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Chromatographic characterization of molecularly imprinted polymers binding the herbicide 2,4,5-trichlorophenoxyacetic acid

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Abstract

Two polymers binding the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) were prepared by utilising the technique of the non-covalent molecular imprinting polymerisation in an aqueous medium. The polymers obtained were packed in HPLC columns and the effects of the mobile phase composition on the retention of the imprinting molecule and the selectivity of the stationary phases towards several analogous structures were studied by liquid chromatography. The columns showed a good level of selectivity towards the template and strictly related molecules. It was found that the molecular recognition mechanism acting on the columns was dependent on a combination of ion pair and hydrophobic interactions.

Keywords: Molecular imprinting; 2,4,5-Trichlorophenoxyacetic acid; Pesticides

1. Introduction

Polymeric stationary phases obtained with the technique of non-covalent molecular imprinting show molecular recognition properties with selectivity towards the template molecule. This peculiar molecular recognition effect is a consequence of the presence in the polymerisation mixture of monomers able to set up non-covalent interactions with the imprinting molecules. These interactions, typically hydrogen bonds, cause the formation of binding sites in the polymeric matrix which is characterised by the presence of different distinct points of interactions between the polymer and the template, and has a marked complementarity between the shape of the binding site and the shape of the imprinting molecule [1–6].

Limits to the non-covalent molecular imprinting are set by these conditions. In fact, the formation of interactions between monomers and the template are stabilised by hydrophobic environments, while polar environments disrupt them easily. Another limit is represented by the need of several distinct points of interactions: molecules characterised by a single interacting group, such as an isolated carboxyl, generally give imprinted polymers with very limited molecular recognition properties, that are of little interest in practical applications [7].

Recently, an alternative method has been proposed, in which 2,4-dichlorophenoxyacetic acid – a hydrophobic molecule – is used as a template in an imprinting procedure in polar medium [8,9]. This

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approach uses a polar medium to elicit hydrophobic binding sites in the polymer, while the interaction between template and binding site is modulated by an ionic pair between the acid and the basic nitrogen of 4-vinylpiridine, used as interacting monomer. So far, this polymer has been studied as an element of recognition in immunoassay-like studies and sensing devices [8–12], but no attempts have been made to use it as a stationary phase for liquid chromatography or solid-phase extraction. In this work we consider the chromatographic behaviour and the molecular recognition mechanism of polymers prepared using a strictly related molecule as template: the 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). This substance is a broad range herbicide, whose use has been banned in Europe and the USA owing to the danger of dioxin contamination connected with the commercial product [13,14]. Thus, it may represent an interesting environmental contaminant of anthropic origin.

2. Experimental

2.1. Materials

2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,3-dichlorophenoxyacetic acid (2,3-D) 3,4-dichlorophenoxyacetic-acid (3,4-D), 4-chlorophenoxyacetic acid (4-CPA), 2-methyl-4-chlorophenoxyacetic acid (MCPA), phenoxyacetic acid (PA), 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB), (±)-2-(2,4,5-trichlorophenoxy)propionic acid (fenoprop), (±)-2-(2,4-dichlorophenoxy)propionic acid (dichlorprop), (±)-2-(2-methyl-4-chlorophenoxy)propionic acid (mecoprop), ethylene dimethacrylate and 4-vinylpiridine were from Sigma-Aldrich-Fluka (Milano, Italy), all others reagents and organic solvents were supplied by Merck (Darmstadt, Germany).

4-vinylpyridine and ethylene glycol dimethacrylate were distilled at reduced pressure immediately before use. Phenoxyacid stock solutions were prepared by dissolving 20 mg of substance in 20 ml of acetonitrile and stored in the dark at −20°C.

The HPLC apparatus (pump L-6200, UV-VIS detector L-4200 and integrator D-2500) came from Hitachi-Merck (Darmstadt, Germany). The reverse phase HPLC column (C_{18} Macrosphere, mm 250× 4.6) came from Alltech (Milano, Italy).

2.2. Synthesis of methyl-2,4,5-trichlorophenoxyacetate (2,4,5-T-OMe)

The esterification of 2,4,5-T was carried out according to the general procedure indicated by Hassner et al. [15]. In a 50 ml round-bottom flask 0.200 g of 2,4,5-T, 0.177 g of *N*,*N*-dicyclohexylcarbodiimide and about 1 mg of 4-(*N*,*N*-dimethylamino)pyridine as catalyst were dissolved into 20 ml of anhydrous methanol. The mixture was stirred overnight at room temperature, then the *N*,*N*-dicyclohexylurea formed was separated by filtration in a G4 Buchner, and the methanol evaporated in a rotavapor. The residue, a clear viscous oil, was dissolved into 50 ml of dichloromethane, then washed three times with 20 ml of 10 m*M* aqueous hydrogen chloride and three times with 50 ml of water. The organic layer was dried over anhydrous sodium sulphate and evaporated under a gentle stream of nitrogen. The raw powder obtained was recrystallised twice in absolute ethanol, giving the pure product as a white powder (0.162 g, 78% yield), deemed pure by reverse phase liquid chromatography (mobile phase: methanol–water–acetic acid (69:30:1, v/v).

2.3. Synthesis of 2,4,5-trichlorophenoxyacetamide (2,4,5-T-$CONH_2$)

The amidation of 2,4,5-T was carried out according to the procedure indicated by Miron et al. [16]. In a 50 ml round-bottom flask 0.200 g of 2,4,5-T, 0.177 g of *N*,*N*-dicyclohexylcarbodiimide and 0.099 g of *N*-hydroxysuccinimide were dissolved into 20 ml of anhydrous tetrahydrofuran. The mixture was stirred overnight at room temperature, the *N*,*N*-dicyclohexylurea formed was separated by filtration in a G4 Buchner, and then 5 ml of ammonium hydroxide (25% w/v) was added. The mixture was stirred for 30 min, then evaporated in a rotavapor. The residue, a clear viscous oil, was dissolved into 50 ml of dichloromethane, then washed three times with 20 ml of 10 m*M* aqueous hydrogen chloride and three times with 50 ml of water. The organic layer was dried over anhydrous sodium sulphate and

evaporated under a gentle stream of nitrogen. The raw powder obtained was recrystallised twice in absolute ethanol, giving the pure product as a white powder (0.145 g, 73% yield), deemed pure by reverse phase liquid chromatography (mobile phase: methanol–water–acetic acid (69:30:1, v/v)).

2.4. Polymer preparation

In a 10 ml thick wall glass test tube a solution was prepared by dissolving 0.200 g (0.783 mmoles, polymer A) or 0.600 g (2.35 mmoles, polymer B) of 2,4,5-T into 3.20 ml of methanol–water (3:1, v/v). Then, 0.243 ml (2.35 mmoles) of 4-vinylpiridine, 2.95 ml (15.7 mmoles) of ethylene dimethacrylate and 0.040 g of 2,2′-azobis-(2-methylpropionitrile) were added. The mixtures were purged with nitrogen and sonicated in a water-bath for 5 min. The vials were sealed with parafilm, then the mixtures were left to polymerise overnight at 60°C. The polymers obtained were broken with a steel spatula, mechanically ground in a mortar and wet-sieved to 30–90 μm particle size. The particulates were extensively washed with ethanol–acetic acid (9:1, v/v). No efforts were made to measure the amount of template molecule recovered. Blank polymers were prepared and treated in the same manner, omitting 2,4,5-T.

2.5. Column packing

An adequate amount of polymer was suspended in a ethanol–water mixture (1:1, v/v) and the slurry packed in a 100 mm stainless-steel HPLC column (I.D. 3.9 mm, geometrical volume 1.19 cm^3). The packing of the stationary phase was performed by gradually adding the slurry of the polymer to the column and eluting it with the mobile phase (ethanol–water (1:1, v/v)) at constant pressure of 10 MPa. The packed column was washed at 1 ml/min with ethanol–acetic acid (9:1, v/v) until a stable baseline was reached (286 nm). After equilibration, the pressure in the column was of 2–5 MPa using organic solvents as a mobile phase and at a flow-rate of 1 ml/min.

2.6. Liquid chromatography

Columns were equilibrated at a flow-rate of 1 ml/min with 40 ml of proper mobile phase; then, 20 μl of stock solution of 2,4,5-T (or related substance) diluted with acetonitrile (1:9, v/v) were injected and eluted at 1 ml/min, and the absorbance recorded at 286 nm. Each elution was repeated three times to assure the chromatogram reproducibility. Column void volumes were measured for each mobile phase formulation by eluting 20 μl of acetone 0.1% v/v in acetonitrile, and the absorbance recorded at 286 nm.

The retention factor (k') was calculated as $(t - t_o)/t_o$, where t is the retention time of the eluted substance, and t_o the retention time corresponding to the column void volume. The selectivity factor (α) is defined as an index of polymer selectivity towards analogues of the template molecule. It was calculated as $k'_{analogue}/k'_{template}$.

3. Results and discussion

3.1. Effect of the mobile phase composition on 2,4,5-T retention

The effect of the mobile phase polarity was studied by eluting 2,4,5-T with mixtures of acetonitrile–water, added with 1% (v/v) of acetic acid in order to limit the peak tailing effect. The results obtained (Fig. 1) can be explained by considering the nature of the stationary phase. In fact, an imprinted

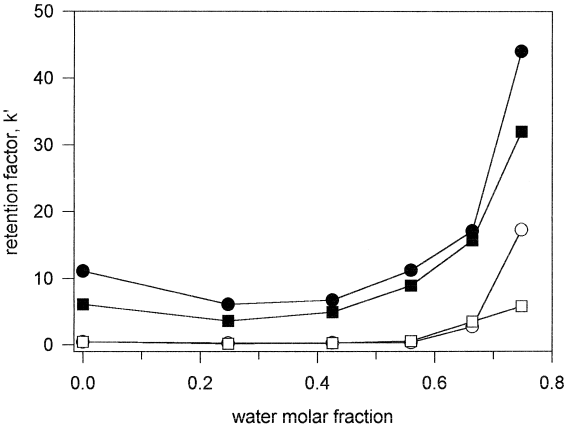


Fig. 1. Effect of the mobile phase composition on the retention of 2,4,5-T. Solid circles: imprinted column A, open circles: blank column A, solid squares: imprinted column B, open squares: blank column B.

polymer prepared in hydrophilic media shows a different binding behaviour with respect to imprinted polymers prepared in more classical hydrophobic media. Such a difference is due both to the presence of an ionic pair interaction between the polymer and the imprinting molecules and to the hydrophobic cavity generated by these molecules in the polymeric matrix. Thus, it follows that the polarity of the mobile phase influences directly the partition of 2,4,5-T in the stationary phase, so that the column behaves like a reversed-phase system in which an increasing molar fraction of water in the mobile phase causes an increase of the retention times.

It is worthwhile noting that blank polymers do not show significant retention for 2,4,5-T in mobile phase mixtures with a water content lower than 30% v/v (molar fraction lower than about 0.6). Thus, the partition of 2,4,5-T appears to be strongly enhanced by the presence of highly hydrophobic binding sites generated by the imprinting process and complementary to the most hydrophobic part of the imprinted molecule.

As with many reversed-phase systems in which the mobile phase is composed of acetonitrile–water mixtures, a minimum in the retention factors is present when acetonitrile is the main component of the eluent mixture. This effect is due to the solvating power of acetonitrile–water mixtures for organic anions, as carboxylic acids, that have their maximum in the water molar fraction range 0.2–0.3 [17]. This maximum of solvating power shifts the partition of 2,4,5-T towards the mobile phase, reducing the overall time of persistence in the column and, in consequence, the retention factor.

The effect of the mobile phase was examined by considering also different organic solvents provided with variable polarity and hydrogen bond donor/acceptor properties. Acetonitrile (MeCN), four aliphatic alcohols: methanol (MeOH), ethanol (EtOH), propanol (PrOH) and isopropanol (iPrOH), and three ethers: tetrahydrofuran (THF), 2-methoxyethanol (MEG) and 1,2-dimethoxyethane (DMEG), were considered. Their effect on the elution of 2,4,5-T in presence of 1% v/v of acetic acid was measured. The values of retention factor, reported in Fig. 2, show that there is not a direct relation between the retention of 2,4,5-T and the properties of the solvents considered. Moreover, an effect linked to the formulation of the polymer composition is also present. In fact, the column A shows a maximum retention factor for 2,4,5-T when eluted with less polar solvents as acetonitrile or isopropanol, while column B shows the same maximum with more polar solvents as ethanol or propanol.

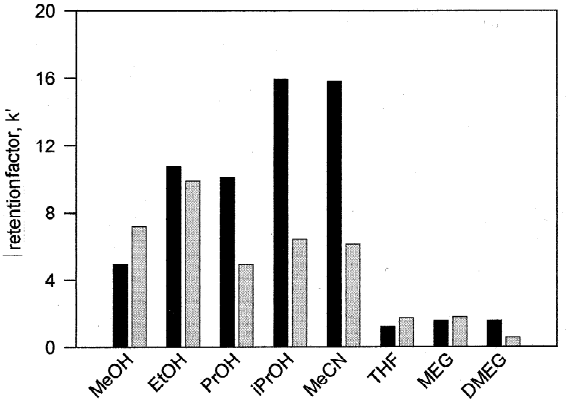


Fig. 2. Effect of different solvents as mobile phase on the retention of 2,4,5-T. Black bars: imprinted column A, grey bars: imprinted column B.

To explain these results, it is necessary to take into account the different amount of pyridine rings present in the two kinds of stationary phase. The polymer A is characterised by an equimolar ratio 4-vinylpyridine–2,4,5-T, and the pyridine rings are mostly included in specific binding sites because they come from the ion pairs formed during the polymerisation process. In these hydrophobic binding sites the driving force of the interaction is based on the ability to stabilise the ion pair, enhanced by less polar solvents, as isopropanol or acetonitrile, and reduced by relatively more polar solvents, as methanol or ethanol.

On the contrary, the polymer B is characterised by a threefold molar excess of 4-vinylpyridine with respect to the templanting 2,4,5-T, so that 2/3 of the pyridine rings are settled out of the binding sites, because they come from the excess of 4-vinylpyridine present in the polymerisation mixture. Thus, during the elution, the 2,4,5-T molecules have more probability to interact with these ''off-site'' pyridine rings than with those into the binding sites. Due to the absence of a stabilising hydrophobic environment, this interaction is more prone to the

reverse phase mechanism of partition. So, the partition equilibrium is shifted towards the mobile phase with less polar solvents, i.e. isopropanol and acetonitrile, and towards the stationary phase with more polar solvents, i.e. methanol and ethanol.

From the experimental data it is clear that ethers greatly suppress the molecular recognition, apparently without remarkable difference between the polymer A and the polymer B. It should be noted that THF and related ethers are weak basic, non acidic solvents, with hydrogen acceptor properties only. For this reason, we think that they could compete with the stationary phase for the acidic hydrogen of 2,4,5-T suppressing the interaction with the pyridine rings.

3.2. Effect of mobile phase additives on 2,4,5-T retention

As a consequence of the proposed mechanism of molecular recognition, those additives able to interfere with the ion pair formation between the pyridine ring and the carboxyl should reduce the retention of 2,4,5-T on the imprinted columns. Acetic acid and sodium chloride were chosen as modifiers; the first in a concentration range between 0.1 and 10% v/v in acetonitrile, and the second in a concentration range between 1 and 100 m*M*, using a water–acetonitrile mixture (1:3, v/v) as a mobile phase to overcome the low solubility of sodium chloride in anhydrous acetonitrile.

The effect observed on the retention factor is reported in Figs. 3 and 4. Both modifiers act as strong ion pair perturbing substances. Even if it was not possible to measure the retention factors on columns eluted without modifiers because of the complete retention of 2,4,5-T, limited amounts of modifiers cause an effective reduction of the retention. This reduction is more effective for sodium chloride because the amount of salt necessary to significantly reduce the retention is in the order of magnitude of millimolar concentrations, while acetic acid in the range 20–100 m*M* shows a more limited effect, with higher retention factors for 2,4,5-T.

The effect of ion pair perturbing substances on the blank columns is very limited, without a significant decrease of the per se limited retention factors. This fact implies that the overall molecular recognition of 2,4,5-T by the polymers, i.e. high values of retention factors, can be efficient only if the electrostatic ion pair interaction between the pyridine ring of the polymer and the carboxyl of 2,4,5-T are enhanced and stabilised by the hydrophobic interaction between the imprinted polymer and the aromatic part of the phenoxyacid. In the blank polymers the absence of specific binding sites provided both with hydrophobic character and exposed pyridyl groups does not promote the partition of 2,4,5-T in a stationary phase.

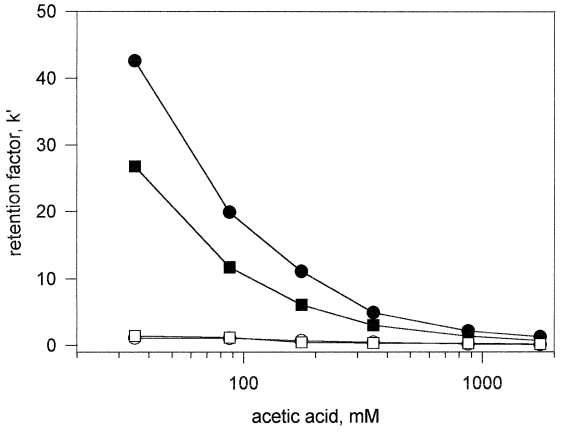


Fig. 3. Effect of the acetic acid as mobile phase modifier on the retention of 2,4,5-T. Solid circles: imprinted column A, open circles: blank column A, solid squares: imprinted column B, open squares: blank column B.

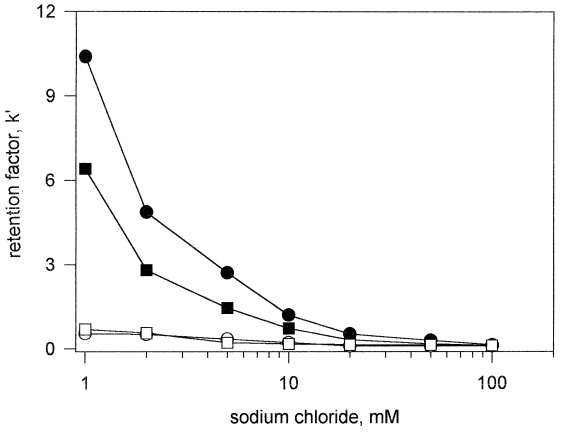


Fig. 4. Effect of the sodium chloride as mobile phase modifier on the retention of 2,4,5-T. Solid circles: imprinted column A, open circles: blank column A, solid squares: imprinted column B, open squares: blank column B.

3.3. Column selectivity

The selectivity of the columns A and B were evaluated by eluting with acetonitrile (added with 1% v/v of acetic acid) several 2,4,5-T-related substances, whose molecular structures are reported in Table 1. The columns show good selectivity, with separation of 2,4,5-T from cogeners in complex mixtures. An example is reported in Fig. 5, where a mixture of 2,4,5-T, 2,4-D, 2,4-DB, fenoprop, mecoprop and MCPA were separated on the column A in three distinct peaks, while the same mixture on the reference column (inset) was eluted without separation of the components. Considering the selectivity factors, reported in Fig. 6, it is possible to observe that molecules strictly related to 2,4,5-T in the aromatic part and different in the acidic sub-structure, are very poorly recognised by the polymers. This effect is present not only with molecules unable to form ion pairs with the pyridine (2,4,5-T-NH_2 and 2,4,5-T-OMe), but also with the 2,4-DB (a molecule characterised by the presence of a butanoic acid sub-structure able to act like a binding site obstructing factor). It is clear that only molecules exactly matching the imprinting molecule in the carboxylic sub-structure can be recognised, and that this interaction is strongly governed by the ion pair formation.

Molecules resembling 2,4,5-T in the carboxylic sub-structure but different in the substitution on the aromatic ring or in the phenoxy sub-structure are recognised proportionally through the similarity with 2,4,5-T. Thus, 2,4-D (chlorine in positions 2 and 4, no chlorine in position 5), 2,3-D (chlorine in positions 2 and 3, the latter being isomeric to the position 5, no chlorine in position 4), and 3,4-D (chlorine in positions 4 and 3, the latter being isomeric to position 5, no chlorine in position 2) are recognised better than 4-CPA (chlorine in position 4, no chlorine in positions 2 and 5), and that 4-CPA is more recognised than PA (no chlorine in positions 2, 4 or 5). The substitution of a chlorine atom with a methyl group reduces the interaction too. Thus, 2,4-D is better recognised than MCPA (chlorine in position 4,

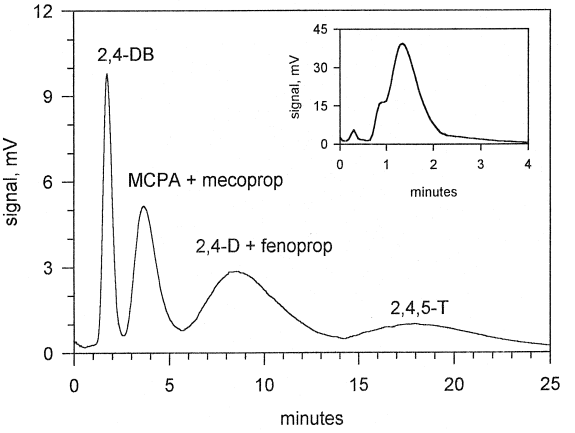
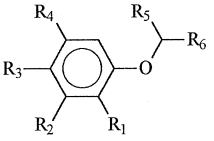


Fig. 5. Example of chromatographic separation of 2,4,5-T from related substances. Stationary phase: column A; mobile phase: MeCN+AcOH 1% (v/v), 1 ml/min. Sample: 20 μl of a MeCN solution containing 0.1 mg/ml of mecoprop, fenoprop, MCPA, 2,4-DB, 2,4-D and 2,4,5-T; absorbance recorded at 286 nm. In the inset the same mixture eluted on a blank column, experimental conditions unchanged.

Table 1
General structure of molecules considered in this work



Substance	R_1	R_2	R_3	R_4	R_5	R_6
2,4,5-T	Cl	H	Cl	Cl	H	COOH
2,4-D	Cl	H	Cl	H	H	COOH
MCPA	CH_3	H	Cl	H	H	COOH
3,4-D	H	Cl	Cl	H	H	COOH
2,3-D	Cl	Cl	H	H	H	COOH
4-CPA	H	H	Cl	H	H	COOH
PA	H	H	H	H	H	COOH
2,4,5-T-OMe	Cl	H	Cl	Cl	H	$COOCH_3$
2,4,5-T-$CONH_2$	Cl	H	Cl	Cl	H	$CONH_2$
Mecoprop	CH_3	H	Cl	H	CH_3	COOH
Dichlorprop	Cl	H	Cl	H	CH_3	COOH
Fenoprop	Cl	H	Cl	Cl	CH_3	COOH
2,4-DB	Cl	H	Cl	H	H	$(CH_2)_3COOH$

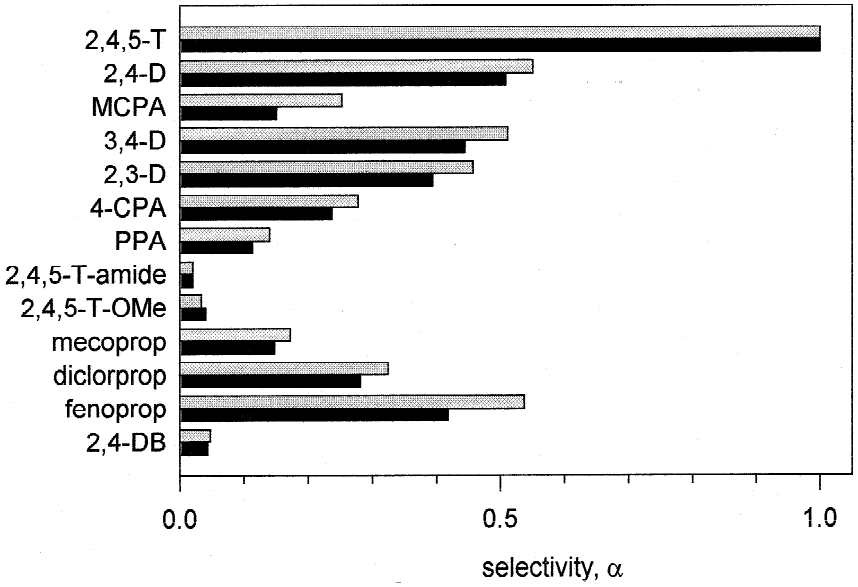


Fig. 6. Selectivity factors for molecules related to 2,4,5-T. Black bars: column A, grey bars: column B.

methyl in position 2). The same happens with 2-phenoxypropanoic acids: fenoprop (chlorine in positions 2, 4 and 5) is less recognised than 2,4,5-T, but is better recognised than dichlorprop (chlorine in positions 2 and 4, no chlorine in position 5), and dichlorprop is better recognised than mecoprop (chlorine in position 4, methyl in position 2, no chlorine in 5).

4. Conclusions

The technique of molecular imprinting in hydrophilic solvent is based on the generation of hydrophobic binding sites in a polymeric matrix by imprinting molecules provided with a hydrophobic structure. The results reported in this work indicate that polymers obtained in this manner have a binding behaviour different from the polymers obtained by molecular imprinting in hydrophobic solvent. In particular, the columns packed with polymers prepared in this work act as true reverse phases provided with molecular recognition properties, allowing us to observe an increment of the retention factor related to the solvating properties of the mobile phase. The presence of an ion pair interaction between the imprinting molecule and the stationary phase, capable of modulating the molecular recognition interaction, is demonstrated by the marked effect on the retention factor caused by ion pair perturbing substances, such as sodium chloride and acetic acid.

The recognition of several analytes strictly related to the imprinting molecule shows that these kinds of polymers could be useful as stationary phases to extract and concentrate halogenated phenoxyacids from complex or very diluted aqueous samples. At the present studies are in progress in our laboratory.

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