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FULL SHORT COMMUNICATION

Optimal Template Removal from Molecularly Imprinted Polymers by Pressurized Hot Water Extraction

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Abstract An optimal extraction method for the removal of templates from molecularly imprinted polymers (MIPs) is presented. The extraction method is based on pressurized hot water extraction (PHWE). PHWE was evaluated by application to three distinctly colored MIPs for chlorophyll (green), quercetin (yellow) and phthalocynine (dark blue) with subsequent monitoring of template removal and template bleeding by an ultraviolet spectrophotometer. The templates were washed-off and the extraction efficiency (EE) was compared to that of soxhlet and ultrasonic extraction methods. PHWE employed hot water at an optimal temperature of 220 °C, pressure of 50 bars and flow rate of 2 mL min^{-1} to thoroughly wash-off the respective templates from their MIPs. The EE evaluated for PHWE was over 99.6% for all the MIPs with no subsequent or minimal template bleeding (<0.01%). The washing procedure was simple and relatively fast as it was achieved in 70 min at the most. At 95% confidence level ($n = 3$), soxhlet and ultrasonic recorded EE that was not significantly different (<94.5% in all cases) from that of PHWE (>99.6% in all cases). Soxhlet and ultrasonic had washing procedures that were slower (over 18 h) and employed large quantities (400 mL) of organic solvents modified with acids. The percentage relative standard deviations (%RSD) for the EE and recovery results were less than 2.3% in all cases indicating the high reproducibility of the method. Overall, the three methods performed comparably in extracting templates. PHWE seems to be the method of choice as it employed water which poses no environmental threat.

Keywords Pressurized hot water extraction · Green method of extraction · Molecularly imprinted polymers · Template removal

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Introduction

Molecularly imprinted polymers (MIPs) are synthetic polymeric materials with specific recognition sites complementary in shape, size and functional groups to the template molecule, which is usually the target molecule in analysis [1]. MIPs are obtained by polymerisation of chosen functional and cross-linking monomers around a template molecule. The template–monomer interaction can be through non-covalent interactions (noncovalent imprinting), reversible covalent bonds (covalent imprinting) or mixed combinations of the two bonding methods (semi-covalent imprinting) [2]. In recent years, the development of MIPs for solid-phase extraction (MISPE) has been extensively reported in the areas of pharmaceuticals [3] environmental [4], and food [5] analysis including their use as selective sorbents for the extraction or clean-up of different classes of compounds from various complex matrices. SPE is the most advanced application area of the MIPs [6].

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 chromatography, template beeding may be insignificant whereas in analytical applications like MISPE, bleeding of the non-extracted template during the

elution step of SPE procedure as reported by Martin et al. [7] is likely to cause quantification inaccuracies. This challenge hampers the use of MIPs for chromatographic analysis. To overcome this limitation an often necessary compromise is to use a closely related structural analogue of the target analyte as the template [8] which is not always available. Thus, a practical approach to overcome the challenge of bleeding is to increase the efficiency of removing the templates from MIPs so as to facilitate their application in sample clean-up or preconcentration prior to chromatographic analysis.

Several extraction methods, including soxhlet extraction [9], washing on-line [10], and solid phase extraction [11] have been used to remove templates from MIPs. Ellwanger et al. [12] have reported the removal of templates with supercritical fluid extraction, microwave-assisted extraction and ultrasonic assisted extraction. Recently, accelerated solvent extraction (ASE) using various organic solvents containing acids or base additives has also been reported [13]. ASE had lower extraction efficiencies (up to 94.2%) than the continuous soxhlet extraction (up to 99%) [9] which used the same extraction solvents and additives. While the two extraction techniques had impressive extraction efficiencies for template removal, the selection of a suitable extraction solvent(s) from an array of solvents or combinations during optimization further slowed down their adaptation.

To address the challenges of conventional template removal, the use of pressurized hot water extraction (PHWE) as a feasible green extraction method that utilizes only water at elevated temperature and pressure conditions [14] for the removal of templates from MIPs is proposed in this study. Traditionally, water is not considered a suitable extraction fluid for non-polar or organic compounds at ambient temperature [15]. When the temperature of water is raised, there is a steady decrease in its permittivity, viscosity and surface tension coupled to an increase in its diffusivity characteristics [16]. With enough pressure to maintain water in the liquid phase at elevated temperature, the initial value of the dielectric constant (ε) of 80 at 25 °C decreases to 27 at 250 °C and 50 bar [17], which falls between those of methanol ($\varepsilon = 33$) and ethanol ($\varepsilon = 24$) at 25 °C [18]. Under these conditions, water behaves like some organic solvents as it can dissolve a wide range of medium and low polarity analytes [19]. The other advantage of PHWE is that there is a reduction in the consumption of organic solvents. Moreover, water is easily available, non-toxic and can be recycled or disposed with minimal environmental challenges [20]. To the best of our knowledge, a demonstration of the efficiency of water under subcritical conditions as the sole leaching solvent (without modifiers) in the removal of templates from MIPs is evaluated for the first time in this study.

Experimental

Methacrylic acid (MAA), ethylene glycol methacrylate (EGDMA), tetrahydrofuran (THF), ethanol, methanol (MeOH), chlorophyll, quercetin, kaempferol and azobisisobutyronitrile (AIBN) were supplied by Sigma-Aldrich (Saint Louis, MO, USA). Copper(II) phthalocynine was a gift from the DST/MINTEK Nanotechnology Innovation Centre at Rhodes University (Grahamstown, South Africa). Reagents used were of analytical grade. All water used was obtained from a Direct Q 3UV Milli Q system (Billerica, MA, USA).

Instrumentation and Apparatus

Custom-made PHWE equipment by Prof. C. Turner (Lund University, Sweden) which comprised of a gas chromatographic oven with a maximum temperature of 350 °C was used for the removal of templates. Inside the chamber, a preheated stainless steel coil was present to maintain the programmed temperature followed by the stainless steel extraction cell. All the tubings were made from stainless steel. Ultrapure water was pumped using Bio LC pump Dionex Model GS50 Gradient Pump, Dionex Corporation (Sunnyvale, CA, USA). The working range of pressure was kept at 50 bars. Soxhlet and ultrasonic extractor from Integral systems (Randburg, South Africa) were also used for extraction of templates so as to compare with the PHWE.

A lambda 25 Perkin-Elmer spectrophotometer, by Perkin-Elmer (Santa Clara, CA, USA) was used to detect the concentration of chlorophyll, copper(II) phthalocynine, quercetin and kaempferol at 680, 610, 312 and 350 nm, respectively, using a 1-cm cell.

An MSE Mistral 1000 by Sanyo Gallenkamp (Loughborough, UK), was employed for centrifugation. Standard test sieves by Retsch GmbH & Co. (Haan, Germany), were used to control the average size of the polymer particles by screen analysis. For thermal analysis, a TGA 7 thermogravimetric analyzer Perkin-Elmer (Santa Clara, CA, USA) was employed.

Preparation of the MIPs

For the synthesis of the three model MIPs, a bulk polymerization method [21] was employed with MAA and EGDMA as the functional and cross linking monomers in the ratio, 1:5, respectively. The mixtures were refluxed in either THF or ethanol at 65 or 75 °C for 6 h for the chlorophyll, 4 h for quercetin and 9 h for the phthalocynine MIP. The resultant polymer monoliths were ground to powders with particle sizes of less than 45 μm in diameter, and then introduced to the PHWE set up, soxhlet or

ultrasonic extraction for template removal. Thereafter, the particles were left to dry in open air overnight ready to be used for rebinding experiments. Control polymers referred to as non-imprinted polymers (NIPs), without the imprinting templates for chlorophyll, quercetin and copper(II) phthalocynine were prepared following a similar procedure.

Thermo-Gravimetric Analysis

TGA was performed to determine the stability of the prepared MIPs to ensure that extractions were carried out at temperatures that the MIPs would not result in the degradation of the polymers.

Template Removal Method

Pressurized Hot Water Extraction

To wash off the templates, 800 mg of the MIPs were extracted in a 34 mL PHWE extraction cell with water as the solvent. All extraction procedures were carried out at a flow rate of 2 mL min^{-1}; temperature, 220 °C and pressure, 50 atm for chlorophyll and phthalocynine MIPs. For quercetin, the optimized temperature was slightly higher at 235 °C. Aliquots of the washings from the PHWE set-up were then collected at 10 min intervals until the detected absorbance of the templates in subsequent washings was constant.

Soxhlet Extraction and Ultrasonic Extraction

800 mg of the MIPs were extracted using up to 9 times fresh 80 mL MeOH aliquots at 70 °C for up to 16 h. Washings were collected every 2 h to determine the absorbance of the templates.

Determination of the Absorbance of the Templates in the Washings Using UV Spectrophotometer

The absorbance of the different templates in the washings was determined with a UV spectrophotometer. This was carried out in triplicates for each washing method. Statistical methods were then used to determine the mean values and the %RSD. From the values, plots of absorbance against time of collection for each washing was constructed for each MIP and extraction method (see Figs. 1, 2, and 3).

Template Bleeding Evaluation

To assess if there were any remnants of the templates (template bleeding) in the washed MIPs, 800 mg of the dry, washed MIP powders were extracted by employing the three different extraction methods with water or MeOH modified with acetic acid (9:1 *v/v*). Acetic acid was chosen as the modifier as it has been used to enhance the elution strength of solvents during desorption studies [12, 13].

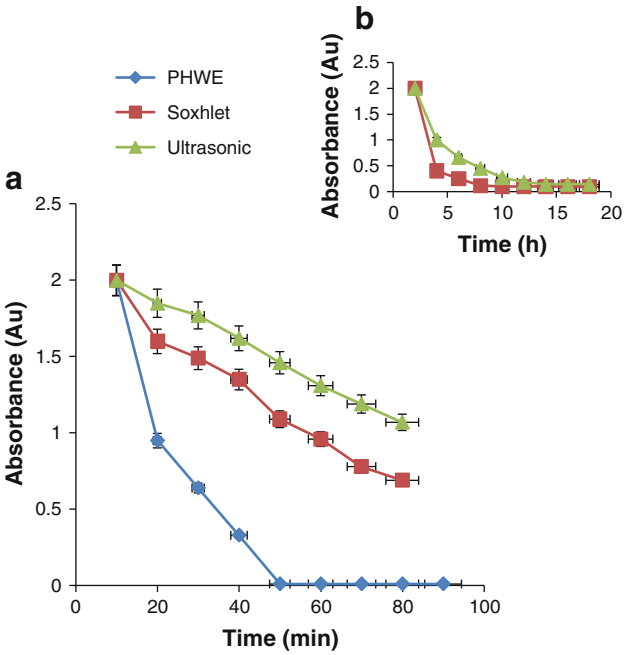

Fig. 1 Absorbance of chlorophyll in each washing at **a** 10 min intervals for the three extraction methods and **b** at 2 h intervals for the two that took longer to complete the extraction

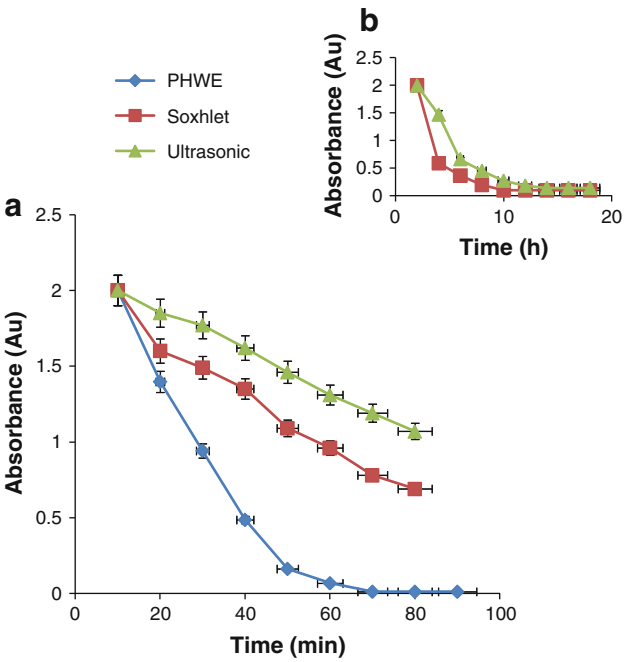

Fig. 2 Absorbance of quercetin in each washing at **a** 10 min intervals for the three extraction methods and **b** at 2 h intervals for the two that took longer to complete the extraction

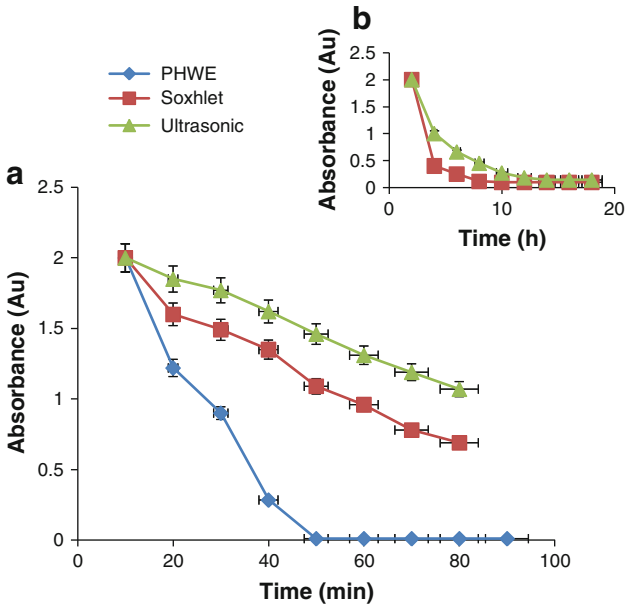

Fig. 3 Absorbance of phthalocynine in each washing at **a** 10 min intervals for the three extraction methods and **b** at 2 h intervals for the two that took longer to complete the extraction

Absorbance of templates from the washings was determined with the UV spectrophotometer so as to ascertain that there was no further change in the template bleeding concentrations detected by each method. The experiments were performed in triplicates.

Rebinding Experiments

Equilibrium rebinding experiments were used to evaluate the binding affinity of the imprinted polymers. An optimal quantity of the washed MIP (800 mg) was mixed with 5 mL aliquots of 10% standards (*w/v*) of each of the templates for an optimal equilibration time of 25 min. The mixtures were then centrifuged for 2 min at 2,000 rpm after which the absorbance of each of the templates in the supernatant was determined and the percentage of the template bound to a relevant MIP calculated to give recovery results. These were performed in triplicates with the accompanying percentage relative standard deviations (%RSD) calculated to give an estimation of the precision of the methods. The same procedure was followed in the control experiments employing 800 mg of the NIP instead. To study the selectivity of the MIPs, the equilibrium rebinding experiments were carried out at the same conditions except that the solutions contained the templates and their structural analogues (competitors). Chlorophyll competed with copper(II) phthalocynine and vice versa; quercetin with kaempferol. From the coupled mixtures, the selectivity coefficients (k) were then evaluated [21].

Results and Discussion

TGA

The TGA results showed that all the MIPs were stable at temperatures where the optimum extraction was realized (220 and 235 °C). The MIPs were stable at these temperatures as it was marked by the initial horizontal sections of the TGA plots up to about 270 °C, after which there were sharp drops indicating to some degradation of the MIPs. During the washing of the MIPs, the temperature of the hot water was maintained at optimal temperatures of 220 and 235 °C to ensure that the integrity of the MIPs was not compromised in the process.

Template Removal Method

Following the procedures described in the experimental section, the templates were thoroughly washed off their MIPs so as to free recognition sites for selective binding during the rebinding experiments. The concentration (absorbance) of the templates as determined by the UV spectrophotometer decreased with time in all cases until it remained constant with continued washing. This marked the point at which the templates were completely removed by a particular method of extraction (see Figs. 1, 2 and 3). The great loss of the distinct, bright colours of the MIPs to slightly white or white after washing also demonstrated that they were thoroughly washed-off their coloured templates.

According to the plots, the complete process of washing-off the templates took under 70 min for all the MIPs when using PHWE (see Figs. 1a, 2a, 3a). This was advantageous as the extraction time was relatively very short compared to that of the soxhlet and ultrasonic extraction methods which took several hours (see Figs. 1b, 2b, 3b). Furthermore PHWE used an environmentally friendly solvent (water) to achieve the same or better results. Conventional methods of removing templates like soxhlet and ultrasonic extraction employ organic solvents to achieve optimal extraction which have detrimental effects to the environment.

Template Bleeding

Figure 4 showed that template removal by PHWE was better than the other two methods employed as marked by the much lower template bleeding concentrations (0.02%) or non-detectable in some cases when using it. The relatively higher bleeding concentrations (over 0.1%) by soxhlet and ultrasonic methods are a clear indication that the methods are not exhaustive in washing-off templates from MIPs.

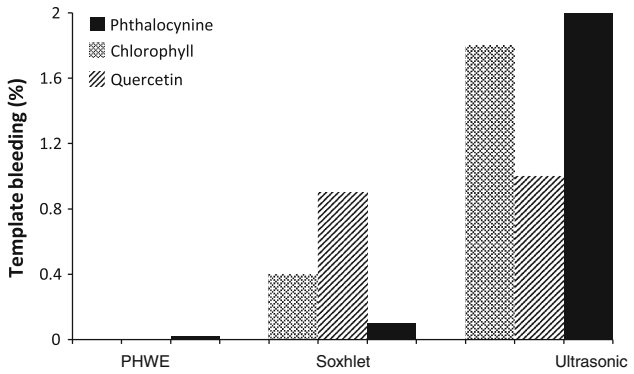

Fig. 4 Template bleeding of the MIPs originally washed by the different extraction methods

Rebinding Experiments

Rebinding experiments were used to evaluate the binding affinity of the MIPs after washing-off the templates. On average, the MIPs adsorbed over 99.6–100% of templates after PHWE template removal and less than 94.5% after the other two methods were used. Statistically, the %recoveries were not significantly different at 95% confidence level using the *t* test hence the perfomance of the three extraction methods were comparable. The two NIPs (one in THF and the other in ethanol) adsorbed 8.9, 7.1, 9.4% for chlorophyll, quercitin and phthalocynine MIP, respectively, which were relatively low and suiting as the NIPs were not templated hence had no recognition sites to rebind highly. Even in the presence of their structural analogues, the MIPs still gave high recoveries resulting in high selectivity coefficients (*k*) of over 12 for all the methods. The *k* value gives an estimation of the effect of imprinting on selectivity [22]. For a value of 12, it means that the prepared MIP selectively adsorbed the intended analyte (template) 12 times better than it absorbed the competing species when the two were exposed to the MIP at the same time. The high percentage recoveries and selectivity coefficients are a demonstration that the recognition sites of the MIPs were not destroyed and still had excellent selectivities even after employing the methods of extraction.

Conclusion

An optimal extraction method to remove templates from MIPs employing hot water and subsequently reducing template bleeding to insignificant levels has been developed. The PHWE method resulted in the most efficient template removal and the lowest bleeding. The recognition sites were maintained even after extraction as marked by the high percentage recoveries during rebinding experiments. PHWE is also the sustainable method of extraction as it employed water which is cheaper, readily available and pose minimal disposal challenges as compared to the organic solvents used by the other methods.

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