

Evaluation of methods aimed at complete removal of template from molecularly imprinted polymers

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Polymers imprinted with clenbuterol were used to study the influence of various post-polymerization treatments [*e.g.*, thermal annealing, microwave assisted extraction (MAE), Soxhlet extraction and supercritical fluid template desorption] on the bleeding of residual template. The aim of the study was to reduce the bleeding to levels that would allow the use of the materials as affinity phases for extraction of clenbuterol from bovine urine at concentrations below 1 ng ml⁻¹. After treatment, the clenbuterol imprinted polymers were packed into solid-phase extraction columns and the bleeding was estimated by quantifying the amount of template released in 10 ml of methanol–acetic acid (9 + 1 v/v). This was followed by an assessment of selectivity and recovery in comparison with non-treated material. The lowest bleeding level was found after MAE using 100% trifluoroacetic acid for 3 × 20 min at 100 °C. The collected eluate contained in this case 3 ng ml⁻¹ of clenbuterol. The same material was subsequently used for the extraction of clenbuterol from spiked bovine urine. The resulting selectivity and recovery were lower compared with those obtained using the untreated material. A milder but still efficient method to reduce the bleeding level was found to be MAE with formic acid. In this case a bleeding level of 14 ng ml⁻¹ was found after only a 1 h extraction time. In a second model system, using a polymer imprinted with L-phenylalanine anilide, the bleeding was reduced to a similar level by extensive on-line washing in good swelling solvents containing acid or base additives and after thermal annealing of the polymers in the dry state.

Introduction

Polymeric network materials capable of recognizing small molecules are available using molecular imprinting techniques.^{1,2} These molecularly imprinted polymers (MIPs) exhibit in many respects antibody-like recognition properties. Their use as alternatives to the biological recognition elements applied in various areas of analytical chemistry is therefore being considered. One potential application that has recently attracted widespread interest is their use for clean-up and enrichment of analytes present at low concentrations in complex matrices.³ Selective solid-phase extraction with molecularly imprinted polymers (MISPE) has previously been described for various pharmaceutical drugs, drugs of abuse and pollutants.^{4–10}

Before the imprinted material can be used in any application, the template molecules have to be removed from the polymer. The necessary extent of template removal depends on the subsequent application. Thus, in preparative applications incomplete removal may be a marginal problem whereas in analytical applications bleeding of non-extracted template is likely to cause quantification inaccuracies. An additional problem is the legal implications of template bleeding when attempting to prosecute for illegal drug use.

Continuous extraction using a Soxhlet apparatus typically results in the removal of up to 99% of the template. Several studies, however, have shown that a small portion of the template remains unextracted even after extensive washing

using various organic solvents containing acid or base additives.^{5–8,11–13} The remaining template can constitute a problem as it might bleed from the polymer during the elution step of the solid-phase extraction (SPE) procedure, giving erroneous results and an increased limit of quantification (LOQ). This problem hampers the use of MIPs for trace level analysis and to overcome the problem an often necessary compromise is to use a close structural analogue of the target analyte as template.⁵ Therefore, it is of prime concern to search for methods capable of reducing bleeding to acceptable levels.

Organic network polymers of the type obtained in molecular imprinting are built up of domains with different cross-linking density.¹⁴ The polymerization conditions (temperature, solvent, type and concentration of monomers, cross-linking level, initiator system) influence the build-up of the porous structure. Common to all materials is that they contain primary particles with a high cross-link density that are agglomerated to different extents with interconnecting segments of various flexibility. At an average level of cross-linking of *ca.* 80%, the materials can range from being non-porous with high swelling factors to being permanently porous with low swelling factors. Non-porous materials are typically obtained when carrying out the polymerization in a good solvent capable of solvating the growing polymer chain, whereas permanently porous are obtained when using a poor solvent.¹⁵ A number of template recovery studies in systems based on covalent attachment of the template to the functional monomer have shown that some

combinations of monomer (usually styrene based) and solvent lead to particularly low recoveries of template.^{16–18} This has been attributed to the low solvent accessibility to the more highly cross-linked domains of these polymers.¹⁸

In non-covalent imprinting using methacrylate based polymers, the template recovery is usually considerably higher and appears in most cases to be quantitative.^{15,19} It is likely that the bleeding observed from these materials is caused by residual template, entrapped in the more highly cross-linked domains of the polymer backbone. This may leach out of the polymer in small portions upon volume changes of the materials. In order to remove the entrapped template, various strategies are possible. First and most straightforward, the use of a solvent that will strongly interact with the polymer may lead to the required swelling of the backbone necessary for template release. Examples of good and poor solvents for linear homopolymers of methacrylic acid (MAA), the minor polymer constituent, and methyl methacrylate (MMA), here assumed as a mimic for the major polymer constituent ethylene glycol dimethacrylate (EDMA), can be found in the *Polymer Handbook*.²⁰ It is notable that chlorinated solvents, which are commonly used as porogens, belong to good solvents for poly(methyl methacrylate) (pMMA). The fact that these porogens result in gel-like materials indicates that they also act as good solvents for the cross-linked polymer. This supports the use of pMMA as a mimic for the cross-linking monomer. The good solvents may then be combined with acid or base additives in order to disrupt the electrostatic interactions between the template and the polymer. Moreover, it may be performed at elevated temperature or assisted by microwaves in order to increase the rate of diffusion.

Rapid diffusion can also be expected when using supercritical fluids for template desorption. It should also be kept in mind that the properties of MIPs are often correlated with the solvent conditions employed during polymerization and thus optimum recognition is frequently observed using this same solvent.^{21–23} Matching the extraction solvent with the synthesis solvent may lead to enhanced recoveries due to better accessibility to the imprinted sites. Yet another alternative is controlled *in situ* decomposition of the template, *cf.*, template calcination in the synthesis of nanostructured inorganic materials.²⁴ In view of the particular requirements that this puts on the template this is not likely to become a general solution to the template bleeding problem since hydrolysis of the polymer backbone has previously been shown to result in higher recoveries but at the expense of selectivity.²⁵ Finally, thermal annealing of the polymer, in which it is treated at temperatures presumably leading to polymer conformational changes, may lead to additional template release.²⁶ This treatment further enhances the stability of the materials, causes a more homogeneous distribution of binding sites and slightly improves the mass transfer properties of the materials.²⁷ However, this treatment is only recommended for materials that are gel-like and non-porous in the dry state since porous materials do not exhibit the required thermal stability.¹⁵

In order to address this problem in more detail, we chose to study two model systems, clenbuterol, an analyte of wide current interest,⁹ and L-phenylalanine anilide (L-PA), a template that has previously been intensively studied.^{15,28–30} These model templates were imprinted using the common protocol of MAA as functional monomer, EDMA as cross-linking monomer and either dichloromethane (DCM) or acetonitrile as solvent (porogen) (Fig. 1).

In one of our laboratories, MISPE is being evaluated for the isolation and clean-up of clenbuterol from urine samples.⁹ Clenbuterol is a synthetic β_2 -agonist, which binds to the β -adrenergic receptor and thereby reduces stress symptoms and asthma. As a side-effect it has been found that at approximately 10 times the therapeutic dose it causes an increase in muscle mass and a decrease in adipose tissue. This effect has led to the

frequent feeding of clenbuterol to cattle during the fattening process. Owing to the potential health risk of residues from β -agonists in meat products, the use of clenbuterol for this purpose is forbidden in the European Community. Detection of β_2 -agonists in biological fluids is difficult as the concentrations are normally low, $<1 \text{ ng ml}^{-1}$. Elimination of bleeding at these levels would therefore allow the direct use of clenbuterol MIPs in SPE for the trace determination of clenbuterol in bovine urine.

Experimental

Chemicals

Clenbuterol hydrochloride and bromoclenbuterol were obtained as a gift from Karl Thomae (Biberach an der Riss, Germany). Acetonitrile (MeCN) and methanol (MeOH) were both of HPLC grade and purchased from Labscan (Dublin, Ireland). Dichloromethane (DCM), acetic acid (HOAc, glacial) (100%) and hydrogen peroxide (30%) were of analytical-reagent grade from Merck (Darmstadt, Germany). The monomers MAA and EDMA and the initiator azobisisobutyronitrile (AIBN) (for synthesis) were supplied by Aldrich Chemie (Steinheim, Germany) and were purified as described previously.¹⁵ Tri-fluoroacetic acid (99%), diethylamine and ethylenediamine were purchased from Aldrich Chemie and acetic anhydride (analytical-reagent grade) and octane-1-sulfonic acid, sodium salt monohydrate (98%), from Acros Organics (Geel, Belgium). The initiator dimethyl-2,2'-azobisisobutyrate (V-601) was obtained from Wako Pure Chemical Industries (Osaka, Japan) and was used without further purification. D- and L-phenylalanine anilide (D- and L-PA) were synthesized as described elsewhere³¹ and purified by crystallization from heptane-chloroform.

All other chemicals were of analytical-reagent grade. The water used was demineralized in-house and purified with a Maxima ultrapure water system (Elga, Salm & Kipp, Breukelen, The Netherlands).

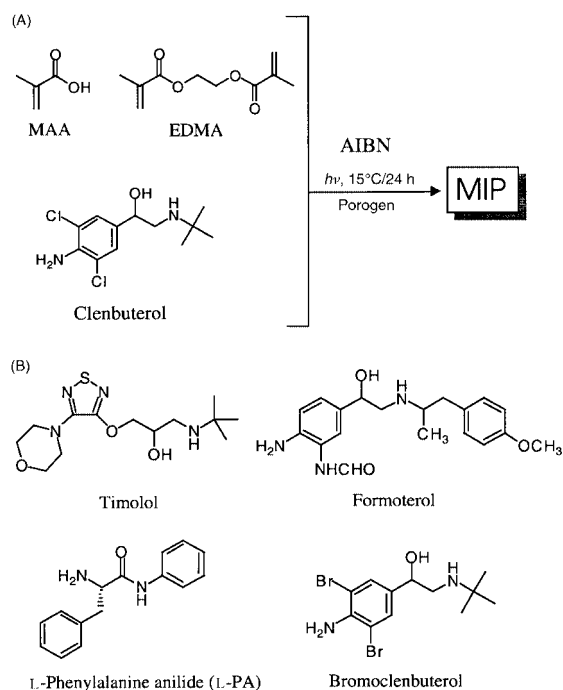


Fig. 1 (A) Protocol used for imprinting clenbuterol or L-PA and (B) structures of L-PA and of amino alcohols used in assessing the selectivity of the materials.

Analysis equipment

Liquid chromatographic (LC) analysis used for quantifying the template in SPE eluates was performed off-line with a system consisting of a pump (Model 2150, Pharmacia/LKB, Uppsala, Sweden) and a WISP 710 A automatic sample injector (Waters, Milford, MA, USA). As detector, an electrochemical cell (Amor, Antec Leyden, Zoeterwoude, The Netherlands) with a glassy carbon working electrode set at +800 mV vs. an Ag/AgCl *in situ* reference electrode was used. The column was a LiChroCART HPLC cartridge (250 × 4 mm id) containing Superspher 60 RP-select B (4 µm) (Merck) with a LiChroCART (4 × 4 mm id) guard column containing LiChrospher 60 RP-select B (5 µm) (Merck). Peak areas and retention times were recorded with an HP 3396 A integrator (Hewlett-Packard, Avondale, PA, USA). The mobile phase consisted of buffer–MeCN (70:30, v/v). The buffer contained 25 mM potassium phosphate, 2.5 mM NaCl, 0.17 mM EDTA and 2.7 mM sodium octanesulfonate and had a pH of 5.5. Prior to use it was filtered (0.2 µm, Schleicher & Schuell, Dassel, Germany) and degassed. The mobile phase flow rate was set to 0.8 ml min⁻¹ and the sample injection volume was 20 µl.

The clenbuterol system

Molecularly imprinted polymers (MIPs) selective for clenbuterol and non-imprinted control polymers (NIPs) were prepared as shown schematically in Fig. 1 using MeCN or DCM as porogens.³²

It should be noted that for each of the following post-polymerization treatments, no prior washing step was imposed on the respective MIP sample, *i.e.*, each sample contained clenbuterol corresponding to the amount added to the monomer mixture, assuming that no template decomposition occurred during polymerisation.

MIP post-treatments. To remove the template molecule, dry polymers (60 mg of particles with a diameter of 25–38 µm) in SPE columns were post-treated in different ways, as shown in Table 1. For the first experiments an SPE column was placed in a Vac Elut SPS 24 unit from Varian (Palo Alto, CA, USA), which was kept at a vacuum of 10 inHg, and 50 ml of washing solution were passed through the column. In the ultrasonication experiments, just enough washing solvent to wet the sorbent was sucked through the column and it was placed in an Erlenmeyer flask containing the same washing solvent. The Erlenmeyer flask was thereafter placed in a B12, ultrasonication bath from Branson Ultrasonics (Danbury, CT, USA), where it was kept for 4 h with the solvent being replaced with fresh solvent every 15 min. Heat treatment at 120 °C was carried out overnight on dry polymers in air at atmospheric pressure in a UT 5042 oven (Hereaus Instruments, Hanau, Germany).

Supercritical fluid template desorption. HPLC columns (25 × 4.6 mm id) were packed with clenbuterol imprinted polymer and mounted on a supercritical fluid chromatography system equipped with a variable-wavelength UV detector (Hewlett-Packard HP 1050 Series, ChemStation software version HP G1850/1855A) as described previously.³³ Each column was washed (unless stated otherwise in Table 2 or in the text) according a protocol as follows. The mobile phase employed was supercritical CO₂ containing 30% of modifier. The modifier composition was either pure methanol or methanol–acetic acid in the proportions indicated in Table 2. The temperature was 100 °C, the flow rate 1 ml min⁻¹ and the back pressure 200 bar. The extraction time was varied as indicated in Table 2 and the baseline of each experiment was continuously monitored at 215 and 260 nm for the respective time.

Microwave assisted extraction (MAE). The MAE experiments were performed on a MARS 5 microwave accelerated reaction system (CEM, Matthews, NC, USA). Unless indicated otherwise, standard MAE conditions were used as follows: 60 mg of MIP and 20 ml of the respective solvent were placed in MAE vessels. The vessels, manually closed and tightened, were exposed to MAE over a 5 min heating ramp up to 100 °C under various conditions as indicated in Table 3. After extraction, the vessels were cooled to room temperature for ~10 min, then the vessels were opened and their contents quantitatively transferred on to an F3-frit connected to a water vacuum pump for removing the solvent. Subsequently the samples were washed with 3 × 10 ml of MeOH which was subsequently also removed with the water vacuum pump.

MIPs that had been treated with bases (MIPs 21–24) were converted into the acid form by the following washing protocol after being exposed to MAE conditions. The experimental set-up for the washing steps was identical with that described above: 1, MeOH 20 ml; 2, HOAc–H₂O–MeOH (5 + 5 + 90) 20 ml; 3, MeOH 20 ml.

Assessment of template bleeding. After each treatment the MIP (60 mg) was transferred to an SPE column that was eluted with 10 ml of MeOH–HOAc (9 + 1 v/v), by placing the column in a Vac Elut SPS 24 unit from Varian (Palo Alto, CA, USA) and keeping it at a vacuum of 10 inHg. The elution solvent, MeOH–HOAc (9 + 1 v/v), was chosen since it had been used successfully for washing of clenbuterol imprints in chromatography.⁹ An aliquot of the eluate, normally 500 µl, was transferred into a new test-tube and evaporated to dryness in a vacuum centrifuge (Univapo 150 H, Genevac, Ipswich, UK). The residue was reconstituted in 200 µl of the mobile phase before analysis by HPLC.

Selectivity measurements. To study MIP selectivity, MeCN containing HOAc (0–10%) was spiked with 10 ng ml⁻¹ clenbuterol. A 10 ml volume was loaded onto the column and the eluate was collected. A 2 ml volume of this was evaporated to dryness and the residue was dissolved in 200 µl of the mobile phase. For comparison, 2 ml of the spiked solution were also evaporated to dryness and the residue was dissolved in the mobile phase and analysed. Between samples the column was washed with 10 ml of MeOH–HOAc (9 + 1 v/v) and dried.

Recovery experiments and MIP affinity evaluation. To study the effects of the various post-treatments on the selectivity of the MIP, the recovery experiments were performed with treated and untreated polymers using the following procedure. The MISPE cartridges were previously washed with 10 ml of water containing 1 M NaOH followed by 20 ml of water. Standards consisted of 5 ml of MeCN spiked with 100 ng of clenbuterol and three structurally analogous compounds (bromoclenbuterol, timolol and formoterol; see Fig. 1). After loading the standards, the cartridges were washed with 10 ml of MeCN–HOAc (99 + 1 v/v) (wash fraction) followed by two fractions of 6 ml of MeOH–HOAc (9 + 1 v/v) (elution fraction). The three fractions were collected and evaporated to dryness in a water-bath at 50 °C under a gentle stream of nitrogen, and the residue was dissolved in the mobile phase and analysed by HPLC.

The L-PA system

Polymer synthesis. L-PA (240 mg, 1 mmol), MAA (0.34 ml, 4 mmol), EDMA (3.8 ml, 20 mmol) and AIBN (40 mg, 0.25 mmol) or V-601 (58 mg) were dissolved in DCM (5.6 ml). The solution was sparged with nitrogen for about 5 min and transferred into a thick-walled glass polymerization tube in

which it was again sparged with nitrogen. The tubes were then sealed with an NMR tube cap, immersed in a water-bath maintained at 15 °C, allowed to equilibrate for 10 min and irradiated using a medium pressure Hg lamp (Original Hanau 800) for 24 h. During this time, they were rotated periodically to ensure even polymerization.

Extraction procedures, heat treatment and assessment of selectivity. At the end of the polymerization, the polymer monolith was crushed in a mortar to give coarse particles and thereafter continuously extracted for 12 h with methanol using a Soxhlet apparatus. The polymer was subsequently further ground and sieved under water to the required size fraction, 25–35 µm. A 125 × 4 mm id stainless steel HPLC column was packed with a slurry of the particles using MeOH–H₂O (80 + 20 v/v) as packing solvent at a maximum pressure of 300 bar. The resulting column contained ~0.5 g dry weight of material.

Evaluation of the polymer selectivity was carried out at regular time intervals throughout the study using MeCN–H₂O–HOAc (92.5 + 2.5 + 5 v/v/v) as the mobile phase.

Different volumes (20–50 ml) of various eluents were then passed through the columns and their effect on the extent of template release was studied. All extracts and eluates were collected for subsequent quantification by reversed-phase HPLC. After this treatment, the polymers were treated in a vacuum oven at 120 °C for 24 h. Thereafter they were subjected to a similar elution procedure as before heat treatment.

Quantification of released template. All collected extracts were evaporated to dryness and the residues reconstituted in 2.0 or 0.5 ml of MeOH. They were then analysed by reversed-phase HPLC using a Hewlett-Packard instrument (HP1050) equipped with a quaternary pump, an auto-sampler and a diode array detector. The HP ChemStation was used for data handling. PA enantiomers eluted after 5 min with typical plate numbers of 3000 using a Prodigy ODS3 column (Phenomenex) and a mobile phase of MeOH–0.02 M potassium phosphate buffer (pH 2.8) (1 + 1 v/v) pumped at 1 ml min⁻¹. After having verified the identity of L-PA, the area was estimated by manual integration and the quantity of L-PA extracted calculated based on the results of double injections (10 µl) and an external calibration curve. As a rough measure of the extent of template removal, the nitrogen contents of the materials were estimated by elemental analysis prior to and after the extraction procedures.

Results and discussion

The clenbuterol system

Wash procedures on-line or in batch mode at room temperature. As discussed in the Introduction, an optimal washing liquid should swell the polymer and suppress interactions between the functional groups of the template and the polymer, but the procedure should also allow fast diffusion of the template molecules from the polymer. In order to remove clenbuterol from the imprinted polymers packed in SPE columns, a washing solution of MeOH–HOAc (9 + 1 v/v) was tried initially.⁹ Although a large volume of washing solution divided over many individual washing steps was used, it was found to be ineffective in quantitatively removing the template. It should be noted in this context that methanol is a poor solvent for pMMA (used as mimic for the major polymer constituent EDMA, see above) but a good one for pMAA (the minor polymer constituent).²⁰ After 20 wash fractions of 10 ml each, the amount of clenbuterol bleeding from the imprinted polymer was ~1000 ng in a 10 ml fraction (Fig. 2). This amount is over 1000 times too high assuming a target maximum bleeding level

of 0.1 ng ml⁻¹, and would preclude its use for the extraction of clenbuterol from urine samples. In Fig. 2 it can also be seen that when the polymer is dried between consecutive washings, a larger amount of template molecule is removed in subsequent washing steps. This observation is in agreement with previous reports^{8,11} and may be attributed to volume changes of the material caused by drying and wetting procedures, a phenomenon that has also been observed when switching between different solvents. The use of even larger volumes of washing solution would be impractical and therefore other washing solvents and procedures were investigated. The first series of treatments were performed on newly packed MISPE columns. After each treatment, 10 ml of MeOH–HOAc (9 + 1 v/v) were percolated through the column and the eluate was collected and subsequently analysed for clenbuterol content. The washing solvents MeCN, DCM and MeOH containing an acid, either TFA or HOAc, were compared (Table 1).

In view of the fact that MeCN was used as a porogen, it is interesting that a significantly higher recovery is seen using MeCN than DCM, in both cases with TFA as a modifier. Given that a further reduction of bleeding was obtained using MeOH–HOAc (9 + 1 v/v) as wash solvent, 100% HOAc was investigated since it was noted that an increasing amount of acid led to reduced bleeding. By treating the column for 4 h in an ultrasonication bath and replacing the solvent with fresh solvent every 15 min the bleeding could be lowered to 6 ng ml⁻¹ in the eluate. It was considered that acetic anhydride would be more effective than HOAc since it can react chemically with

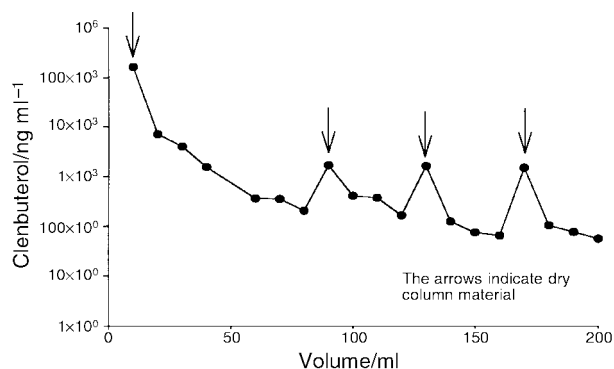


Fig. 2 Washing of clenbuterol imprinted polymers with MeOH–HOAc (9 + 1 v/v). The concentrations are measured in 10 ml eluate fractions.

Table 1 Concentration of clenbuterol in the eluate after different post-treatment procedures using a clenbuterol MIP (porogen: MeCN)

Post-treatment	Clenbuterol/ng ml ⁻¹
None	163 000
DCM–TFA (95 + 5 v/v)	4990
3% H ₂ O ₂	940
MeCN–TFA (95 + 5 v/v)	710
MeOH–HOAc (9 + 1 v/v)	350
100% HOAc	42
100% HOAc ^a	6
100% HOAc ^a	9 ^b
100% HOAc ^a	120 ^c
100% Acetic anhydride ^a	20
1 M HCl ^a	560
5 M HCl ^a	240
10 M HCl ^a	270
1 M NaOH ^a	9870
5 M NaOH ^a	6730
10 M NaOH ^a	260

The post-treatments were performed as described in the Experimental section. ^a Concentration of clenbuterol in the eluate after ultrasonication of a clenbuterol MIP (porogen: MeCN) for 4 h in different solvents and replacement of solvent every 15 min. ^b Porogen: DCM. ^c Heat treated material.

clenbuterol; however, after 4 h of treatment with acetic anhydride, the eluate still contained 20 ng ml⁻¹ (Table 1).

Since HOAc is a weak acid, the use of a strong acid, HCl, was therefore also evaluated, in addition to investigations using a strong base, NaOH, each at concentrations ranging from 1 to 10 M and in the absence of organic solvent. Levels of bleeding from the polymer, however, remained 10–100-fold higher than when 100% HOAc was used in the pretreatment step.

These results can be explained in part by the observation that water is a relatively poor solvent for these polymers, leading to low swelling factors.¹⁵ When the polymer shrinks, its structure will be more compact and the accessibility of smaller pores to the washing solvent will diminish, resulting in less efficient template removal. Zander *et al.* found that the elution of nicotine adsorbed on nicotine-imprinted MIPs decreased to less than 50% when the eluent contained 30% water compared with elution with MeCN and a small amount of TFA.²⁶

Comparison of acetonitrile and dichloromethane as porogen. The diffusion of template molecules from the polymer depends on their morphology. The swellability and porosity of cross-linked polymers depend on the solvents (porogens) used during polymerization. Also, polymers swell and shrink to different extents depending on the solvent in which they are immersed. The degree of swelling and shrinking thus depends on the polymerization conditions (see discussion above). Two porogens, MeCN and DCM, were used in the polymerization process. These are known to give rise to mesoporous moderately swellable and non-porous swellable polymers, respectively.¹⁵ The two types of polymers were treated in 100% HOAc for 4 h in an ultrasonication bath. The bleeding after this post-treatment of these MIPs was found to be 6 and 9 ng ml⁻¹ for the MeCN and the DCM polymers, respectively (Table 2), indicating that the polymer morphology in this case has a marginal influence on the extent of polymer bleeding.

Oxidation of clenbuterol in the polymer. Chemical degradation of the template offers an alternative to exhaustive extraction. Considering the very high sensitivity of electrochemical detection for clenbuterol, oxidation seems to be the most suitable option. It was considered that by allowing an oxidizing agent, *e.g.*, hydrogen peroxide, to penetrate into the polymer, clenbuterol would be oxidized *in situ*, presumably at the aromatic amino group which is most prone to oxidation. Since this functional group is most likely involved in the molecular recognition process,^{1,2} oxidation at this site may lead to release of the template. An SPE column was washed with 5 × 10 ml of 3% hydrogen peroxide. The bleeding from the column after this treatment amounted to 940 ng ml⁻¹ (Table 1). Incubation with 10% hydrogen peroxide either at 50 °C or in an ultrasonication bath for 2 h turned out to be less effective. Oxidation using peroxide dissolved in more effective swelling organic solvent–aqueous mixtures remains to be tested. However, this technique does not offer a general alternative for template removal since it requires that the template is oxidatively sensitive.

Supercritical fluid template desorption. By using supercritical fluids to remove the template molecules, the diffusion coefficient can be increased at least 10-fold and is most suitable for extraction of relatively non-polar compounds from solid matrices.³⁴ Additionally, owing to the near-zero surface tension, porous materials such as MIPs can be penetrated efficiently and thus may give rise to more efficient template removal. The disadvantage in using supercritical fluids for removal of clenbuterol template is the relatively high polarity of clenbuterol. Polar compounds have to be extracted at higher

temperatures and pressures and also require the addition of a polar modifier to the extraction medium.

In the present experiment, a stainless steel column filled with the clenbuterol MIP was washed continuously using an SFC instrument under the above conditions (Table 2). As expected, the signal decrease was fastest at the beginning and, after 21 h of washing with supercritical CO₂ containing 30% MeOH, no further decrease of the baseline was observed. The MeOH content was then lowered to 10% and held constant for another 18 h; however, no significant change of the baseline was observed. Thereafter the modifier content was raised again to the original 30% MeOH for another 24 h. Analogous to the washing procedures described in Fig. 2, the signal initially increased slightly for a short period of time but quickly reverted to the earlier baseline level. The impact of the different MeOH concentrations on the washing procedure for the polar clenbuterol template molecules is not surprising, since in SFC the polarity of the mobile phase is governed by the amount of modifier added.

After transferring 60 mg of the washed MIP into an SPE column, the clenbuterol content of the eluate [10 ml of MeOH–HOAc (9 + 1, v/v)] was found to be 106 ng ml⁻¹. This amount of clenbuterol template indicates that supercritical fluid washing conditions with modifier concentrations between 10 and 30% of pure methanol did not sufficiently reduce bleeding from MIPs.

Further reduction can be achieved, however, by the use of stronger modifiers (Table 2). Bleeding levels down to *ca.* 50 ng ml⁻¹ were achieved by employing HOAc as additive and longer extraction times (> 9 h).

In general, however, this technique is not expected to be widely applicable as a routine post-treatment step for MIPs. Obvious disadvantages are the complexity, in terms of optimization, and the instrumentation required. Other unattractive features are the long operation times, high costs and high solvent consumption.

Soxhlet extraction. This treatment appeared to be less efficient than simple sonication or microwave assisted extraction (see below) but could be improved somewhat by extraction in glacial HOAc for 24 h. This procedure resulted in bleeding levels of *ca.* 42 ng ml⁻¹, a significant decrease (50 times lower) compared with extraction in MeOH alone (Table 2), indicating once again the efficiency of acetic acid for template removal. The poor result obtained using TFA may be due to the lower

Table 2 Concentration of clenbuterol in the eluate after supercritical fluid template desorption (SFD) and continuous Soxhlet extraction (Soxhlet) of clenbuterol MIPs

Extraction mode	Modifier/solvent	Extraction time/h	Clenbuterol/ng ml ⁻¹
SFD ^a	MeOH–HOAc (90 + 10)	18	53
SFD ^a	MeOH	9	147
SFD ^a	MeOH–HOAc (95 + 5)	9	49
SFD ^a	MeOH–HOAc (90 + 10)	9	50
SFD ^a	MeOH–HOAc (90 + 10)	1	360
SFD ^a	MeOH–HOAc (90 + 10) ^c	9	94
SFD ^a	MeOH–HOAc (50 + 50)	9	46
Soxhlet ^b	HOAc	4	68
Soxhlet ^b	HOAc	24	42
Soxhlet ^b	TFA	4	109
Soxhlet ^b	MeOH	4	2145

^a The mobile phase was CO₂ containing 30% of modifier as indicated above, the flow rate was 1 ml min⁻¹, the extraction temperature was 100 °C and the outlet pressure was 200 bar unless stated otherwise. The MIP was prepared using DCM as porogen. ^b 180 mg batches of the clenbuterol MIP were extracted with 200 ml of solvent in each experiment. The MIP was prepared using MeCN as porogen. ^c Outlet pressure: 150 bar.

boiling point of this acid, resulting in a lower temperature of extraction. The decrease in bleeding of only 30% with a six-fold increase in extraction time indicates the limitation of this technique; however, employing other acids, *e.g.*, formic acid, may lead to a more efficient extraction.

Microwave assisted extraction. Microwave assisted extraction (MAE) turned out to be the most effective tool in achieving efficient extraction of the template. As shown in Table 3, bleeding levels as low as 3 ng ml⁻¹ could be achieved using stronger organic acids such as TFA. The time of extraction, the number of solvent exchanges and the type of acid all affect the bleeding level. Hence repeated exchange of solvent is an important factor. However, it should be noted that the weight loss was larger for the samples treated with the stronger acids. This indicates that partial hydrolysis of the polymer has occurred.

Interestingly, formic acid (HCOOH) was found to be much more efficient than HOAc, giving a bleeding level of 14 ng ml⁻¹ after only 1 h with no solvent exchange. An additional advantage with this treatment is that it leads to a smaller loss of material than with treatment using stronger acids, indicating that it is less harsh to the polymer. Possibly the smaller size of this acid facilitates easier access to the micropores associated with the imprinted sites of highest binding energy.

Investigations using basic solvents as extraction media, amine bases and NaOH, gave mixed results. Amine solvents in general were very poor although ethylenediamine (EDA), a diamine capable of chelating two-point interactions with two acid groups, resulted in half the level of bleeding compared with that for triethylamine (TEA). However, the bleeding was still far higher than that achieved with the acidic washes. Although not as efficient as acidic washing, encouraging results were achieved using the previously suggested NaOH based wash solvent (5 M NaOH–H₂O–MeOH (20 + 30 + 50 v/v/v)).⁵ This resulted in significantly lower bleeding compared with that obtained using the amine bases.

In conclusion, the MAE procedure resulted in the most efficient template removal and should therefore be considered as a routine post-treatment step for imprinted polymers, preferably in combination with 'milder' solvents such as formic acid.

MIP retention properties after post-treatment. To verify whether the MIPs retained recognition properties after the various treatments, they were packed in cartridges and tested for selectivity and recovery of the template and analogues. First, the polymer treated in acetic acid by sonication (Table 1) was checked for rebinding of clenbuterol in MeCN with different amounts of HOAc (0–10%). The binding of clenbuterol to the imprinted polymer was compared with the binding of clenbuterol to a blank polymer. The blank polymer was prepared in the same way, but without clenbuterol present during polymerization. In pure MeCN clenbuterol bound completely to both polymers, but on adding 1% HOAc the binding to the blank polymer decreased to 33% whereas complete binding to the clenbuterol imprinted polymer was still observed (Fig. 3). This shows that the templated sites in this polymer are capable of selective rebinding even after the wash treatment. At an HOAc concentration of 10% all clenbuterol bound to the polymer was removed. Corrections for the bleeding were made in the calculations.

The materials treated by MAE were tested in a similar manner but were also compared with a corresponding untreated non-washed material. First, blank wash fractions obtained from the polymers showing the lowest bleeding levels were compared (Fig. 4). Although the template bleeding was significantly reduced, the C₁₈ chromatograms revealed other bleeding peaks that partly can be attributed to the harsh treatment subjected to the material. However, this type of bleeding is not uncommon for polymer based adsorbents. Thereafter, an aqueous sample, spiked with four amino alcohols (Fig. 1), was applied to the columns, which were washed and then eluted (Fig. 5 and Table 4).

Table 3 Microwave assisted extraction of clenbuterol MIPs in various solvents^a

MIP No.	Porogen	Solvent	Extraction time ^b /h	Remaining weight ^c /%	Clenbuterol ^d /ng ml ⁻¹
1	DCM	HOAc	1		161
2	DCM	HOAc	4		135
3	DCM	HOAc ^e	4		138
4	DCM	TFA	4		6
5	MeCN	MeOH–TFA (90 + 10)	1	89	88
6	DCM	TFA	20 min	79	41
7	MeCN	TFA	20 min	77	48
8	DCM	TFA	1	77	20
9	MeCN	TFA	1	83	27
10	DCM	TFA	2	71	15
11	MeCN	TFA	2	83	21
12	DCM	TFA	4	79	17
13	MeCN	TFA	4	84	16
14	DCM	TFA	3 × 20 min	70	4
15	DCM	TFA ^f	1	82	3
16	DCM	HCl	1	77	32
17	DCM	HCO ₂ H	1	89	14
18	DCM	H ₂ SO ₄	1	0	—
19	DCM	MeOH	1	97	1433
20	DCM	TFA	3 × 20 min	67	10
21	DCM	MeOH–TEA (99 + 1)	1	87	514
22	DCM	MeOH–EDA (99 + 1)	1	122	231
23	DCM	MeOH–EDA (90 + 10)	1	122	218
24	DCM	5 M NaOH–H ₂ O–MeOH (20 + 30 + 50)	1	94	20

^a 60 mg (MIP Nos. 1–4 and 6–18) or 100 mg (MIP Nos. 5 and 19–24) of MIP in 20 ml of solvent were used and the temperature was 100 °C in each experiment unless indicated otherwise. ^b Extraction time in hours unless indicated otherwise. ^c Approximate % weight remaining after extraction in relation to prior to extraction. ^d Level of clenbuterol bleeding. In the case where 60 mg of MIP were used in the extraction the amount of polymer remaining after extraction was not sufficient to fill the extraction cartridge (full cartridge: 60 mg). In this case the bleeding value has been corrected for the actual weight (*m*) with a factor 60/*m*. ^e The volume of solvent was 50 ml. ^f The temperature was 140 °C.

It should be noted that the results for the closely related analogue bromoclenbuterol should reveal whether the templated sites had been significantly damaged upon the wash treatment. As expected, the untreated material showed considerable bleeding, resulting in a recovery of clenbuterol which was 10 times larger than the injected amount (Table 4). Clearly, the lower bleeding seen in the treated MIPs is obtained at the expense of selectivity and affinity for the template. Considering the close structural analogue bromoclenbuterol, no breakthrough was observed in the wash fraction on the non-treated material whereas significant breakthrough was seen on the post-treated materials. The breakthrough level seemed to increase in the order of decreasing bleeding. Moreover, the breakthrough of clenbuterol increased in this order. Particularly noteworthy is the increased breakthrough seen for the less structurally related

amino alcohols timolol and formoterol. Hence the TFA treatment, although dramatically reducing the bleeding problem, also gives rise to a certain loss in both binding affinity and selectivity. Most likely this loss outweighs the benefits of the reduced bleeding.

The L-PA system

The extraction procedure employed for the L-PA MIPs differed from that employed for the clenbuterol system. Two columns, packed with L-PA imprinted polymers prepared using either AIBN or V601 as initiator, were subjected to a series of different elution solvents and post-treatments after which the effect on the incremental recovery of template was studied (Fig. 6). The columns exhibited high enantioselectivity, in agreement with previous reports, and the washing procedures affected neither the selectivity nor the performance of the columns significantly. Thus at a sample load of D,L-PA of 100 nmol, the separation factor (α) prior to and after wash treatment was 5.3 and 5.8, respectively [$k'(L)$ was 5.3 prior to the elution].

A polymer prepared using the azoinitiator V-601 was used for comparison owing to the attractive properties of this initiator compared with AIBN. Thus, although they exhibit similar dissociation energies and half-lives, V-601 is associated with significantly less toxic side products. It is freely soluble in more solvent systems than AIBN and it leaves no residual nitrogens in the polymer after initiation. The latter should simplify the assessment of template recovery by nitrogen analysis. As observed in our previous work,¹⁵ the initial extraction in the Soxhlet apparatus results in incomplete recovery of the template. The discrepancy between the chromatographic determination (61%) and the result from elemental analysis of nitrogen (> 90%) is likely to be due to decomposition of the template during the prolonged reflux extraction. After column

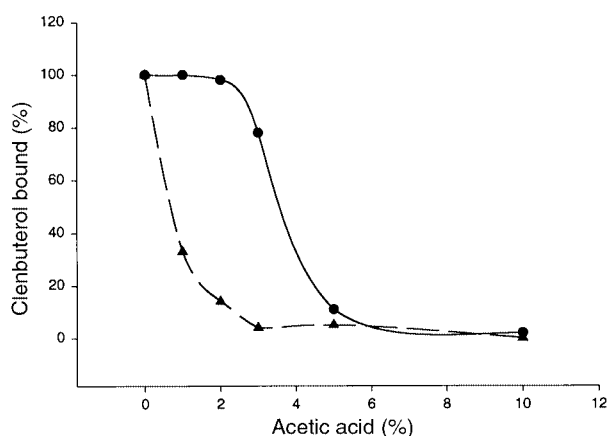


Fig. 3 Binding study using 10 ml of clenbuterol in MeCN (10 ng ml^{-1}) modified with 1–10% HOAc. The solid curve shows binding of clenbuterol to the imprinted polymer and the dashed curve shows binding to the blank polymer.

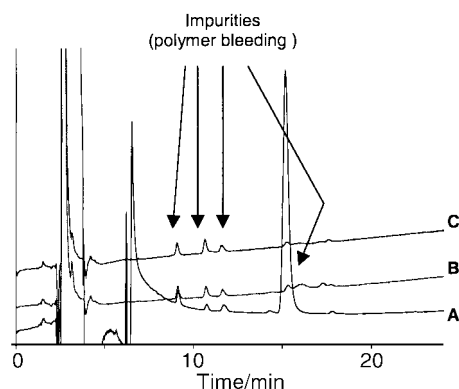


Fig. 4 C_{18} elution profiles of the fractions eluted using 10 ml of MeOH–HOAc (9 + 1 v/v) from the cartridges. (A) After spiking with 100 ng of clenbuterol; (B) cartridge packed with MIP No. 14; and (C) cartridge packed with MIP No. 15. Mobile phase: MeCN–phosphate buffer (30 + 70 v/v). Electrochemical detection: +800 mV vs. Ag/AgCl.

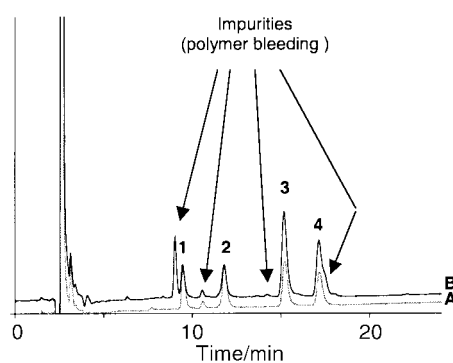


Fig. 5 C_{18} elution profiles of (A) the wash fraction [MeCN–HOAc (99 + 1 v/v)] and (B) the elution fraction [MeOH–HOAc (90 + 10 v/v)] obtained in MISPE after having passed a sample of the amino alcohols 1–4 [100 ng each of timolol (1), formoterol (2), clenbuterol (3) and bromoclenbuterol (4) in 5 ml of MeCN] through a cartridge packed with MIP No. 14. For more details, see Experimental section.

Table 4 Percentage recovery of four analytes in duplicate SPE experiments. The cartridges contained 60 mg of MIP except for MIP Nos. 14 and 15 that contained ca. 40 mg MIP. For details see Experimental section and captions of Figs. 4 and 5

Analyte	Untreated MIP		MIP 20		MIP 14		MIP 15	
	Wash	Elute	Wash	Elute	Wash	Elute	Wash	Elute
Timolol (1)	< 1	89 ± 2	47 ± 4	47 ± 2	26 ± 3	65 ± 7	55 ± 8	37 ± 3
Formoterol (2)	6 ± 3	92 ± 6	37 ± 7	65 ± 6	50 ± 7	41 ± 4	53 ± 5	32 ± 7
Clenbuterol (3)	198 ± 57	1014 ± 174	44 ± 14	137 ± 15	23 ± 8	63 ± 12	42 ± 3	50 ± 9
Bromoclenbuterol (4)	3 ± 1	87 ± 5	18 ± 2	85 ± 4	21 ± 3	65 ± 4	23 ± 1	59 ± 6

packing, each wash fraction, with a maximum volume of 50 ml, contains significantly less than 1% of the theoretical maximum amount of template. Apparently most of the template is removed upon washing of the polymer in MeCN containing HOAc or TFA and water.

After these eluents have been passed, some additional release is seen upon washing of the polymer using nitrogen bases as additives instead. After switching to DCM, a more swellable solvent which also corresponds to the porogen used in the synthesis of the MIP, no additional recovery is seen using HOAc as additive. However, on switching to the stronger acid TFA an additional release is observed. This release occurs after having passed more than 0.5 l of organic solvent containing acid and base additives. Switching to base additives did not lead to additional release in this solvent. It should be noted that the maximum extent of bleeding (*ca.* 6 nmol g⁻¹ dry packing collected in a 20 ml fraction corresponding to *ca.* 36 ng ml⁻¹ of L-PA) in these fractions is of the same order as that observed in the clenbuterol system.

After having passed the various eluents through the column, the latter was emptied and the recovered material was dried and treated under vacuum at 120 °C for 24 h. Thereafter the column was subjected to a similar elution series as prior to the heat treatment (Fig. 7). It appears that after the heat treatment the extent of template bleeding is significantly lower. For the material prepared using AIBN as initiator, after an initial elution leading to additional recoveries of up to 0.003% (based on added template), subsequent washes did not contain detectable levels of L-PA with the exception of the wash fractions in dichloromethane and TFA. However, these contained less L-PA (*ca.* 0.0013%) compared with those prior to heat treatment (*ca.* 0.003%). Interestingly, MeCN-TFA as wash solvent did not result in detectable release, which may be due to the mismatch with the porogen. This contrasts with the results for the clenbuterol system where MeCN-TFA resulted in a larger release than DCM-TFA. This agrees with the fact that MeCN was used here as the porogen.

After the DCM-TFA wash it is interesting that the subsequent wash in DCM containing EDA as additive displaced a relatively large amount of L-PA. This shows that the template removal is still incomplete and that other solvent systems may

be more efficient for the release of the remaining template. Nevertheless, the heat treatment seems to lead to a reduced overall bleeding, which agrees with previous observations.²⁶ Combined with the enhanced stability of the heat treated materials, it is likely that this type of protocol will increase the performance of the gel-like type of MIPs.²⁷ Since only one column was used in this study, the effect of this treatment needs to be verified in other systems.

Conclusions

Promising post-treatments and wash procedures have been found that, after additional optimization, may lead to procedures for reducing template bleeding to levels acceptable for trace level analysis. The most efficient wash solvents all have in common their ability to solvate efficiently linear homopolymers of the constituent monomers. Furthermore, a match between the porogen and the wash solvent seems important for achieving higher recoveries. For instance, MIPs prepared using dichloromethane and acetonitrile as porogens show lower bleeding when they are washed with their respective porogens containing acid or base additives. However, methanol containing the acid or base additive is more efficient in this regard. Even lower bleeding levels were achieved after washing in pure organic acid where the bleeding decreased with the strength or size of the acid employed. MAE using solvents such as TFA or HCOOH gave the lowest bleeding levels (3 and 14 ng ml⁻¹, respectively). Such treatment in combination with on-line coupled column techniques employing a minimum amount of MIP sorbent at present appears to be the method of choice to obtain MISPE with minimal template bleeding. With a target bleed level of less than 10% of the normal analyte concentrations, the obtained levels are, however, more than two orders of magnitude too high. At present, the bleeding problem therefore appears to be best solved by the use of a target analogue as template.⁵

Nevertheless, alternative ways to increase the removal need to be investigated. These may consist of reducing the particle size or preparing the polymers as thin layers on solid supports.

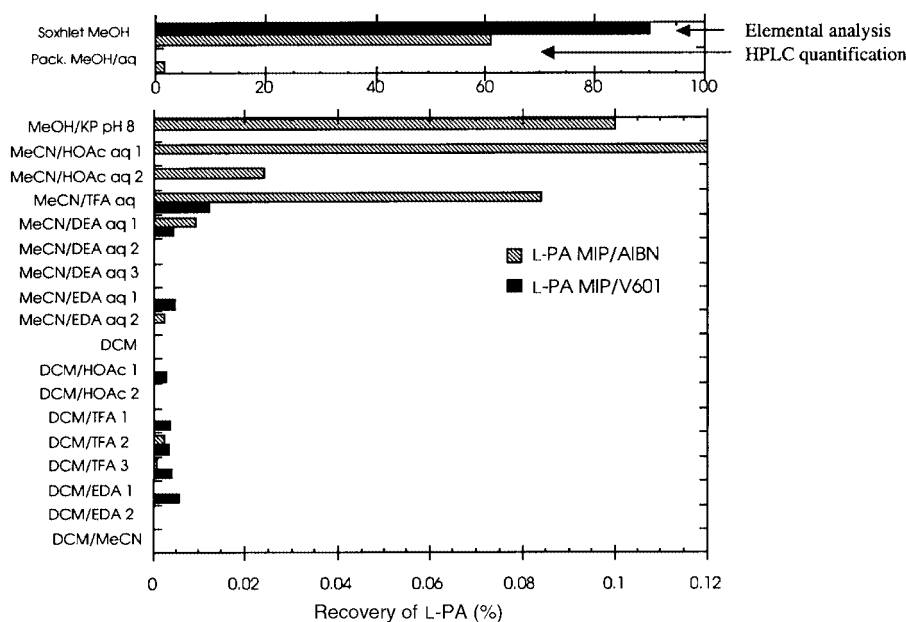


Fig. 6 Recovery of L-PA on consecutive extraction of L-PA imprinted polymers prepared using either AIBN or V-601 as free radical initiator. The volumes of the first five extracts were 100, 150, 50, 50 and 50 ml whereas for the remaining extracts the volumes were 20 ml. The recovery was calculated based on the amount of template added to the monomer mixture. DCM = dichloromethane; TFA = trifluoroacetic acid; DEA = diethylamine; EDA = ethylenediamine; MeCN-HOAc aq = 92.5 + 5 + 2.5 v/v/v; KP = potassium phosphate buffer, 0.05 M. All other additives were added to 1% v/v concentration. Packing solvent = MeOH-H₂O (80 + 20 v/v).

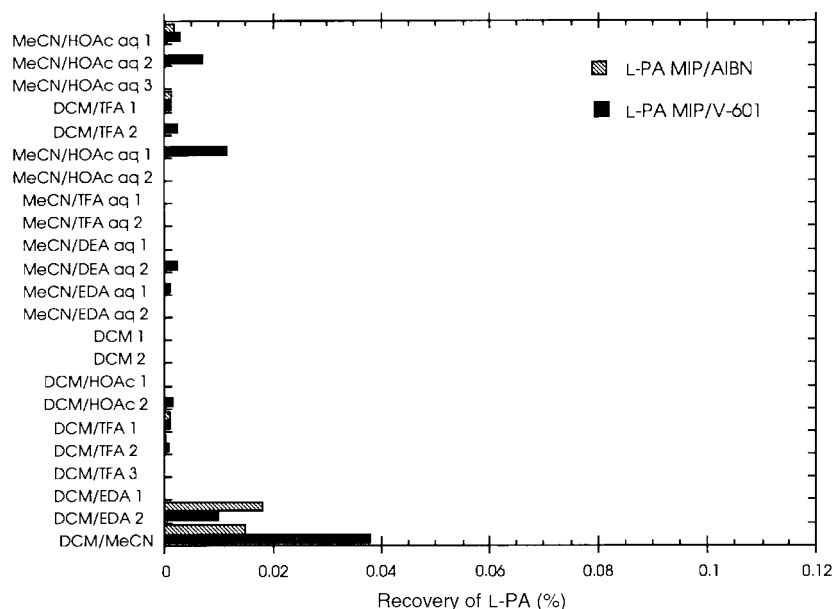


Fig. 7 Recovery of L-PA on consecutive extraction of heat-treated L-PA imprinted polymers prepared using either AIBN or V-601 as free radical initiator. The volumes of the extracts were 20 ml. For other conditions, see Fig. 6.

In combination with the optimum treatment found in this work, this may result in MIPs showing acceptable bleeding levels.

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