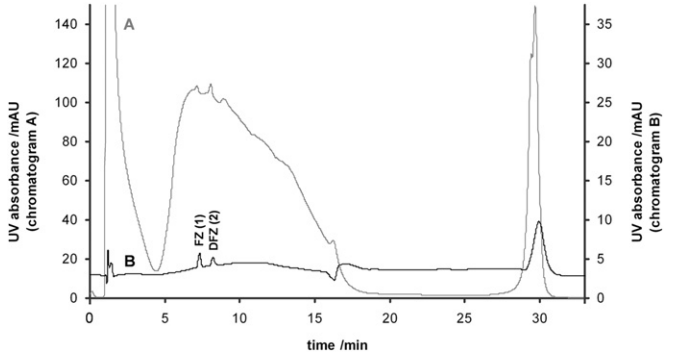

Fig. 7. Chromatograms of soy sauce before (A) and after (B) MISPE. The sample is analyzed in HPLC on PGC column with UV detection at 275 nm. Peaks corresponding to FZ (1) and DFZ (2) are observed in extracts after MISPE.

phenyl boronic acid, lead to low affinity. It is the case for methylpyrazine (**3**, IF < 1.2), 2-methylpyrazine-5-carboxylic acid (**4**, IF < 1.2), 2-hydroxy-3-methylpyrazine (**5**, IF < 1.8), 3-amino-pyrazine 2-carboxylic acid (**6**, IF < 1) and pyrazine 2,3-dicarboxylic acid (7, IF < 1). On the contrary, P-1 showed higher affinity for diols-containing analytes (**8**, **9**, **10**). Indeed, 1,2-benzenedimethanol (**8**) and neopterin (**9**) rebind 32% and 39% with IF of 1.6 and 1.2, respectively. These results reveal that the recognition mechanism is governed by the strong covalent interaction between boronic acid and diols and the role of acrylamide is less significant. As expected, between all interfering analytes, P-1 showed the highest affinity for 1-(2-quinoxalinyl)-1,2,3,4-butanetetrol (**10**, IF < 1.3) which is the closest structurally related to FZ and contains two diols able to interact with boronic acid. The imprinting factor between P-1 and NIPa-1 is much lower, this verifies the presence of specific cavities for FZ inside the MIP. In all cases, binding by NIPb-1 was very weak and NIPa-1 had affinity only for diols that confirms the role of boronic acid. Additionally, NIPa-1 showed less affinity for the analytes containing diols than P-1 which indicates high selectivity of the MIP and validates the presence of specific cavities. Finally, in real samples, we can assume that only molecules containing diols could compete with the binding of our target molecules.

3.6. Extraction of DFZ and FZ from soy sauce

Purification of DFZ and FZ on imprinted polymer P-1 was performed from soy sauce. Chromatograms of soy sauce (Fig. 7) before and after MISPE showed that both FZ and DFZ were present in the eluting samples. First, the identification of both molecules was based on correct retention times on porous graphitic carbon (PGC) column. Then, to confirm their presence the eluting fractions were spiked that results in an increase of the peaks areas. On the chromatogram of the eluting fraction of soy sauce, we can see that the peaks corresponding to FZ and DFZ were separated from the complex matrix. The fraction eluted by water contains mostly FZ. Indeed, the extraction yield is 89% for FZ and 22% for DFZ. DFZ is mostly recovered in another eluting fraction using ethanol/ammonium acetate buffer (80/20, v/v) at pH 11 with 38% extraction yield. As a result, FZ and DFZ can be extracted from soy sauce by P-1 and recovered by two different eluting fractions with high extraction yields (89% and 60%, respectively).

4. Conclusions

For the first time, a selective and water-compatible molecularly imprinted polymers of fructosazine and 2,5-deoxyfructosazine were synthesized with a covalent approach. VPBA showed a rapid reversible binding of diols of the template. Elements that compete in the synthesis of MIP were optimized and MISPE could be successfully performed to extract these two compounds from soy sauce with high selectivity. Eco-friendly parameters were also employed for the choice of porogen solvent of the MIP and extraction solvents. The resulting MIP shows a polar behavior with highly cross-linked polymer. This behavior is usually specific of hydrogel MIPs. Because of a simple and very specific extraction procedure combined with a simple separation method, MIP allows the extraction of fructosazine and 2,5-deoxyfructosazine from aqueous samples in routine analysis. The imprinted polymer P-1 allows the purification and separation of FZ and DFZ from foods samples by removing most of interfering peaks.

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