Development of a molecularly imprinted polymer (MIP) for the analysis of avermectin.

NOTE TO USERS

DEVELOPMENT OF A MOLECULARLY IMPRINTED POLYMER (MIP) FOR THE ANALYSIS OF AVERMECTIN

By

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A Dissertation

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Abstract

Molecularly imprinted polymers (MIPs) are a common tool in the field of separations. The presence of highly specific binding sites that are complementary to the target molecule in size, shape and functionality provide MIPs with a very high selectivity for the analyte of interest. Because of this high selectivity, they are ideal for the isolation and concentration of target analytes from complex matrices. Most MIPs thus far have been prepared for small analyte molecules in solvent-based applications.

To determine if an MIP can be prepared to selectively isolate avermectin, a large molecule, in aqueous samples, several MIPs were synthesized using different methods, monomers, and polymerization solvents. The polymers were evaluated using chromatography and different mobile phase solvents containing various levels of water. The retention of avermectin on the imprinted polymers was compared to the retention on non-imprinted control polymers. Specificity was evaluated by comparing the retention of avermectin to the retention of a compound with a very similar structure. The most successful imprinted polymer with very high selectivity (>18.0) for avermectin was prepared non-covalently in chloroform using methacrylic acid monomer and evaluated in chloroform mobile phase. An imprinted polymer prepared non-covalently in acetonitrile was not as selective for avermectin when evaluated in chloroform compared with the polymer that was prepared in chloroform. Adding water to the mobile phase diminished the selectivity of all the imprinted polymers for avermectin. The MIP prepared in acetonitrile was evaluated for use in solid phase extraction of avermectin in aqueous samples.

Results show that MIPs can be prepared for the selective recognition of a large, complex molecule. The best selectivity was demonstrated when the polymer was prepared non-covalently in chloroform and also evaluated in chloroform containing a small amount of acetic acid.

For evaluation of a more polar acetonitrile matrix, the best selectivity was observed when the polymer was prepared non-covalently in acetonitrile. Covalent preparation of the polymer followed by recognition of the analyte by non-covalent interactions did not improve the selectivity. The addition of a small amount of water to the sample matrix reduced the selectivity of all MIPs.

Introduction

Molecular imprinting is a rapidly developing technique for preparing polymeric materials that are capable of very specific molecular recognition. The method usually involves crosslinking functional monomers in by radical polymerization in the presence of an analyte, followed by removal of the analyte to leave behind cavities in the polymer which are complementary in shape to the analyte molecule. The imprinted polymer can then selectivity rebind the analyte.[1] Two general approaches to molecular imprinting are: covalent, in which the functional monomers are chemically bound to the analyte molecules prior to polymerization; and non-covalent, in which the functional monomers participate in weaker non-covalent interactions with the analyte molecules during polymerization.[2]

In both methods, a polymer is prepared which contains cavities that recognize a specific analyte molecule in terms of shape, size and chemical functionality. The molecule of interest, called the "template," is incorporated during the synthesis of the polymer. When the synthesis is complete, the template is extracted, leaving behind a three dimensional cavity that is complementary both physically and chemically to the template.[3]

Molecularly imprinted polymers have unique characteristics that are beneficial in analytical applications. Imprinted polymers provide an analytically powerful and inexpensive alternative to conventional technologies by enabling the identification of a target molecule in the presence of numerous interfering species. Imprinted polymers exhibit good specificity for various compounds of interest, and in some cases, the selectivities and binding affinities achieved from the molecular imprinting process

approach those demonstrated by antigen-antibody systems.[4,5,6] They have excellent chemical and mechanical stability over long-term use, and are resistant to chemically harsh environments.[4,7]

Molecularly imprinted polymers do, however, have several disadvantages. The template must be able to tolerate the polymerization process to the extent that it can impart useful structural information to the polymer. Some polymers, although selective for their templates, are mechanically not suitable for chromatographic applications and undergo significant compaction under flow conditions.[8] The potential for leakage of the imprint molecule from the polymer is also an issue if template removal from the finished polymer is difficult. This can be a significant problem when using the polymer for trace analysis, such as in solid phase extraction (SPE).[9] This can also result in an imprinted polymer with low capacity and binding site heterogeneity. However, this can sometimes be circumvented by the use of a compound with a very similar structure to the analyte for the imprinting process.[10,11] The imprinted sites should recognize the analyte of interest, and leakage of the molecule used for imprinting should not interfere with quantitation of the target species as long as the separation using the polymer is followed by analysis using a technique that can distinguish between the two species. This approach also avoids the difficulties of finding a pure sample of the analyte to be used as template.[3]

Traditionally, MIPs have been prepared and used only in solvent-based environments. For non-covalently prepared MIPs, alcohols and water compete with the hydrogen bonding interactions that are the main interactions providing selectivity between the template and monomers. For covalent MIPs, many potential target molecules are soluble only in solvents.[12] However, many analytes, especially those with biological relevance, need to be dissolved in aqueous medium due to lack of solubility or

change in conformation in organic medium.[13] Therefore, research in ways to create and use MIPs in an aqueous environment is needed.

Molecularly imprinted polymers have been prepared for various analytes of importance and have been used successfully in applications such as chromatography, capillary electrophoresis, solid phase extraction and other separation methods.[14,15] However, MIPs thus far have been used primarily for small analytes, and the practical use of MIPs for selective recognition of large molecules has been limited.[16,17] Avermectin is a pharmaceutical compound that was introduced to the marketplace in the 1980's as an antiparasitic drug and an agricultural pesticide.[18] It is a large, complex molecule with a molecular weight of 872 amu. It is not very soluble in water, but the analysis of avermectin in wastewater at very low concentrations is important for environmental concerns due to its high toxicity to aquatic life. Because avermectin is toxic at concentrations lower than the detection limit of the analytical method currently used for its analysis in water, development of a method to improve the sensitivity of the analysis would be very beneficial. The use of an MIP to separate the analyte from the complex aqueous sample matrix and to concentrate it for further analysis would provide a valuable and practical application of an MIP for this molecule.

The overall objectives of this research project are A) to develop an MIP, prepared either non-covalently or covalently, that is selective for a large, complex molecule, and B) to optimize the preparation of the MIP for use in aqueous medium.

The applied goal of the project is the preparation of an imprinted polymer for the recognition and isolation of avermectin in an aqueous medium. To approach this goal, several specific aims will be addressed:

1. the preparation of an MIP for avermectin using non-covalent imprinting to evaluate the possibility of making an imprinted polymer for this compound.

2. the evaluation of the possibility of using an imprinted polymer that relies on hydrogen bonding interactions to recognize avermectin in organic media.

3. the evaluation of the possibility of using an imprinted polymer that relies on hydrophobic interactions to recognize avermectin in aqueous media.

4. the covalent preparation of an MIP to improve the selectivity of the polymer for avermectin in an aqueous medium.

5. the use of the most selective polymer for development of a solid phase extraction method for avermectin in an aqueous sample.

Background

History

Molecular imprinting is becoming an established technique for the preparation of polymeric materials capable of recognizing small molecules.[19,20] The first appearance of molecularly imprinted polymers (MIPs) dates back to the early 1930's when silica gels

were used in the initial investigations. In the 1940's, fundamental studies were performed to determine the mechanism of antibody antigen interactions using molecular imprinting technology. However, due to the limitations of silica for molecular imprinting, there was a decline in molecular imprinting research. In 1972, covalent molecular imprinting technology was introduced by Wulff et al.[21] and in the early 1980's the successful preparation of an MIP using non-covalent imprinting technology was presented by Mosbach et al.[22] These techniques offered a means of preparing molecular imprints in organic matrices and avoided the limitations of stability and reproducibility of silica material. Since the 1980's, there has been an almost exponential increase in published papers on molecular imprinting technology.[3]

Non-covalent Versus Covalent Preparation Methods

Molecular imprinting can be approached in two ways: the self-assembly (non-covalent) approach and the pre-organized (covalent) approach. A schematic representation of these two approaches, which differ with respect to the mechanism of interaction in pre-polymerization, are shown in Figure 1.[4]

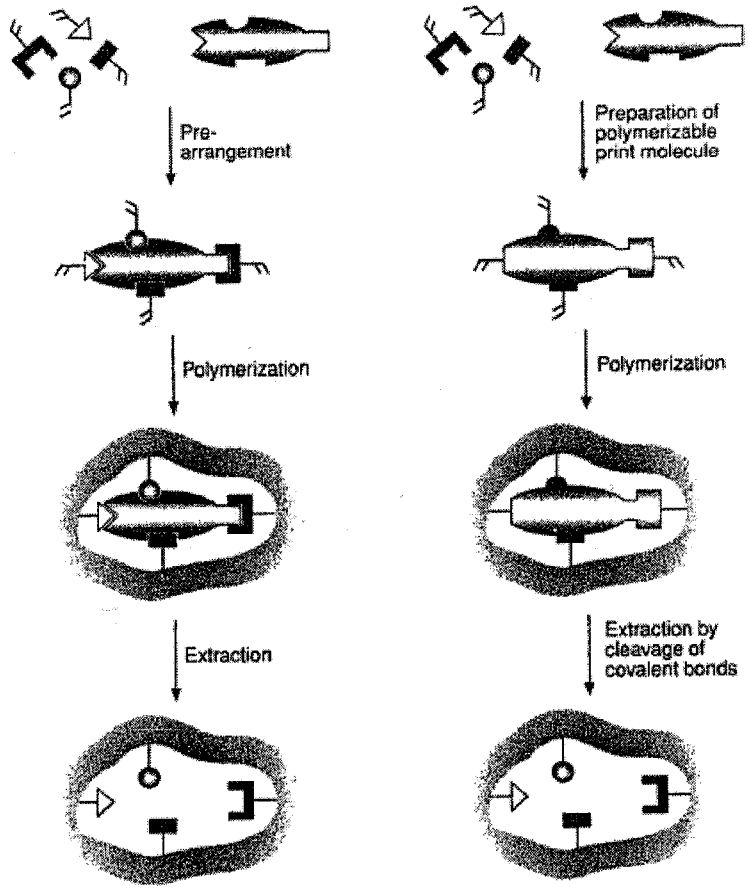


Figure 1. Schematic representation of molecular imprinting by the self-assembly (non-covalent) approach (left) and the pre-organized (covalent) approach (right).[4]

The self-assembly approach is referred to as non-covalent because it utilizes non-covalent interactions between the template and the monomers during the formation of the polymer. These same interactions are then used in the rebinding of the template to the polymer during later use to recognize the template. These interactions can be one or more of the following interactions: pi-pi interactions, hydrogen bonding, hydrophobic/van der Waal's interactions, transition metal-ligand binding, crown ether ionic interactions, and ionic bonding.[23] These interactions (as well as a covalent

interaction) are represented in Figure 2. The type of interaction exploited for imprinting depends on the functionalities present on the template and the monomer chosen for polymerization.

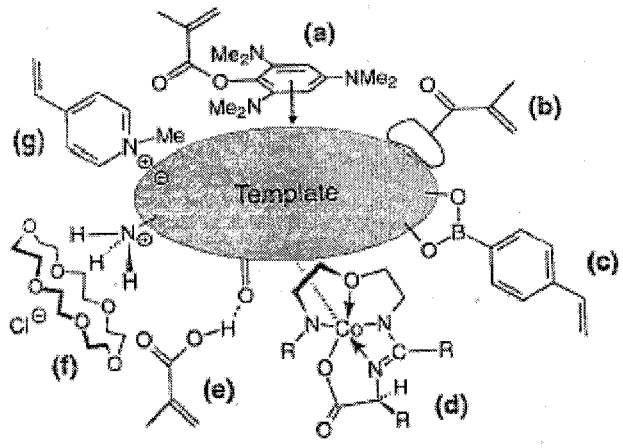


Figure 2. Types of binding interactions exploited during templating: a. pi-pi interaction, b. hydrophobic or van der Waals interaction, c. covalent bonds, d. transition metal-ligand binding, e. hydrogen bonding, f. "crown ether" ionic interaction, g. ionic interaction.[23]

To prepare a non-covalently imprinted polymer for a given template molecule, a functional monomer or combination of monomers is selected with chemical functionality complementary to that of the template. The template and monomer are combined in an aprotic, nonpolar porogenic solvent that does not interfere with the interaction (for example, hydrogen bonding) of the analyte and monomers. The solvent induces a porous structure in the polymer, thus allowing the template molecule to access the imprinted sites later during rebinding.[24] One or more of the weak intermolecular interactions causes the monomers to assemble around the template molecule. An excess of a cross-linking monomer is added which forms the backbone of the polymer

structure. An initiator is added which is activated by heat or UV radiation to begin the polymerization process. During polymerization, self-assembled complexes are spontaneously established in the liquid phase and then sterically fixed by polymerization with a high degree of cross-linking in the polymer network. After the polymer is formed, which usually takes about 24 hours, the template is extracted by grinding the polymer and washing out the template with an appropriate solution, leaving vacant cavities that are complementary sterically and contain functionality corresponding to the imprinted molecule. Because there is no covalent bonding between the template and monomers, most of the template is easily extracted by washing. The shape of the sites, maintained by the polymer backbone, and the arrangement of functional groups in the cavities result in recognition sites that are specific and have affinity for the template. Subsequent use of the polymer for recognition of the imprinted molecule results in the selective rebinding of the imprint structure from a matrix by the same non-covalent interactions that were used in the preparation of the polymer.[25,26,27,28]

In covalent imprinting, the monomer is first linked to the template through a labile covalent bond. The monomer-template complex is then copolymerized with an excess of crosslinker in the presence of a porogenic solvent. Polymerization is completed as in non-covalent imprinting, and the monomer-template complex becomes bound as part of the polymer. After polymer formation, the polymer is dried, ground, and sieved; the bond holding the template to the polymer is chemically cleaved to free the template from the polymer, leaving a cavity that is complementary to the template in shape. Recognition of the template by the polymer relies on the same covalent interaction by which the template was first bound to the monomer.[26,27,28]

More recently, a hybrid of these techniques called "semi-covalent" imprinting has been developed.[29] In this type of imprinting, the monomer and template are first linked

by covalent bonds as in the covalent approach. Following cleavage of the template from the polymer, only non-covalent interactions are used for the rebinding of the template to the polymer during recognition.[30,31]

Advantages and Disadvantages of the Different Methods of Preparation

Both the non-covalent and covalent imprinting methods have advantages and disadvantages.[32] Most of the research to date has involved the non-covalent preparation of imprinted polymers.[4,25] The non-covalent approach is simple and easy because the reagents are simply mixed, allowing the functionalized monomers to "prearrange" around the template molecules by non-covalent interactions prior to polymerization. Covalent modification of the print molecule is not required, and a variety of different binding interactions may produce the monomer-template interactions. The kinetics of the non-covalent binding, analogous to enzyme-substrate binding, compare favorably to the reversible covalent binding approach.[33] Imprinted polymers can be prepared for templates without specific knowledge of their structure or reactivity.[8] However, in non-covalent imprinting, the interactions between the template molecule and monomers are not specific and only limited control may be exerted over the exact formation of the binding cavities. As a result, non-covalently prepared polymers contain a heterogeneous distribution of binding sites throughout the polymer, which can lead to non-specific binding and poor molecular recognition of the template.[34,35] If the polymer is used for chromatography, peak broadening and tailing may result.[8,36]

The covalent bonding approach may, on the other hand, provide the best imprinted polymers due to the more homogeneous binding sites that can result from the more stable monomer-template interactions defined prior to the imprinting process.[37]

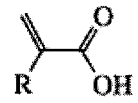
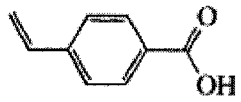
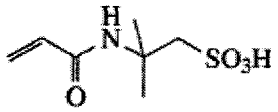
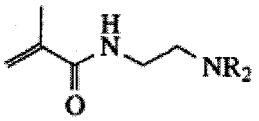
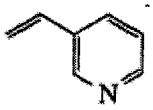
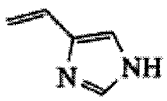
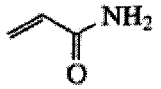
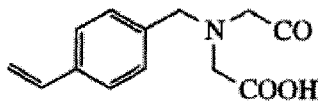
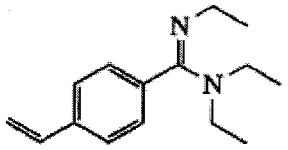
However, several disadvantages limit the use of this approach. Although enhanced selectivities can sometimes be obtained with covalently imprinted polymers, not all templates can form reversible covalent linkages with the monomers used in polymerization. Imprinting of these molecules must utilize the non-covalent method, which is not as limited by the functionality of the template.[38] After polymerization is complete, the template must be cleaved from the polymer, which typically requires refluxing the polymer in sodium hydroxide because simple washing is not usually sufficient to disrupt the covalent bond between the template and the monomer incorporated into the polymer network.[4,33] In most cases when using the polymer for template recognition, the rebinding of the template to the polymer needs to be rapid, such as in chromatographic separations in which the covalent bond formation and cleavage need to be readily reversible under conditions mild enough to perform chromatography.[39,40] Because the repeated interaction of the analyte with the polymer stationary phase needs to be quick but the covalent interactions required for recognition are often slow, poor chromatography may result.[23,29]

The "semi-covalent" approach combines the benefits of better binding site integrity provided by the covalently bound monomer-template precursor with the versatility and more kinetically favorable non-covalent interactions used for the rebinding of the template to the polymer during recognition.[29,33,41] However, this approach also requires the synthesis of a covalent monomer-template derivative as in the covalent approach.

Preparation of MIPs

Because most of the research with MIPs has involved the preparation of non-covalently imprinted polymers, the synthesis of MIPs using this approach will be discussed in more detail. In preparing a non-covalent polymer for a chosen analyte, a number of choices must be made. The choice of an appropriate functional monomer for the optimum non-covalent interactions can affect the efficiency of the imprint. The monomer must be able to form a non-covalent bond with the template molecule during polymerization.[42] A list of the most common functional monomers used in the synthesis of MIPs is shown in Table 1.

Table 1. Examples of the most commonly used functional monomers for the synthesis of MIPs.[43]

Functional monomer	Structural formula of the monome
Acrylic acids (R = H, CH_3, CF_3, CH_2COOH)	
Vinylbenzoic acids	
Acrylamidosulfonic acids	
Aminomethacrylamides (R = H_2, C_2H_5)	
Vinylpyridines	
Vinylimidazoles	
Acrylamides	
4-(Vinylbenzyl)iminodiacetic acid	
N,N'-Diethyl-4-vinylbenzamidine	

Currently, the carboxylic acid group, as found in methacrylic acid (Figure 3), is the most commonly used hydrogen bonding functional group in molecular imprinting. Although some screening methods are being developed for improving the process of

choosing a monomer, the choice is usually made by trial and error, or based on past experience and existing knowledge. A large number of functional monomers have been studied for various template molecules, and methacrylic acid (MAA) has demonstrated a general utility for a wide range of compounds.[19,42] Methacrylic acid is capable of acting as both a hydrogen bond donor and acceptor, and as such can promote the hydrogen bonding required for retention of many analytes during rebinding studies.[44,45]

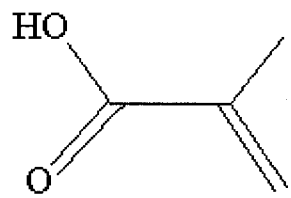


Figure 3. Methacrylic acid (MAA) monomer used in the preparation of non-covalent molecularly imprinted polymers.

Although MAA can interact strongly with basic functional groups, the hydrogen bonding ability of this functional group is not very strong in polar solvents. Molecularly imprinted polymers made in a polar solvent using carboxylic functional monomers and print molecules which can only form hydrogen bonds have often exhibited weak recognition and, in some cases, no recognition at all.[46] Several articles on the imprinting of molecules in solvents more polar than chloroform, such as acetonitrile, have indicated that some analytes, especially protected amino acids, were better imprinted in acetonitrile using acrylamide as the functional monomer instead of MAA. The authors suggested that acrylamide (Figure 4), although less acidic than MAA, is more polar and can therefore undergo stronger hydrogen bonds in polar media with the template.

Derivatives of the amino acids tryptophan, tyrosine and phenylalanine were shown to exhibit better selectivity and enantiomeric recognition when imprinted with acrylamide compared with MAA.[47]

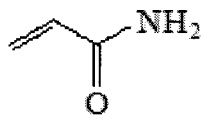
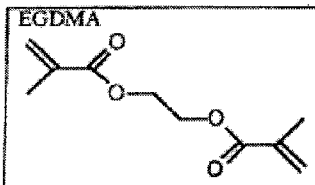
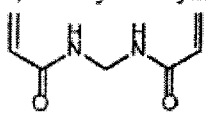
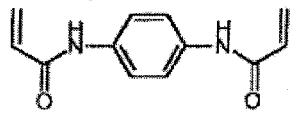
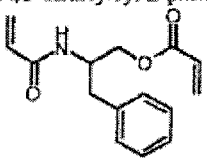
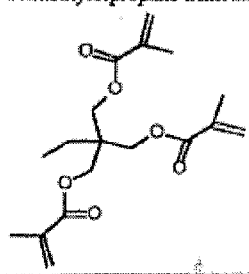
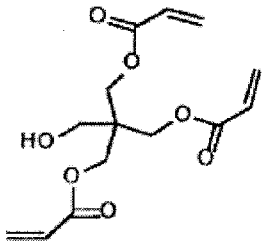
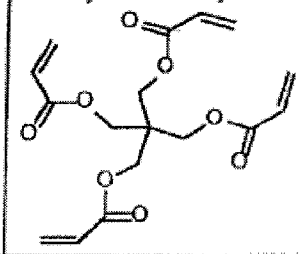


Figure 4. Acrylamide monomer used in the preparation of non-covalent molecularly imprinted polymers.

The selection of crosslinker is the next important choice to be made when preparing an MIP. The role of the crosslinker is to form a polymer network that is rigid enough to retain a memory of the imprint after the template has been removed.[48] Table 2 is a list of crosslinkers which can be used for the preparation of MIPs. Ethylene glycol dimethacrylate (EGDMA) is a commonly chosen crosslinker for initial investigations.

Table 2. Commonly used crosslinkers for preparation of MIPs.[49]

EGDMA 	p-DVB 
N,N'-methylendiacrylamide 	N,N'-1,4-phenylendiacrylamide 
N,O-bisacryloyl-L-phenylalaninol 	Trimethylolpropane trimethacrylate (TRIM) 
Pentaerythritol triacrylate 	Pentaerythritol tetraacrylate 

To determine the ratio of crosslinker and monomer to template, trial and error is again the most frequently used method. The use of non-covalent interactions for molecular imprinting is inherently limited by the stability constant(s) for the formation of adducts in solution. The monomer must be present in sufficient excess compared to the template to provide enough functionality to form non-covalent interactions with as many locations on the template as possible to improve the formation of the cavities.[38] However, using higher functional monomer:template ratios to increase the number of non-covalent complexes formed leads to increased numbers of randomly oriented

functional groups, which in turn leads to increased levels of non-specific binding. Using much lower ratios, a non-imprinted polymer being the extreme case, can lead to fewer useful polymer cavities with shapes complementary to the analytes.[41] The crosslinker must be in much greater excess with respect to the template and monomer to ensure a high degree of crosslinking to stabilize the functional group arrangement.[38] A ratio of 40:15:1 crosslinker:monomer:template is a typical ratio for initial investigations.

The final decision to be made in the synthesis of an MIP is the choice of polymerization solvent. The porogenic solvent used for molecular imprinting is one of the most important factors in determining effective molecular recognition, especially in non-covalent molecular imprinting, because the "tightness" of the assembly of the polymer around the template molecule is determined by physical and chemical characteristics of the solvent used for polymerization.[36] The structural integrity of the polymer network must be "tight" enough to withstand the conditions in which the polymer is later used for recognition of the analyte, without swelling or otherwise deforming. However, the polymer must be "loose" enough to allow the formation of cavities in the polymer that maintain the shape and functionality to recognize the analyte during later use. Generally, MIPs prepared by polymerization in a relatively nonpolar organic solvent exhibit better recognition than those prepared using a polar organic solvent. The hydrogen bonding strength is significantly affected by the polymerization medium, and the functional group of the monomer associates more weakly with the template molecules in a polar solvent.[47] The vast majority of MIPs reported have been prepared using organic nonpolar solvents for polymerization and evaluation.[41]

Applications of Imprinted Polymers

Molecularly imprinted polymers have been used to selectively recognize molecules in a wide range of pharmaceutical, analytical and biological applications.[25] The number of investigations into the use of MIPs for many analytical techniques, including liquid chromatography, capillary electrophoresis, capillary electrochromatography, solid phase extraction, and immunoassay has been steadily increasing.[5,50] Molecularly imprinted polymers have also been used in thin layer chromatography, membrane separations and bubble fractionation and have been introduced as a complement to biological antibodies in different types of binding assays. Another application of increasing interest is the use of an MIP as the recognition element in sensors.[3] One of the most exploited areas of application is in tailor-made chiral chromatographic stationary phase development, in which the high selectivity of these materials for a predetermined ligand makes them versatile systems for custom chromatographic method development.[7] Materials prepared by molecular imprinting have been used successfully for chiral separations of amino acid derivatives, drugs and sugar derivatives, for specific recognition of steroids, proteins and protein analogues, as antibody and receptor mimics, as ion selective absorbents, and as enzyme mimics to direct organic reactions.[47] The versatility of the imprinting methodology can also be exploited to generate polymers for uses beyond those involving simply the recognition or separation of small organic molecules. Researchers have begun to explore the use of imprinted polymers in synthesis, with a view to creating novel and robust reactive supports as "protective groups" and catalysts.[51] One of the easiest and most practical uses of MIPs is for applications in solid phase extraction.

Solid Phase Extraction (SPE) Applications

During the past few years, much effort has been put into the development of MIP materials for solid phase extraction (SPE).[3] Solid phase extraction is a widely used sample-preparation technique for the isolation of selected analytes, usually from a mobile phase (gas, fluid or liquid). SPE was initially developed as a complement or replacement for liquid-liquid extraction.[52,53] It is now the most common sampling technique in many areas of chemistry, including environmental, pharmaceutical, clinical, food and industrial chemistry.[54] The analyte, usually in a complex matrix, is transferred to the solid phase in which it is retained. The solid phase is then washed to isolate the analyte by removing undesirable components from the sample.[55] The analyte is then recovered by elution using a small amount of liquid, which also serves to concentrate the target analyte for further analysis. The principal goals of SPE are trace enrichment (concentration), matrix simplification (sample clean-up) and medium exchange (transfer from the sample matrix to a different solvent).

SPE using MIPs has been shown to be superior to ordinary SPE and liquid-liquid extraction in its ability to produce much cleaner extracts. The selectivity and stability of MIPs also offer faster method development and cost efficiency.[3] However, the use of MIPs for SPE is not always straightforward for providing both good recovery and selectivity. To optimize the extraction procedure for the analyte of interest, a good knowledge of the retention mechanism is required to identify the nature of the interactions developed between the analyte and the MIP during the extraction process.[56]

Areas for Further Investigation

Although successful MIPs for many applications have been demonstrated, several areas need more investigation. Exploitation of MIPs has primarily been for applications involving small, low-molecular weight molecules in solvent matrices.[5,8,23,25] However, the use of MIPs for biological applications requires the preparation of MIPs for large macromolecules, and the study of MIP preparation using these large templates has been limited. Biological molecules are also usually found in aqueous media, often have poor solubility in nonpolar organic solvents, and may become denatured and change conformation in such solvents. Development of molecularly imprinted polymers that work in aqueous solution would open new applications in the fields of life sciences.[1,7]

The use of MIPs for recognition in aqueous media has received limited attention.[57] For non-covalent and semi-covalently prepared MIPs, the main interactions that are developed between the template and the imprinted polymer are weak non-covalent interactions, such as hydrogen bonding.[56] These interactions are strongest in hydrophobic environments, and therefore interactions between the template and MIP will be greater in nonpolar environments compared with polar environments.[58] The presence of water in the environment quickly overwhelms the ability of a template to hydrogen bond with the polymer.

However, in an aqueous environment, an additional type of interaction, the hydrophobic interaction, can become important in rebinding the analyte to the polymer.[59] As the aqueous portion of the medium increases, the balance between hydrogen bonding interactions become less important, and hydrophobic interactions can become a stronger force in binding the analyte to the polymer binding sites. The selectivity is

changed such that in nonpolar organic solvents, the imprints recognize subtle differences in polar functionalities of the molecule, and in aqueous media they are more efficient at recognizing the hydrophobic parts of the molecule. However, the presence of water can lead to strong, non-specific binding due to adsorption at the hydrophobic polymer surface, which is undesirable when trying to isolate the analyte from a complex matrix. This contributes to the challenge of using MIPs in aqueous media.[5,60]

Evaluation of an MIP for Avermectin

Research on the preparation of an MIP for avermectin addresses several of the challenges of using MIPs for some practical applications. Avermectin is a very large molecule (872 amu) with very few functional groups available for the synthesis of either non-covalently or covalently prepared MIPs (Figure 5). The hydroxyl group at the 5 carbon is the most reactive site on the molecule, followed by the hydroxyl group at the 4" carbon. The hydroxyl group at the 7 carbon is too sterically hindered to be reactive.

Avermectin is soluble in most organic solvents, including chloroform, but has very low solubility in water (6-9 ppb). However, due to its extreme potency, it is very toxic to freshwater aquatic life at very low concentrations (48 hr LC50 for Daphnia magna is 25 ppt).[18] The most sensitive analytical method for the detection of avermectin has a limit of detection of ~2 ppb, which is higher than the concentration of avermectin that is toxic in aquatic environments.

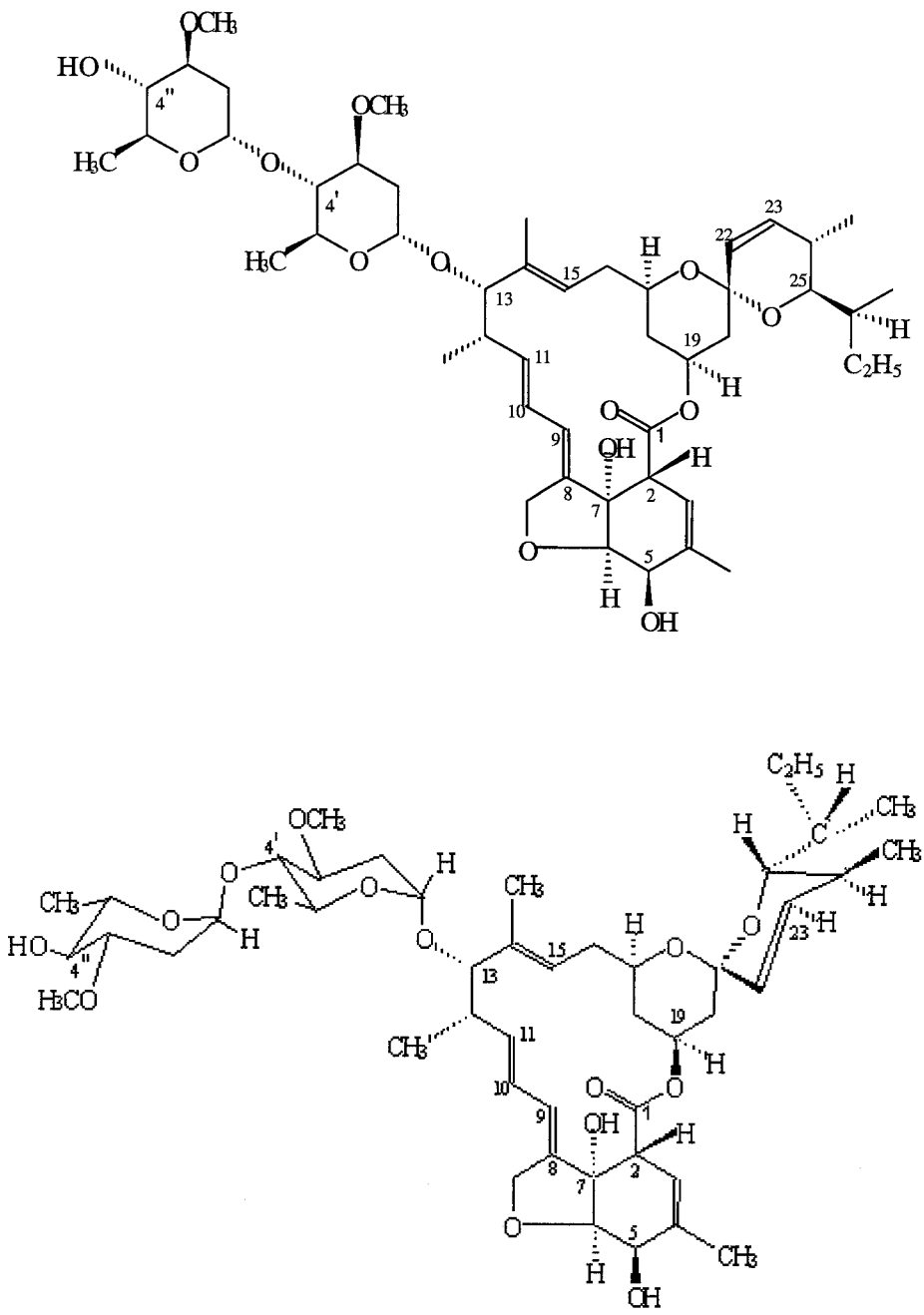
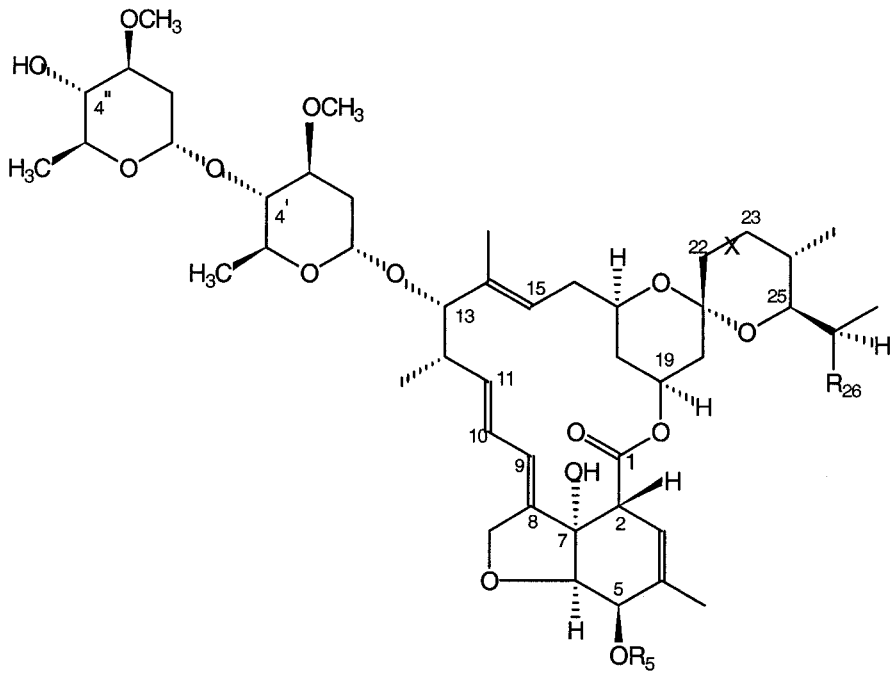


Figure 5. Structure of avermectin. The bottom model is a closer representation of the three dimensional structure. [18]

Avermectin is actually a family of eight closely related large complex molecules that each contain a 16-membered macrocyclic lactone (see Figure 6).[18] In all eight structures, the macrocyclic backbone is further substituted with a spiroketal unit (C-17 to C-28), a hexahydrobenzofuran unit (C-2 to C-8a), and a disaccharide substituent at C-13. Avermectin is made by fermentation of the actinomycete Streptomyces avermitilis, which produces four homologous pairs of closely related compounds: avermectin A1, A2, B1, and B2. The A-compounds have a 5-methoxy, whereas the B-compounds have a 5-hydroxy substituent; the 1-compounds have a 22,23-double bond that is formally obtained by dehydration of the axial 23-hydroxy group of the 2-compounds. The four pairs are further divided into the major components A1a, A2a, B1a, B2a with a secondary butyl sidechain at carbon position 25 and minor components (1-20%) A1b, A2b, B1b, B2b with an isopropyl substituent at carbon 25. Of the components, B1a is the most important because it has high potency against a broad spectrum of endo- and ectoparasites in farm animals and many agricultural mites and insect pests. It also serves as a starting material for several other closely related compounds (eprinomectin and emamectin benzoate) for both prevention and cure of parasitic infections of animals.[18]



Avermectin	R_5	R_{26}	C_{22}-x-C_{23}
A_{1a}	CH_3	C_2H_5	-CH=CH-
A_{1b}	CH_3	CH_3	-CH=CH-
B_{1a}	**H**	**C_2H_5**	**-CH=CH-**
B_{1b}	H	CH_3	-CH=CH-
A_{2a}	CH_3	C_2H_5	OH ▼ -CH_2-CH-
A_{2b}	CH_3	CH_3	OH ▼ -CH_2-CH-
B_{2a}	H	C_2H_5	OH ▼ -CH_2-CH-
B_{2b}	H	CH_3	OH ▼ -CH_2-CH-

Figure 6. Structures of the eight avermectins.[18]

Currently, the most sensitive analytical method for the detection of avermectin is high performance liquid chromatography (HPLC) with fluorescence detection after derivatization with a fluorophore. A limit of detection of ~2 ppb for this method is achievable by extracting a large water sample three times with solvent, which must then be evaporated. The sample is reconstituted in a small amount of solvent and then derivatized. The alternative to this analytical method for aqueous environmental samples is the use of a biotoxicity assay, which measures the toxicity of a sample on aquatic life by determining the death rate of a particular microbe in a sample being analyzed. However, this technique is not specific for any one analyte, is expensive, and requires a minimum of 48 hours to obtain the results. Therefore, an analytical technique that is quicker and specific for avermectin in an aqueous environment would be highly beneficial. Preparation of an MIP for avermectin has the potential to improve the analysis by either direct analysis by packing a chromatographic column with the MIP, or by providing a more efficient concentration step by solid phase extraction using the MIP, followed by a simple chromatographic analysis. Even if the limit of detection cannot be improved, the use of an MIP has the potential to improve the speed and efficiency of the analysis, potentially eliminating the need for derivatization. However, there are several challenges for this application. Avermectin is a large molecule, but has few functional groups that would lend themselves to either the weaker non-covalent interactions, such as hydrogen bonding, or to a stronger reversible covalent bond that could be exploited for a covalent MIP preparation.

A three dimensional space-filling model and a structural model of avermectin are shown in Figure 7. There are three hydroxyl groups on the molecule (not visible in the space-filling model). The secondary allylic hydroxyl group at the 5 carbon is the most reactive, followed by the secondary hydroxyl group at the 4" carbon. The tertiary allylic

hydroxyl group at the 7 carbon is relatively unreactive.[18] The two reactive hydroxyl groups, as well as any of the other potential hydrogen bonding oxygens that may be exposed to the surface of the molecule, may be utilized for a non-covalent preparation of an MIP for avermectin.

A covalently-prepared polymer may also be a possibility for application of the MIP technology for the recognition of avermectin. The hydroxyl group on either the 5 carbon or the 7 carbon, or both, could be used to make a covalent bond with a monomer as a precursor. This could then be polymerized and once the avermectin is removed from the polymer, non-covalent interactions could be used for avermectin recognition, utilizing the "semi-covalent" approach.

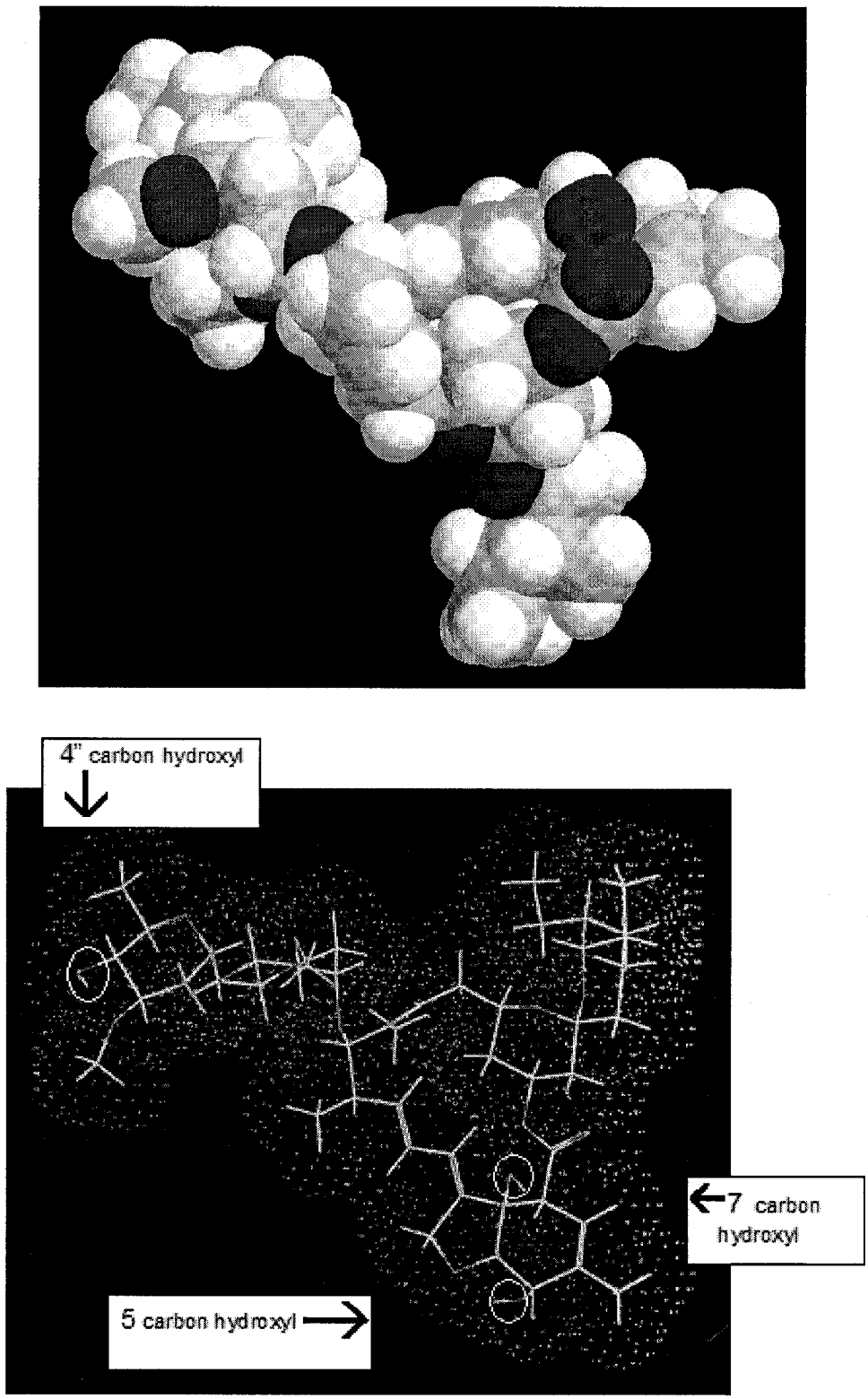


Figure 7. A three-dimensional space-filling model (top) and a computer-generated structural model (bottom) of avermectin. The three hydroxyl groups are circled in white.[18]

If an imprinted polymer can be prepared by one of these methods, the use of the polymer to recognize avermectin in an aqueous medium is the next important challenge to using the polymer in a practical situation. The use of the MIPs prepared by either method may rely on non-covalent interactions, specifically hydrogen bonding of the hydroxyl groups to the polymer, for the rebinding of avermectin. The presence of water in the sample can potentially interfere with these interactions, although hydrophobic interactions may also be present. Because avermectin is very hydrophobic, it may preferentially bind to the polymer rather than remain in the polar aqueous environment. If the correct combination of aqueous and solvent environment can be determined, a successful polymer for the recognition of avermectin may be prepared.

Experimental Section

Reagents

Methacrylic acid (MAA) inhibited with 100-250 ppm hydroquinone, acrylamide (ACRYL), ethylene glycol dimethacrylate (EGDMA) inhibited with 100 ppm monomethyl ether hydroquinone, 2,2"-azobisisobutyronitrile (AIBN), methacryloyl chloride, and dimethylaminopyridine were obtained from Aldrich and used as received. Chloroform stabilized with amylenes was obtained from Aldrich and was dried over 4A° molecular sieves before use. Acetonitrile, toluene and acetone were HPLC grade and were obtained from Fisher Scientific. Acetic acid, sodium hydroxide, sodium bicarbonate, disodium hydrogen phosphate and phosphoric acid were obtained from Fisher Scientific. Ethanol was obtained from AAPER Alcohol and Chemical Co. Avermectin bulk and eprinomectin were synthesized by Merck Manufacturing Division (Danville, PA). Avermectin bulk contains at least 80% B1a and about 95% B1a and B1b combined, which differ only in a methyl group at the 26 carbon (Figure 6). Because avermectin bulk was used for all studies, further references to avermectin are understood to contain the functionalities of the B1a structure. Avermectin bulk, which contains 6.4% water, was dried at 50 °C under vacuum for at least 24 hours to a water content of 0.1-0.6% before use.

Instrumentation

The HPLC systems used for analysis of avermectin in polymer washes and for evaluation of polymers packed into columns consisted of a ThermoSeparations P4000 pump, UV2000 detector at 254 nm, and AS3000 autosampler controlled by an SN4000 controller. All chromatography was performed at room temperature. Because some packed columns did not provide sufficient backpressure on the HPLC system to allow the autosampler to operate properly, an extra length of tubing was often added between the pump and injection valve to increase the overall pressure on the system. The chromatographic data analysis was performed using ChromQuest software version 2.51.

Synthesis of Non-Covalently Prepared Polymers

Non-covalently prepared polymers were synthesized by combining avermectin (1 mmol), EGDMA (40 mmol), functional monomer (15 mmol), 10 mg AIBN initiator, and 10.0 mL of dry polymerization solvent (chloroform or acetonitrile) in a 25-mL glass scintillation vial. A control polymer was also prepared for each test polymer using the identical procedure but eliminating the avermectin. The vials were purged with nitrogen for five minutes, then capped tightly and placed between two UV lamps (365 nm) in a refrigerator at 5-8 °C for at least 16 hours. Vials were then transferred to an 80 °C oven for at least two hours to complete the polymerization. The vials were broken away from the resulting solid polymers, which were ground using a hand-held coffee grinder and a

mortar and pestle. Ground polymers were washed in a scintered glass funnel with 1 L of 10% acetic acid in acetonitrile followed by 1 L of acetonitrile.

To determine recovery of avermectin from the polymer, washes were analyzed for avermectin content by HPLC using a 25 x 0.46 cm Zorbax ODS column, a mobile phase of 84/16 methanol/water, a flow rate of 1.0 mL/min for 20 minutes, a 20 µL injection volume, and UV detection at 254 nm.

Synthesis of Covalently Prepared Polymers

Avermectin-Methacrylate Synthesis

The methacrylic acid-avermectin monomer-template was by prepared by esterification of the avermectin at the more reactive 5 carbon hydroxyl group. Avermectin was combined with 1.2 molar ratio of methacryloyl chloride in dry toluene under nitrogen. Dimethylaminopyridine (DMAP) was added to remove hydrochloric acid formed during the reaction. The progress of the reaction was monitored by TLC in 2:1 ethyl acetate:heptane and plates were charred for viewing. The product was extracted with 5% phosphoric acid to remove excess DMAP, followed by 5% sodium bicarbonate to remove excess acid. The product, which remained in the toluene, was dried on a rotary evaporator. The product was then isolated from the di-methacrylated product by flash chromatography on silica with 2:1 ethyl acetate:heptane. Product identity was confirmed by proton, carbon and distortionless enhancement by polarization transfer (DEPT) NMR.

NMR Identification of Product

Proton, carbon, and DEPT NMR spectra of avermectin and of the avermectin-methacrylate template were collected on a Bruker Avance 300 NMR. All samples were dissolved in deuterated chloroform containing 0.03% TMS. (Spectra are shown in Figures 42, 43, 45, 46, 47 and 48.)

Polymer Preparation

The first covalently prepared polymer was synthesized by combining the avermectin-methacrylate (1 mmole) with 40 mmole EGDMA, 10 mg AIBN initiator, and 10.0 mL acetonitrile in a 25-mL glass scintillation vial. A control polymer was prepared with 1 mmole methacrylic acid instead of avermectin-methacrylate. The vials were purged with nitrogen for five minutes, then capped tightly and placed between two UV lamps (365 nm) in a refrigerator at 5-8 °C for at least 16 hours. Vials were then transferred to an 80 °C oven for at least two hours to complete the polymerization. The vials were broken away from the resulting solid polymers, which were ground using a hand-held coffee grinder and a mortar and pestle.

The test polymer was refluxed in 50/50 methanol/2M aqueous sodium hydroxide for 12 hrs to remove the avermectin. Imprinted ("test") and non-imprinted ("control") polymers were then washed with 10% acetic acid in acetonitrile and dried. The extent of template removal from the test polymer was evaluated by Fourier transform infrared (FTIR) spectroscopy.

A second set of covalently prepared polymers was synthesized by the same procedure except 14 mmole additional MAA was added to each vial.

Fourier Transform Infrared (FTIR) Evaluation of Template Removal from Covalently Prepared Polymer

FTIR spectra of the washed test polymers, control polymers and control polymers spiked with avermectin were collected to determine the percent removal of avermectin from the test polymers after reflux. Spectra were collected from 650 to 4000 cm^{-1} on a Nicolet Magna-IR spectrometer 750 equipped with a SensIR ATR Durascope, at a resolution of 4, using 25 scans/sample. (Spectra are shown in Figures 49 and 53, and details of the method used to determine percent removal of avermectin are discussed in the Results and Discussion section.)

Column Preparation

The washed polymer particles were slurried with acetonitrile and passed through 38 and 25 μm sieves; about one gram of particles between 25 and 38 μm in size was collected. Particles were re-suspended in acetonitrile and packed into 10 x 0.46 cm stainless steel HPLC columns from Alltech. Sieving of the particles a second time was usually required prior to packing the column to ensure the maximum pressure allowed by the pump was not exceeded due to excess fines in the column. Columns were packed using a Waters Delta Prep 3000 preparatory-scale pump and system controller. A custom-made adapter, which consisted of a 25 x 0.46 cm empty stainless steel column welded onto a threaded joining union, was attached to the top of a 10 cm column that was capped at the bottom, and the combined columns were filled with the polymer slurried in acetonitrile. Acetonitrile was pumped through the columns at

incrementally increasing flow rates for about 30 minutes with intermittent vibration of the column provided by an electronic engraver held against the outside of the column during packing. The adaptor was removed and the 10 cm column was capped after leveling off the polymer packing. Columns were then attached to an HPLC system and washed with at least ten column volumes of 10% acetic acid in acetonitrile followed by at least ten column volumes of 100% acetonitrile prior to analysis.

Polymer Evaluation by HPLC

All non-covalently prepared and covalently prepared polymers and controls were evaluated by preparing a solution of avermectin (~100 μg/mL), 0.01 vol % toluene, and 0.1 vol % acetone in the polymerization solvent. Selectivity for each polymer for avermectin was determined by injecting a similar solution containing eprinomectin (Figure 13) instead of avermectin. The solutions (20 μL = 0.002 μmole avermectin) were injected onto the columns at room temperature, using a mobile phase flow rate of 0.8 mL/min and UV detection at 254 nm. To determine retention times of closely eluting components, the non-retained component (toluene or acetone) and the components being evaluated (avermectin and eprinomectin) were injected as separate solutions.

Evaluation of Non-Covalently Prepared Polymer by SPE

Preparation of the Solid Phase Extraction (SPE) Cartridges

One non-covalently prepared polymer was evaluated for potential use in SPE. A portion (0.5 g) of the washed and sieved polymer particles was slurried with acetonitrile and poured into an empty 6-mL SPE cartridge with a frit at the bottom. This quantity occupied about one third of the cartridge volume. A frit was placed on top of the polymer, acetonitrile added to the top, and vacuum applied to the bottom to pack the polymer into the cartridge. One cartridge was prepared for the imprinted polymer and one for the control polymer.

Polymer Evaluation by SPE

The non-covalently prepared polymer was evaluated by first conditioning the cartridge with at least ten column volumes of acetonitrile, followed by at least ten column volumes of eluting solution, followed by at least ten column volumes of acetonitrile. This final portion was collected and analyzed to ensure no residual avermectin from the imprinting step was still eluting before the cartridges were used for evaluation. Other than the first SPE study (which utilized 12.0 µg avermectin prepared in 25% acetonitrile in water), avermectin (2.0 µg/mL) was prepared in 1% acetonitrile in water, and 10.0 mL (20 µg = 0.020 µmole of avermectin) was loaded by pipet onto the top of each cartridge (one test and one control per study) by applying vacuum and allowing the cartridges to run dry. Eluents from this step were collected and analyzed for avermectin. Cartridges

were washed with 4.0 mL of the chosen wash solution, followed by elution of avermectin with 4.0 mL of eluting solution. Final cartridge washes consisted of 2.0 mL of acetonitrile. During each step, 1.0 mL portions were collected for analysis of avermectin. All analyses for avermectin were performed by HPLC using a 25 x 0.46 cm Zorbax ODS column, a mobile phase of 84/16 methanol/water, and UV detection at 254 nm.

Results and Discussion

Comparison of Polymerization Solvent and Monomer in the Synthesis of Non-Covalently Prepared Polymers

Preparation of Polymer using Methacrylic Acid in Chloroform and Evaluation in Chloroform-Based Mobile Phase

The first set of polymers for avermectin was prepared to evaluate the possibility of synthesizing an imprinted polymer for the selective recognition of avermectin. A non-covalent preparation technique was chosen initially for several reasons. The technique is relatively easy because all the reagents are simply mixed, and the functional monomers arrange around the avermectin molecule by whatever non-covalent interactions occur naturally prior to polymerization.

Methacrylic acid was chosen as the functional monomer to be used in the first synthesis, because it is capable of acting as both a hydrogen bond donor and acceptor. The avermectin molecule has numerous functionalities with the potential to hydrogen bond. However, the most promising functionalities for hydrogen bonding with the MAA are the two accessible hydroxyl groups: the secondary alcohol group at the 5 carbon in

the more rigid region of the molecule, and the secondary alcohol at the 4" carbon in the more flexible region of the molecule.

The crosslinker chosen for polymerization was EGDMA because it is commonly chosen as a crosslinker for initial investigations. The ratio of crosslinker:monomer:template for the first polymer preparation was 40:15:1. This ratio was selected to ensure sufficient crosslinker to form a stable polymer that can support the required cavities, and sufficient monomer to interact with as many functionalities on the large avermectin molecule as possible.

The solvent chosen for the first MIP preparation was chloroform, the less polar of the two aprotic polymerization solvents evaluated. Because hydrogen bonding is believed to be the predominate non-covalent binding force between the avermectin and methacrylic acid, nonpolar solvents should provide an environment for stronger hydrogen bonding than a polar solvent.

A representation of the preparation of the polymer with the polymer is shown in Figure 8.

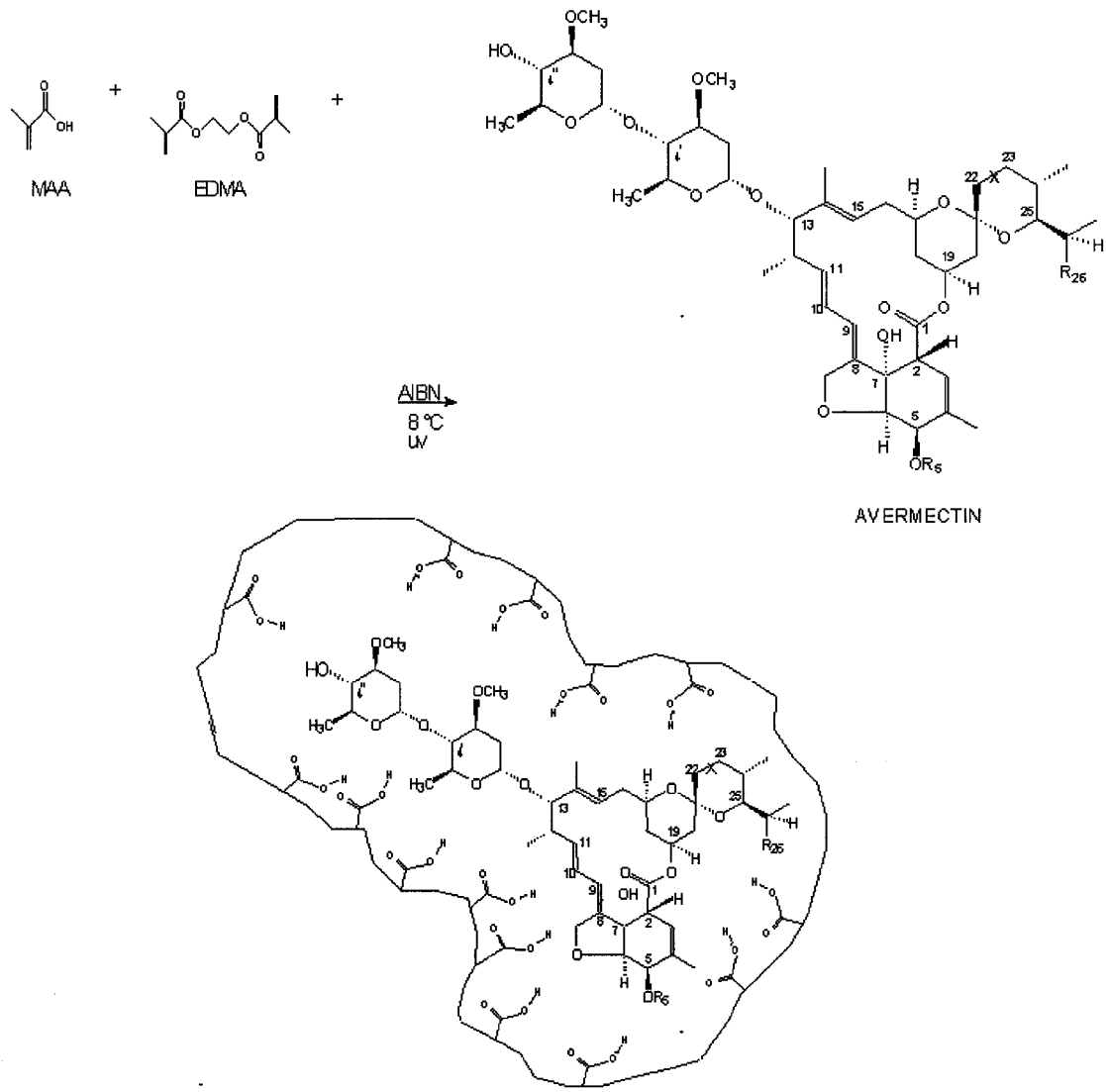


Figure 8. Representation of the preparation of a non-covalently-prepared polymer for avermectin.

After polymerization, the avermectin was removed from the polymer by grinding and washing. Evaluation of the washes by HPLC analysis for avermectin confirmed 100 % recovery of the avermectin added during polymerization. This indicates that the avermectin molecule remained intact during polymerization and that all of the template was recovered, leaving all of the cavities formed in the polymer vacant. The imprinted polymer was packed into an HPLC column ("test column") to evaluate its ability to

selectively retain avermectin compared with a non-imprinted polymer ("control column") which was prepared by the same procedure but without the avermectin template.

Evaluation of the polymers was performed using the columns and HPLC analysis to determine if the imprinted polymer retained the avermectin compared with a non-imprinted polymer prepared without the avermectin template as a control. Chloroform was chosen as the mobile phase because it has been largely demonstrated that MIPs offer the highest selectivity when samples are evaluated in the solvent used for the MIP preparation.[36,56] A small amount of acetic acid (0.1 vol% and 0.05 vol%) was added to the mobile phase during the evaluations to compete with the hydrogen bonding of the avermectin with the methacrylic acid residues on the polymer and to ensure elution of the avermectin from the polymer. The different levels of acidic modifier incorporated in the eluent mixtures should affect the proportion of carboxylic acid residues in the polymer that are in their protonated form, which in turn influences the displacement of avermectin from the MAA residues in the polymer. Because toluene is nonpolar and should not be retained in either column, it was included in the avermectin solutions as a void marker for the initial evaluations to determine the time required for a non-retained compound to elute from the columns.

Figure 9 shows a chromatogram from the control column after injection of a solution of 0.01% toluene and 100 µg/mL avermectin in the mobile phase, which was 0.1% acetic acid in chloroform. Figure 10 is the chromatogram of the same solution injected on the test MIP column. Comparison of the two chromatograms indicates that the control column did not retain the avermectin, because it eluted at the same time as the non-retained toluene. The test column did retain avermectin, demonstrated by the separation of toluene and avermectin as two peaks, indicating recognition of the analyte by the imprinted polymer.

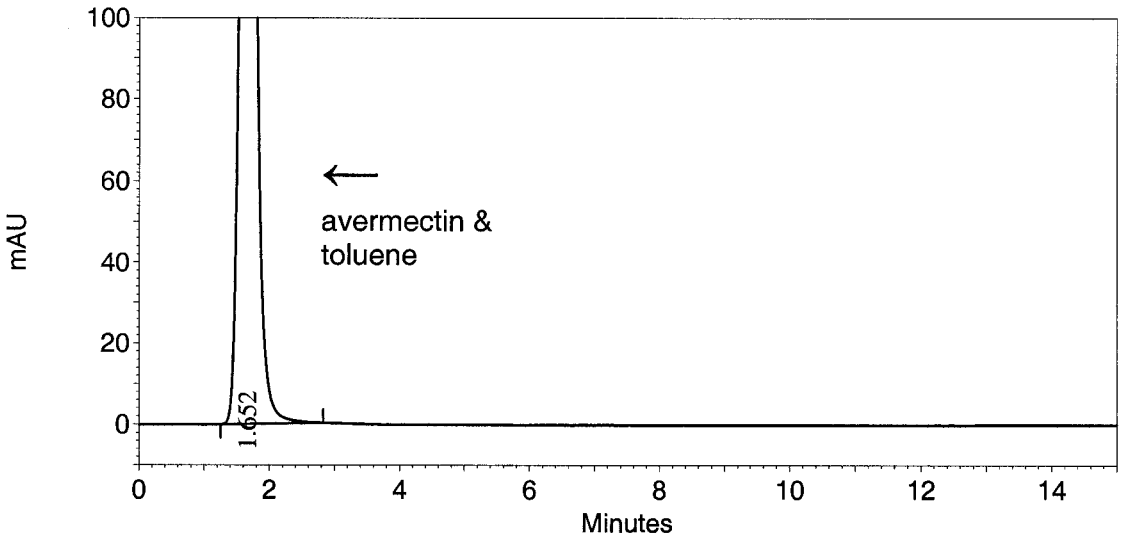


Figure 9. Chromatogram of a solution of 0.01% toluene and 100 µg/mL avermectin diluted in mobile phase of 0.1% acetic acid in chloroform, evaluated on the control column.

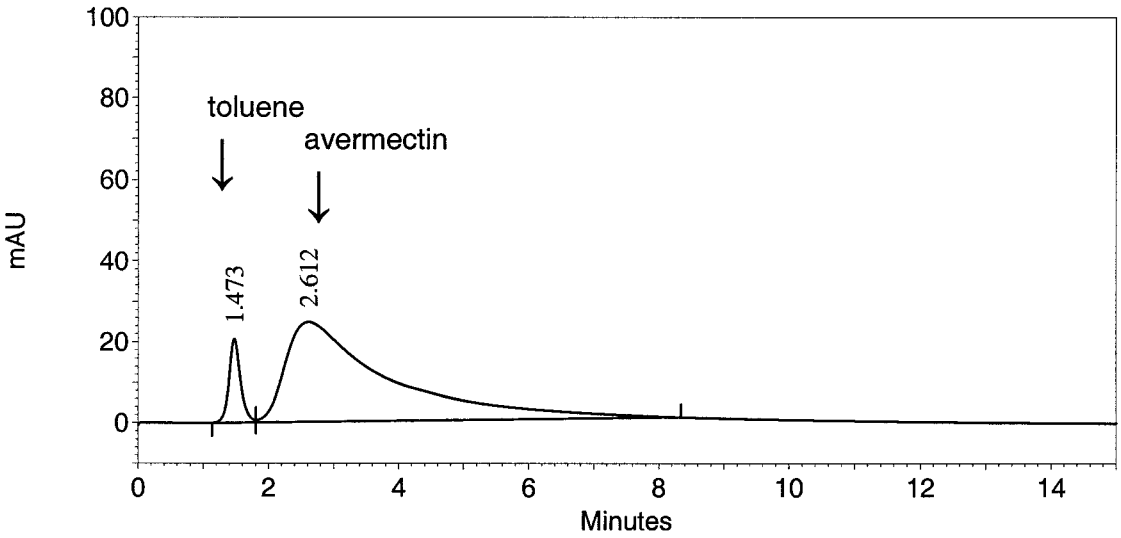


Figure 10. Chromatogram of a solution of 0.01% toluene and 100 µg/mL avermectin diluted in mobile phase of 0.1% acetic acid in chloroform, evaluated on the test column.

In an attempt to increase the retention of avermectin on the test column, the amount of acetic acid in the mobile phase was decreased from 0.1% to 0.05% in chloroform to reduce the competition with the avermectin for hydrogen bonding with the

methacrylic acid residues on the polymer. Figures 11 and 12 are chromatograms from the control and test columns, respectively, after injection of a solution of 0.01% toluene and 137 µg/mL avermectin using a mobile phase of 0.05% acetic acid in chloroform. As expected, there was no separation of avermectin from toluene on the control column, and slight improvement in the separation of avermectin from toluene on the test column.

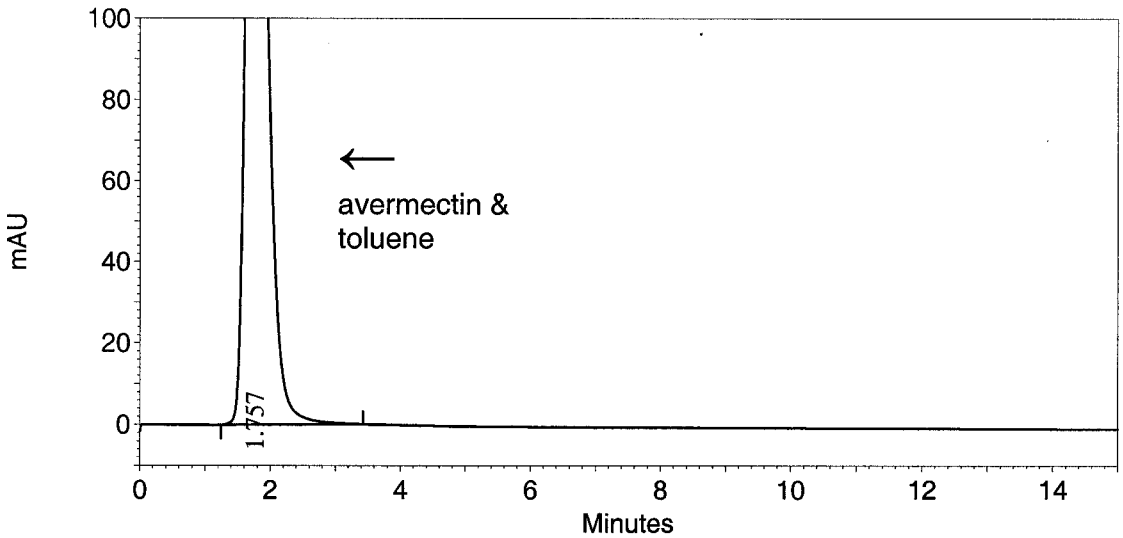


Figure 11. Chromatogram of a solution of 0.01% toluene and 137 µg/mL avermectin in a mobile phase of 0.05% acetic acid in chloroform, evaluated on the control column.

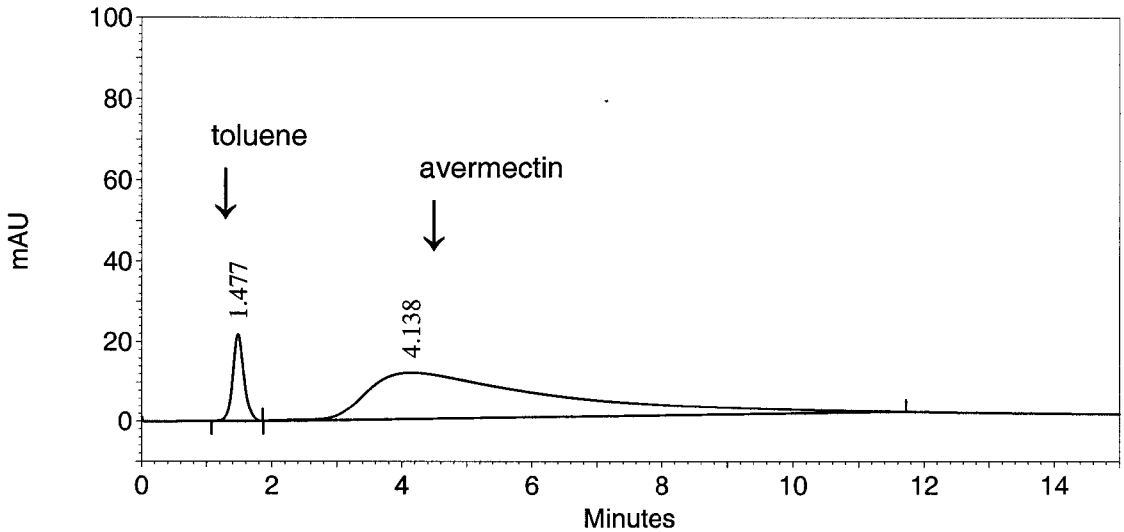


Figure 12. Chromatogram of a solution of 0.01% toluene and 137 µg/mL avermectin in a mobile phase of 0.05% acetic acid in chloroform, evaluated on the test column.

Because differences in retention times may result from differences in column packing efficiencies, the following values are used for comparison:[61]

t_0 = retention time of unretained compound

tr(avm) = retention time of avermectin

t'r(avm) = tr(avm) - t_0 = adjusted retention time of avermectin

k'(avm) = t'r(avm) / t_0 = capacity factor for avermectin

α = k'(2) / k'(1), in which 2 is the test column and 2 is the control column

The capacity factor (k'(avm)) is used to compare retention of a component when evaluating the effectiveness of different columns and different column elution conditions. Comparisons of these values for avermectin in two different mobile phases are shown in Table 3.

Table 3. Comparison of retention of avermectin on the control and test columns in chloroform mobile phase containing different levels of acetic acid. (N/A indicates no separation of avermectin and toluene so no value for k' could be calculated. For calculation of α values, N/A is assumed to be <0.1.)

Mobile Phase	**t'r(avm) control**	**t'r(avm) test**	**k'(avm) control**	**k'(avm) test**	**α(avm) test/ control**
0.1% acetic acid in chloroform	0.00	1.14	N/A	0.77	>7.7
0.05% acetic acid in chloroform	0.00	2.66	N/A	1.80	>18.0
100% chloroform	2.04	6.82	1.22	4.80	3.93

Although the avermectin was not retained on the control column in a mobile phase with either level of acetic acid (Figures 9-12), the avermectin was retained significantly more strongly on the imprinted polymer column in a mobile phase containing the lower level of acetic acid (0.05%) compared with the higher level (0.1%). This is indicated by the data in Table 3 which shows that the capacity factor (k') for avermectin in chloroform containing 0.05% acetic acid (1.80) is more than twice as high as the capacity factor in 0.1% acetic acid (0.77). The capacity factor for avermectin in chloroform containing no acetic acid is even higher (4.80). The lower level of acetic acid in the mobile phase increases the hydrogen bonding of the avermectin with the methacrylic acid residues in the polymer and decreases the level of displacement of avermectin from the residues, increasing the retention. Past studies discussed in the literature support these findings and suggest that chloroform has a lower potential relative to polar modifiers added to the mobile phase for interrupting the hydrogen bonds between the ligand and MIP. Data from these studies also show an increase in the capacity factor (k') with a decrease in the concentration of the polar modifier.[58] This correlates with the increase in hydrogen bonding with a decrease in acetic acid observed in the results of this study.

The increase in retention of avermectin with a decrease in acetic acid concentration in the mobile phase is observed again in the data from evaluation of the polymers in 100% chloroform with no acetic acid (Table 3). Retention of avermectin on the test column was even greater in this mobile phase. However, in 100% chloroform, the retention of avermectin on the control column also increased. Because the control column does not contain imprinted sites for the avermectin, the retention on the control column is due to non-specific interactions between the avermectin and the polymer. As

a result, the selectivity was lower for avermectin in 100% chloroform than in chloroform with acetic acid modifier.

When using HPLC to analyze MIPs, one common problem is tailing of the broad analyte peaks (see Figure 12). This may be due to site heterogeneity created during non-covalent molecular imprinting due to the random arrangement of the template and monomer molecules, resulting in a distribution of binding sites with different affinities for the sample molecule.[47] Slow mass transfer and binding kinetics may also contribute to the poor peak shape.[4] Not only can tailing considerably increase the time needed to complete one analysis, but peak asymmetry also makes it difficult to measure HPLC chromatogram parameters accurately. Although the chromatographic separation of the avermectin from the toluene is baseline resolved (Figure 12), the avermectin peak exhibits the poor peak shape that is common in non-covalently prepared MIPs.

Another variable which is used to compare the efficiencies of columns is alpha (α) which is defined as k'(2) / k'(1), in which 2 and 1 refer to two different compounds or two different columns. In Table 3, α is used as a measure of the "imprinting effect" with the same analyte (avermectin) on the test versus control columns, which is also referred to as "selectivity." Table 3 shows the comparison of the selectivity of the test column to the control column for avermectin (α(avm) = k'(avm)test / k'(avm)control = 1.80 / <0.1 = >18.0). (When no separation of toluene and avermectin was observed, N/A is indicated in the table because no value for k' could be calculated. Based on multiple injections of toluene and avermectin on the control column, the error of determination in capacity factors was 0.1. As a result, for calculation of α values, N/A is assumed to be <0.1). This large α value of >18.0 for avermectin indicates that the imprinted polymer has a large non-aqueous selectivity for avermectin in a nonpolar mobile phase, at least 18 times greater than the selectivity of the control column. Because the avermectin

contains two very accessible hydroxyl groups, it is most likely that hydrogen bonding of one or both of these groups with the methacrylic acid residues in the avermectin-shaped cavities of the polymer is responsible for the retention of the avermectin on the test column. The retention is not seen on the control column because the avermectin template was not present during polymerization to form cavities in the polymer with methacrylic acid residues at the proper location for rebinding of avermectin during analysis.

To evaluate the specificity of the polymers for avermectin, comparison of retention of avermectin with the retention of a compound which has a structure similar as avermectin is useful. A compound that has a structure that is very similar to avermectin is eprinomectin (4"-epi-acetylamino avermectin) which is an insecticide synthesized from avermectin. The solubility of eprinomectin is similar to avermectin; it is only slightly more soluble than avermectin in water. This compound is used to evaluate the specificity of the polymers for avermectin. The structure of eprinomectin is the same as avermectin with the exception of an acetamide group (-NH-CH=O) replacing the hydroxyl group on the 4" carbon (Figure 13). Like avermectin, eprinomectin has a hydroxyl group at the 5 carbon, which is in the more "rigid" portion of the molecule. The 4" carbon acetamide group is on the more "flexible" end of the molecule. Comparing the specificity of eprinomectin versus avermectin may provide information regarding which end of the avermectin molecule is more important in recognition by the imprinted polymer.

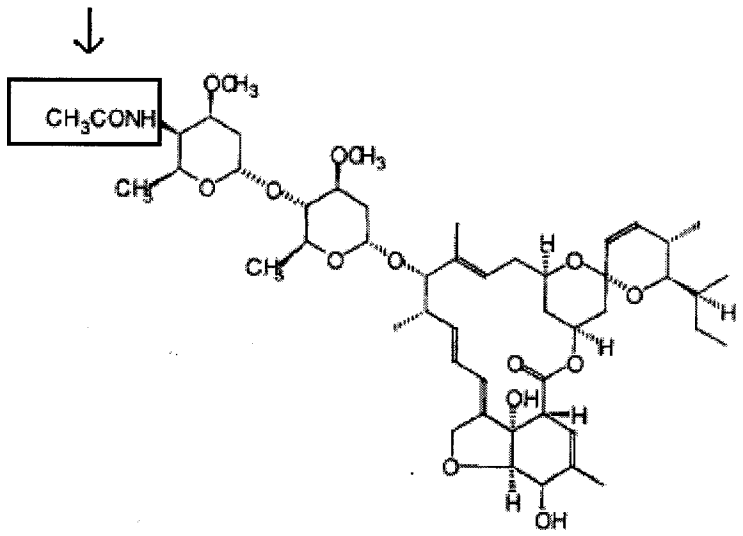


Figure 13. Structure of eprinomectin. The portion of the molecule that differs from avermectin is shown in the square.

Eprinomectin was injected on the columns to determine if the test column retained avermectin more strongly than a similarly structured compound in the same mobile phase. Figures 14 and 15 are chromatograms of the control and test columns, respectively, after injection of a solution of 0.01% toluene and 87 µg/mL eprinomectin in a mobile phase of 0.05% acetic acid in chloroform.

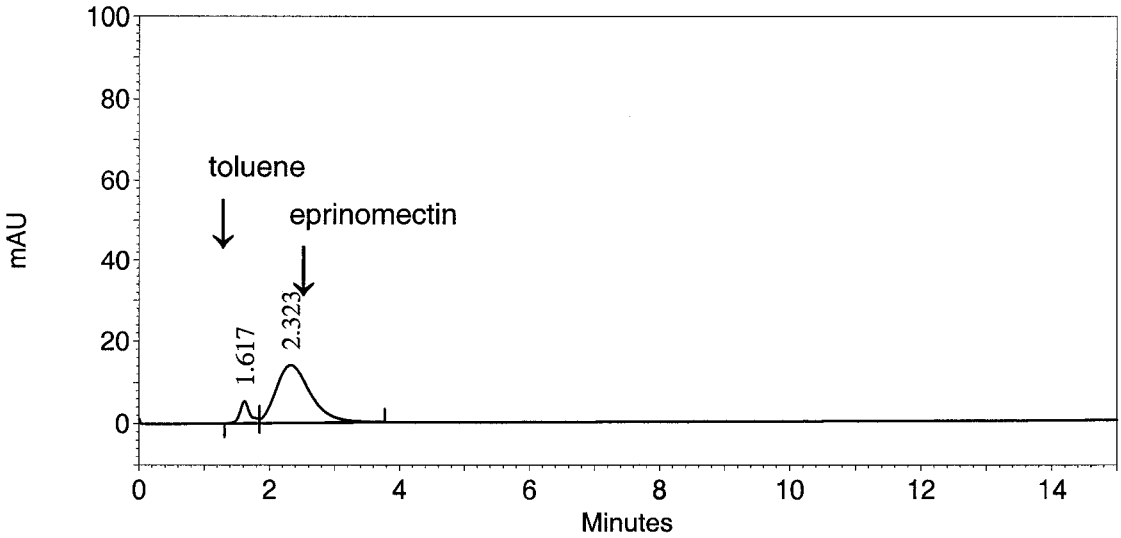


Figure 14. Chromatogram of a solution of 0.01% toluene and 87 µg/mL eprinomectin in a mobile phase of 0.05% acetic acid in chloroform, evaluated on the control column.

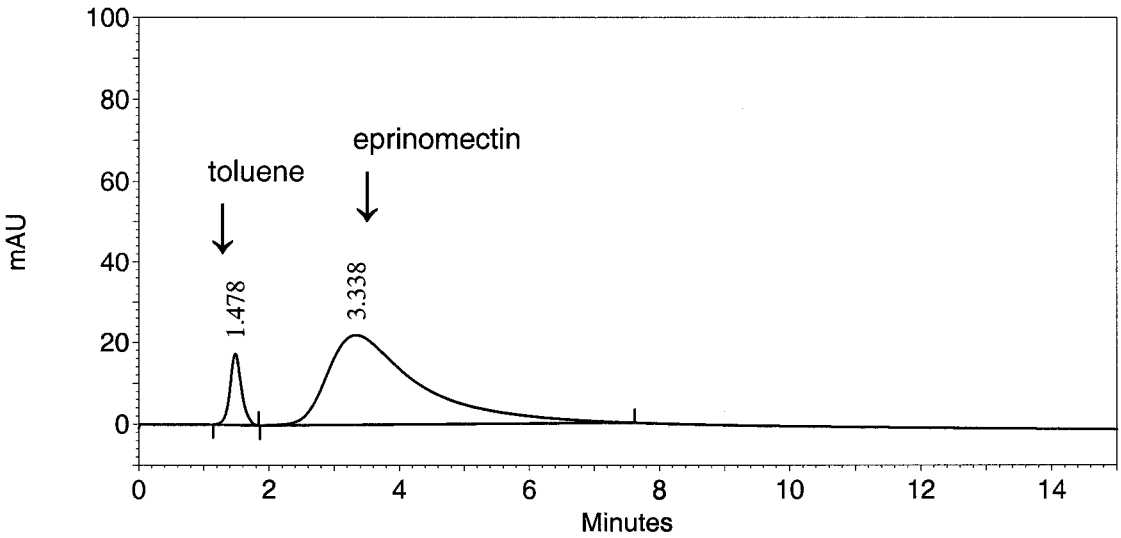


Figure 15. Chromatogram of a solution of 0.01% toluene and 87 µg/mL eprinomectin in a mobile phase of 0.05% acetic acid in chloroform, evaluated on the test column.

The eprinomectin was retained somewhat on the test column (k' = 1.24, Table 4), but not as long as the avermectin was retained on the same column in the same mobile phase (k' = 1.80, Table 3). This indicates that the imprinted polymer has some specificity for avermectin over a compound with a similar structure. Some retention of

eprinomectin on the test column is expected due to the structural similarities of the two compounds. However, because the structure of eprinomectin is slightly different than avermectin, retention of eprinomectin on the test column is expected to be less than avermectin due to imperfect fit of eprinomectin into the cavity in the polymer formed during the imprinting process with avermectin. Also, because hydrogen bonding is believed to be the non-covalent interaction responsible for the retention of avermectin by the imprinted polymer, the difference in the functionality at the 4" carbon is also expected to result in less retention of eprinomectin compared with avermectin on the imprinted polymer. The lower capacity factor for eprinomectin on the test polymer (1.24, Table 4) compared with the capacity factor for avermectin in the same mobile phase (1.80, Table 3) indicates a successful imprinting effect on the test polymer. Because the only difference in the two molecules is the substitution at the 4" carbon, the results indicate that the hydroxyl group at the 4" carbon at the more flexible end of the avermectin molecule is important in binding the avermectin to the imprinted polymer during recognition.

Unlike avermectin, however, eprinomectin is also retained somewhat on the control column (k'(epr) = 0.41, Table 4). The retention of the eprinomectin on the control column indicates there are some non-selective interactions occurring in both the control and test columns that are unrelated to the imprint of the polymer. Because avermectin is not retained on the control column (k'(avm) = N/A, Table 3), this indicates that the acetamide group on the 4" carbon of eprinomectin has more non-selective interaction with the MAA residues in the polymers than the 4" hydroxyl group on the avermectin, leading to slight retention of the eprinomectin on both the control and test columns.

Table 4. Comparison of retention of eprinomectin on the control and test columns.

Mobile Phase	t'r(epr) control	t'r(epr) test	k'(epr) control	k'(epr) test	α(eprin) test/ control
0.05% acetic acid in chloroform	0.66	1.83	0.41	1.24	3.02

Comparing the selectivity of the imprinted polymer in 0.05% acetic acid in chloroform for avermectin (α(avm) = 1.8 / < 0.1 = >18.0, Table 3) with selectivity for eprinomectin (α(epr) = 1.24/0.41 = 3.02, Table 4) indicates that, even though eprinomectin binds stronger non-selectively, the imprinted polymer is > 6 times (>18.0/3.0) more selective for avermectin. Because a difference in the selectivity of the imprinted polymer for avermectin over eprinomectin is observed, and the only difference in the two structures is at the 4" carbon functionality, this suggests that the hydroxyl group at the 4" carbon of avermectin is important in the selective interactions that occur during recognition, either due to shape or hydrogen bonding ability.

Evaluation of the MIP Prepared in Chloroform in Acetonitrile-Based Mobile Phase

Although the imprinted polymer for avermectin demonstrated a large non-aqueous selectivity (α >18.0) in chloroform, the eventual practical application of the polymer is selectivity for avermectin in an aqueous medium. The binding of this polymer in an aqueous environment was investigated. The pH and organic/aqueous ratio of mobile phase are important for retention of analyte.[62] It was demonstrated in the previous study that the acidic content of the mobile phase significantly affects the retention of avermectin in a nonpolar mobile phase, probably by affecting the extent of

hydrogen bonding between the avermectin and the MAA residues in the imprinted cavities, which is the driving force for recognition in this mobile phase. However, in a mobile phase containing water, the ability of the avermectin to hydrogen bond with the polymer decreases due to the replacement of the hydrogen bonding of the avermectin to the polymer with hydrogen bonding of the greater number of water molecules in the mobile phase. However, as the aqueous portion of the mobile phase increases, hydrophobic interactions begin to become more important. Because the avermectin molecule is hydrophobic, these interactions may become more important as water content increases, and recognition of the more hydrophobic portions of the molecule by the polymer become the driving force for recognition.

To determine the extent of recognition of avermectin in an aqueous medium, evaluation of retention in a mobile phase containing increasing portions of water was carried out. However, the mobile phase used in the initial studies was chloroform, the polymerization solvent. Because water is not miscible with chloroform, the mobile phase was changed to acetonitrile, which is a water-compatible solvent and is slightly more polar than chloroform.

The two columns (test and control) were evaluated for the retention of avermectin and eprinomectin using acetonitrile containing 0.05% acetic acid as the mobile phase. Solutions of avermectin and eprinomectin containing toluene were analyzed on the control and test columns to determine first if the selectivity changes with an increase in solvent polarity. Figure 16 is an overlay of the chromatograms of the control column after injection of a solution of 137 µg/mL avermectin and 0.01% toluene (dark line), and a solution of 87 µg/mL eprinomectin and 0.01% toluene (gray line), in a mobile phase of 0.05% acetic acid in acetonitrile. Figure 17 is the overlay of chromatograms of the analysis of the same two solutions on the test column.

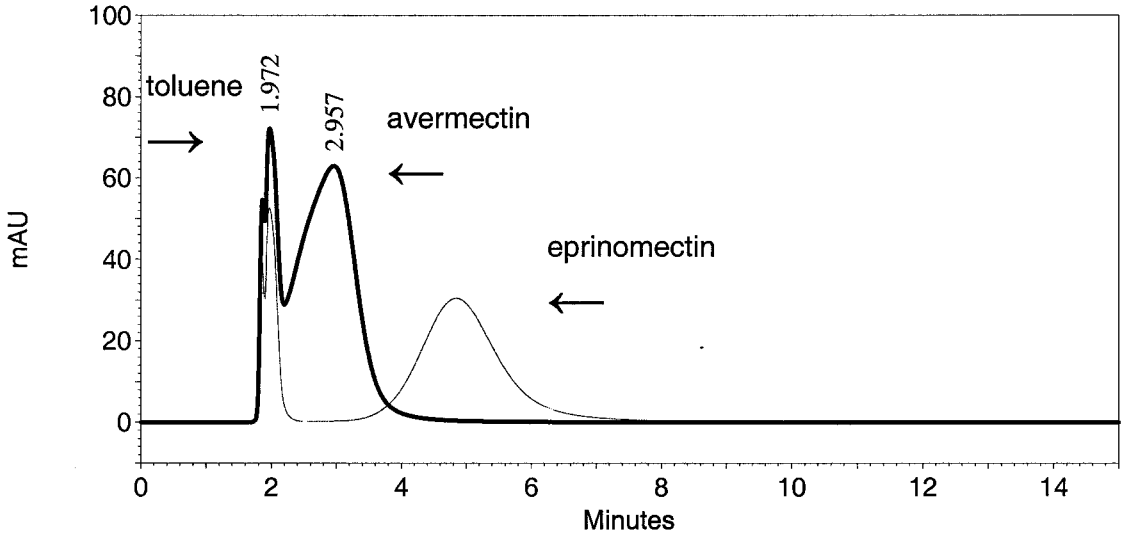


Figure 16. Overlay of chromatograms of 137 µg/mL avermectin and 0.01% toluene (dark line), and 87 µg/mL eprinomectin and 0.01% toluene (gray line), in a mobile phase of 0.05% acetic acid in acetonitrile, evaluated on the control column.

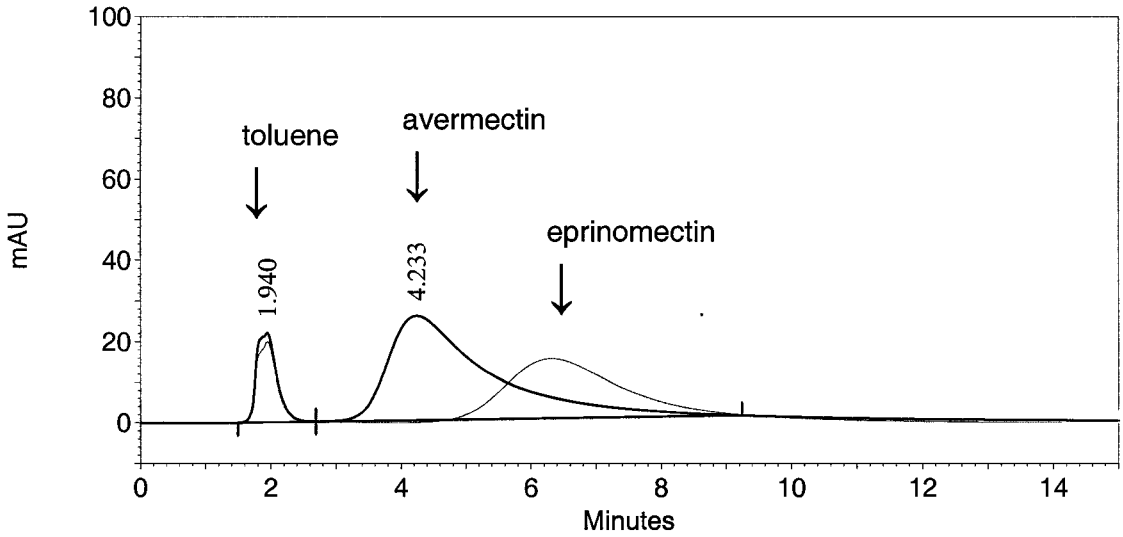


Figure 17. Overlay of chromatograms of 137 µg/mL avermectin and 0.01% toluene (dark line), and 87 µg/mL eprinomectin and 0.01% toluene (gray line), in a mobile phase of 0.05% acetic acid in acetonitrile, evaluated on the test column.

Results show that in the more polar acetonitrile mobile phase, the avermectin was retained slightly on the control column compared with no retention in chloroform

mobile phase (see Table 5). In acetonitrile, the avermectin was retained longer on the test column than on the control column, but the selectivity of the polymer for avermectin was 2.43, which is significantly lower than the selectivity of >18.0 in the chloroform mobile phase. The loss of most of the selectivity may be due to an increase in non-selective interactions (seen in the increase in k' of the control) and some loss in selective interactions (seen in the decrease in k' of the test MIP). This occurs when changing from chloroform to acetonitrile because acetonitrile is more polar and may destroy some of the interactions upon which the selective retention of avermectin relies. Based on the discussions in the literature, the solvent affects the microenvironment of the binding sites created in the polymer during polymerization. Because there is enhanced binding in polymers immersed in the solvent in which they were polymerized, ideal rebinding conditions for a given template should include the solvent used as the porogen during polymerization.[19,63,64] Another hypothesis from the literature suggests that during the imprinting/polymerization stage, what was actually imprinted was the imprint molecule plus a number of solvating molecules rather than the imprint molecule alone.[65] When the imprint molecule rebinds in the same solvent, the imprinted site accommodates the presence of the solvent molecules. If the rebinding solvent is not the same as that used for polymerization, then the solvent molecules could interfere with rebinding at or close to the imprinted site.[58] This is why the best selectivity (at least for non-aqueous applications) is obtained when rebinding is performed in the polymerization solvent. This suggests that preparation of an MIP in acetonitrile may give better selectivity if the polymer is then used for binding in aqueous acetonitrile. However, using the current polymer, the selectivity of 2.43 is sufficient for further investigations, and the presence of some water in the mobile phase might improve

recognition by introducing a hydrophobic interaction, which is strong in the presence of water.[66,67]

Eprinomectin, on the other hand, was retained longer than avermectin on both control and test columns in the acetonitrile mobile phase, which is different than what was observed when using the chloroform mobile phase (see Table 5). Some retention of eprinomectin is expected on the test column because the structure of eprinomectin is very similar to that of avermectin. The non-selective interactions that caused the eprinomectin to be retained on the control column in chloroform mobile phase are also present in the acetonitrile mobile phase, because eprinomectin is again retained on the control column. These non-selective interactions are stronger for eprinomectin than for avermectin, causing longer retention of eprinomectin on the control column than the retention of avermectin. This may be due to stronger hydrogen bonding of the acetamide group on the eprinomectin with the MAA residues on the polymer surface compared with the hydroxyl group on the avermectin. Because the acetamide group of eprinomectin has two sites of potential hydrogen bonding (O: and H atoms) with two locations on the MAA residue (H and O: atoms) compared with only one location on the avermectin 4" carbon hydroxyl group (H atom), the non-selective hydrogen bonding of the amide group on the eprinomectin to the polymer may be stronger than the hydrogen bonding of the hydroxyl group of the avermectin. Retention of any analyte is due to the sum of selective and non-selective binding.[5,68] These non-selective hydrogen bonding interactions, which occur in both the control and test columns, along with the partial retention due to recognition in the imprinted cavities on the test column, combine to cause longer retention of the eprinomectin than the avermectin on the test column.

Table 5. Comparison of retention of avermectin and eprinomectin on the control and test columns in mobile phase with 0.05% acetic acid in chloroform versus acetonitrile. (For calculation of α values, N/A is assumed to be <0.1)

Mobile Phase	**t'r(avm) control**	**t'r(avm) test**	**k'(avm) control**	**k'(avm) test**	**α(avm) test/control**
0.05% acetic acid in chloroform	0.00	2.66	N/A	1.80	>18.0
0.05% acetic acid in acetonitrile	0.95	2.25	0.47	1.14	2.43
	t'r(epr) control	**t'r(epr) test**	**k'(epr) control**	**k'(epr) test**	**α(epr) test/control**
0.05% acetic acid in chloroform	0.66	1.83	0.41	1.24	3.02
0.05% acetic acid in acetonitrile	2.81	4.34	1.40	2.19	1.56

However, the selectivity for eprinomectin on the imprinted column compared with the control column is smaller using acetonitrile mobile phase (α = 1.56) compared with chloroform (α = 3.02, Table 5). Comparing test to control column selectivity for avermectin and eprinomectin in acetonitrile mobile phase containing 0.05% acetic acid:

α(avm) = k'(avm)test / k'(avm)control =

α(avm) = 1.14/0.47 = 2.43
α(epr) = 2.19/1.40 = 1.56

Even though the eprinomectin binds stronger non-selectively to both control and test polymers, the imprinted polymer is 1.6 times (2.43/1.56) more selective for avermectin than for eprinomectin in the acetonitrile mobile phase. This is much lower than the 11.5 times (18.0/ (2.19/1.40) = 11.5) for avermectin over eprinomectin in chloroform mobile phase.

Thus, it has been shown that a non-covalent MIP can be prepared that exhibits selectivity for the large avermectin molecule. Although the best selectivity was demonstrated when the polymer was evaluated using the same solvent that was used

for polymerization, the use of acetonitrile for evaluation does provide enough selectivity to further investigate the potential to use the MIP in aqueous environment.

Evaluation of the MIP Prepared in Chloroform in Acetonitrile-Based Mobile Phase Containing Aqueous Buffer, using Avermectin and Eprinomectin Solutions Prepared in Chloroform Containing 0.1% Acetic Acid

Because the second aim of the study was to evaluate the possibility of using an imprinted polymer that relies on hydrogen bonding interactions to recognize avermectin in an aqueous medium, the effect of the addition of water to the mobile phase was investigated. To evaluate the binding of avermectin to an imprinted polymer in aqueous medium, 2 mM aqueous phosphate buffer adjusted to pH of 2.2 was added to the mobile phase to maintain a constant pH. Avermectin and eprinomectin were each analyzed on the test and control columns in mobile phases of 2% and 5% (v/v) 2 mM aqueous phosphate buffer in acetonitrile.

Figure 18 is the overlay of the control column chromatograms of a solution of 137 µg/mL avermectin and 0.01% toluene (dark line), and 87 µg/mL eprinomectin and 0.01% toluene (gray line). Figure 19 is the overlay of chromatograms of the same two solutions on the test column. Figures 20 and 21 are overlays of the chromatograms of the injection of the two solutions in a mobile phase of 5% aqueous buffer on the control and test columns, respectively. Results show that the retention and separation of avermectin and eprinomectin on both columns were reduced as the aqueous portion was increased.

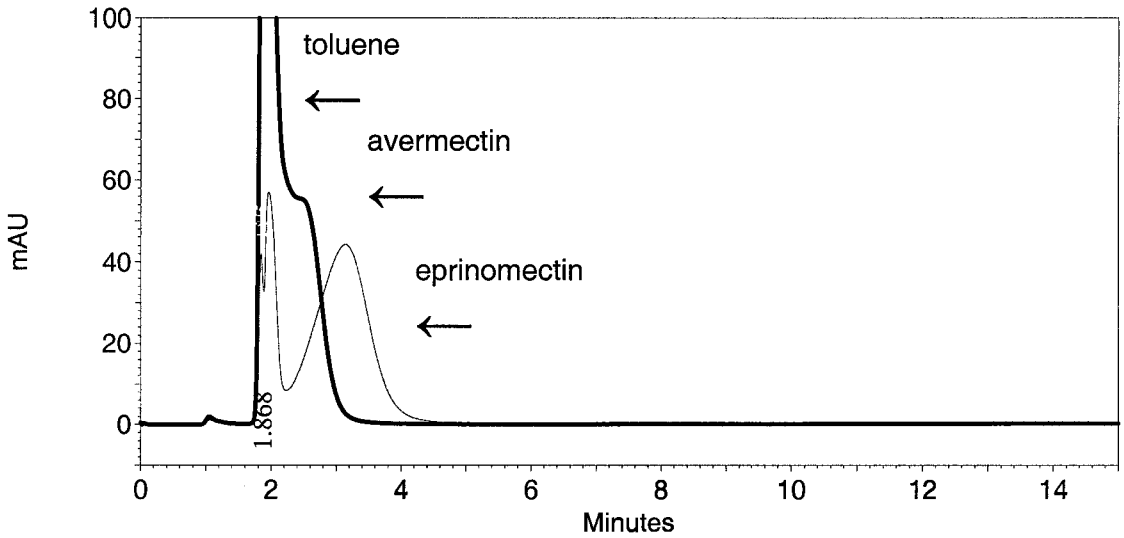


Figure 18. Overlay of chromatograms from the control column of 137 µg/mL avermectin and 0.01% toluene (dark line), and 87 µg/mL eprinomectin and 0.01% toluene (gray line), in mobile phase of 2% 2 mM aqueous phosphate, pH 2.2 in acetonitrile.

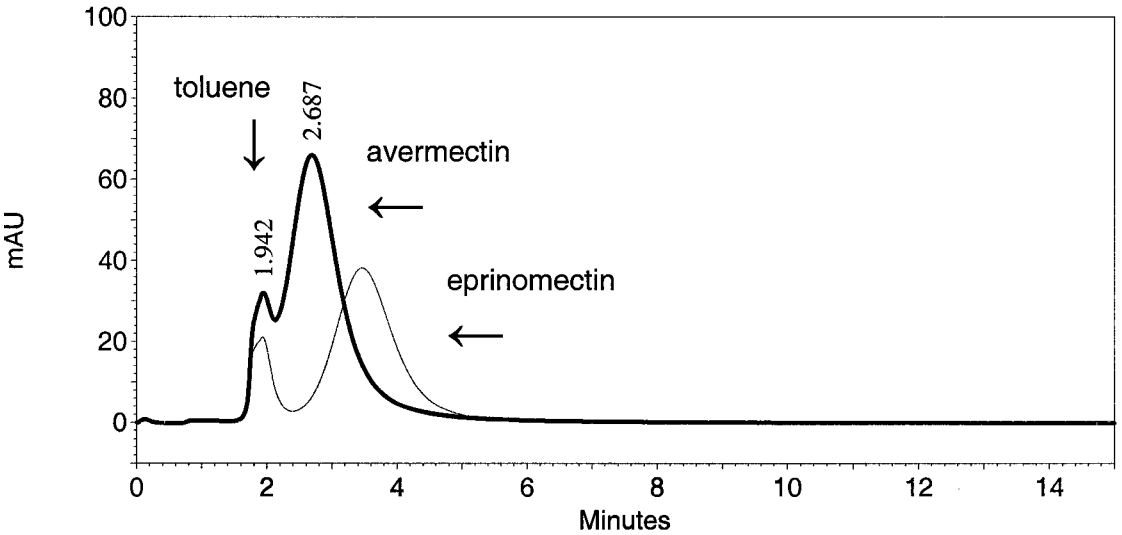


Figure 19. Overlay of chromatograms from the test column of 137 µg/mL avermectin and 0.01% toluene (dark line), and 87 µg/mL eprinomectin and 0.01% toluene (gray line), in mobile phase of 2% 2 mM aqueous phosphate, pH 2.2 in acetonitrile.

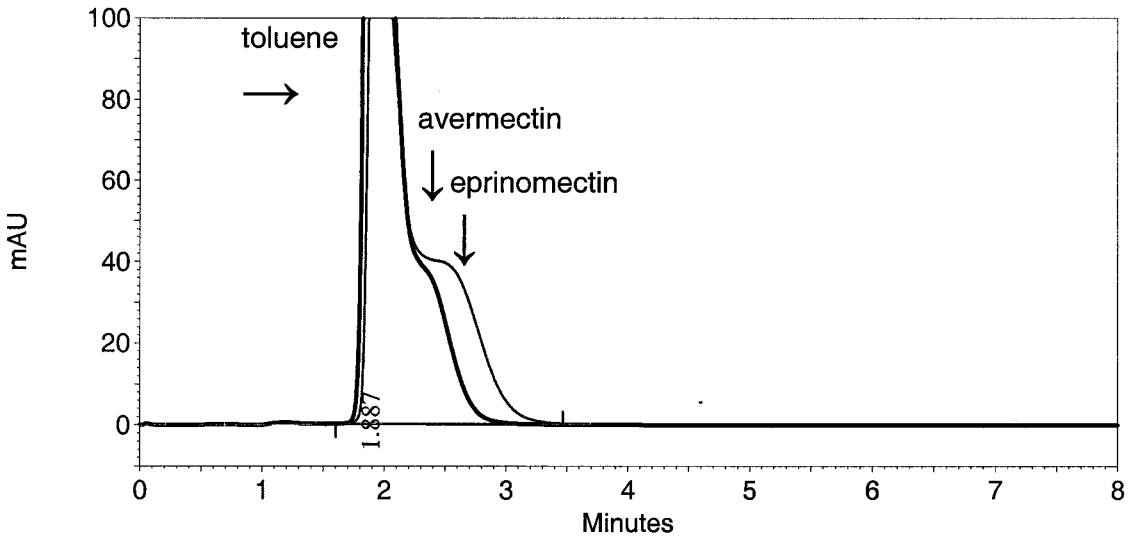


Figure 20. Overlay of chromatograms of 137 µg/mL avermectin and 0.01% toluene (dark line), and 87 µg/mL eprinomectin and 0.01% toluene (gray line), in mobile phase of 5% 2 mM aqueous phosphate, pH 2.2 in acetonitrile, evaluated on the control column.

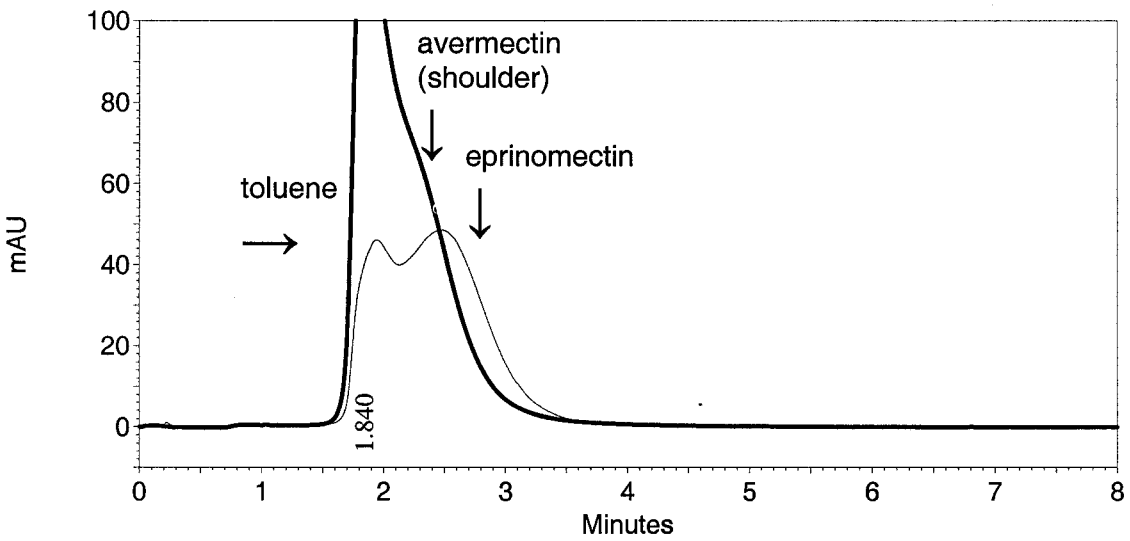


Figure 21. Overlay of chromatograms of 137 µg/mL avermectin and 0.01% toluene (dark line), and 87 µg/mL eprinomectin and 0.01% toluene (gray line), in mobile phase of 5% 2 mM aqueous phosphate, pH 2.2 in acetonitrile, evaluated on the test column.

Table 6. Comparison of retention of avermectin and eprinomectin on the control and test columns made in chloroform, evaluated in acetonitrile mobile phase with aqueous phosphate buffer. (Avermectin and eprinomectin solutions were prepared in chloroform containing 0.1% acetic acid.)

Mobile Phase	t'r(avm) control	t'r(avm) test	k'(avm) control	k'(avm) test	α(avm) test/control
0.05% acetic acid in acetonitrile	0.95	0.95	0.47	1.14	2.43
2% 2 mM phosphate buffer in acetonitrile	0.54	0.54	0.28	0.40	1.43
5% 2 mM phosphate buffer in acetonitrile	0.41	0.41	0.22	0.21	0.95
	t'r(epr) control	**t'r(epr) test**	**k'(epr) control**	**k'(epr) test**	**α(epr) test/control**
0.05% acetic acid in acetonitrile	2.81	4.34	1.40	2.19	1.56
2% 2 mM phosphate buffer in acetonitrile	1.18	1.54	0.60	0.80	1.33
5% 2 mM phosphate buffer in acetonitrile	0.61	0.57	0.32	0.30	0.94

Upon introducing increasing amounts of water into the eluent, a decrease in the retention time was observed for avermectin (Table 6). This result has been observed in past studies of retention of other analytes.[7] Although hydrogen bonding is the major factor for recognition in MIPs prepared in nonpolar solvents, the presence of water significantly lowers the effectiveness of the hydrogen bonding between the analyte and polymer receptor site due to competition for hydrogen bond donor and acceptor sites. This explains why the retention of avermectin (Figures 16-21) decreases as the water content increases, until there is no separation from the non-retained toluene. This indicates that, at these two levels of water (2% and 5%), the loss of hydrogen bonding has a greater detrimental effect on recognition efficiency than any improvement due to a hydrophobic effect.

Although eprinomectin is retained longer than the avermectin, as was observed during earlier studies with no water in the mobile phase, a decrease in the retention of eprinomectin was also seen with increasing water content (Table 6). However, there is still slight separation of the eprinomectin from the non-retained toluene in the 5% buffer (Figure 21). Because eprinomectin is retained longer than the avermectin, the effective elimination of hydrogen bonding in the imprinted polymer by the addition of water does not affect the retention of eprinomectin as significantly as it does the avermectin. This indicates that the retention of eprinomectin in the absence of water may be due to both selective hydrogen bonding interactions with the imprinted sites (as is the case with avermectin), but also to other non-selective interactions with the polymer itself (both control and test) that are not present in the avermectin, and are not completely eliminated in the presence of water. If this non-selective interaction in the eprinomectin is due to the stronger hydrogen bonding of the amide with the MAA residues in the polymer, then increasing the amount of water even further would eliminate this retention.

However, past studies have shown that as the water content continues to increase, retention may begin to increase due to hydrophobic interactions which become more important as the water content of the mobile phase increases above a particular level.[62,69,70] To determine if a further increase in water content forces hydrophobic interactions to become more important and increases the retention of avermectin on the MIP, higher concentrations of buffer in the mobile phase were evaluated.

Evaluation of the MIP Prepared in Chloroform in Acetonitrile-Based Mobile Phase Containing Higher Levels of Aqueous Buffer, using Avermectin and Eprinomectin Solutions Prepared in Acetonitrile

For the evaluations thus far, the avermectin and eprinomectin solutions (containing toluene as the void marker) were prepared in chloroform containing 0.1% acetic acid. These solutions were miscible in acetonitrile containing up to 25% aqueous phosphate buffer, but not in mobile phase containing 50% buffer or higher. To determine if hydrophobic interactions become more important for binding of the analyte to the polymer in mobile phase containing these higher levels of aqueous buffer, the solutions of avermectin and eprinomectin for these evaluations were prepared in acetonitrile instead of chloroform for the evaluation of retention and selectivity.

During initial investigations using the solutions prepared in acetonitrile, the retention of the toluene void marker increased, indicating some interaction of the toluene with the stationary phase. Because toluene is not miscible with water, it was not expected to be a suitable void marker in the presence of increasing concentration of water. Consequently, acetone was used as the non-retained component for the remainder of the studies. The retention time of acetone in 100% acetonitrile mobile phase is approximately the same as in 50% aqueous mobile phase, indicating it should be acceptable as a void marker in mobile phases of increasing water content.

The two columns made from the control and test polymers which were prepared in chloroform were evaluated for retention of avermectin and eprinomectin in acetonitrile mobile phases containing increasing portions (0%, 2%, 5%, 10%, 15%, 25%, and 50% v/v) of 2 mM aqueous phosphate buffer. The insolubility of avermectin in water necessitates that some organic solvent be included in the mobile phase.

To illustrate the retention of avermectin and eprinomectin as a function of water concentration in the mobile phase, graphs (Figure 22 for the test column and Figure 23 for the control column) are used to show that retention of both avermectin and eprinomectin decreases as the water content increases from 0% to 10%. This indicates that the longer retention of eprinomectin than avermectin which occurred in the mobile phases containing 2% and 5% water was most likely due to the stronger hydrogen bonding of the eprinomectin with the MAA residues in both the control and test polymers, because at higher levels of 10% and 15% water, the retention of both eprinomectin and avermectin are reduced to similar values. However, the retention of both avermectin and eprinomectin then increased significantly when the aqueous content was increased from 25% to 50%, indicating hydrophobic interactions are involved in these higher levels of water content in the mobile phase.

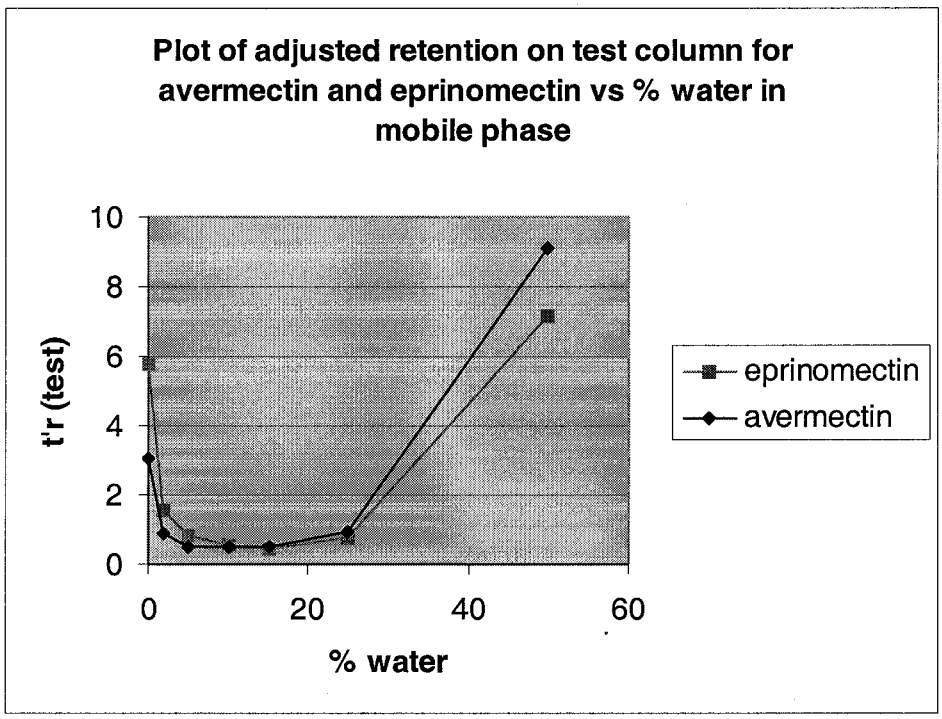


Figure 22. Plot of adjusted retention time [t'r = tr - t_0] versus % water in mobile phase for non-covalently prepared test column synthesized in chloroform.

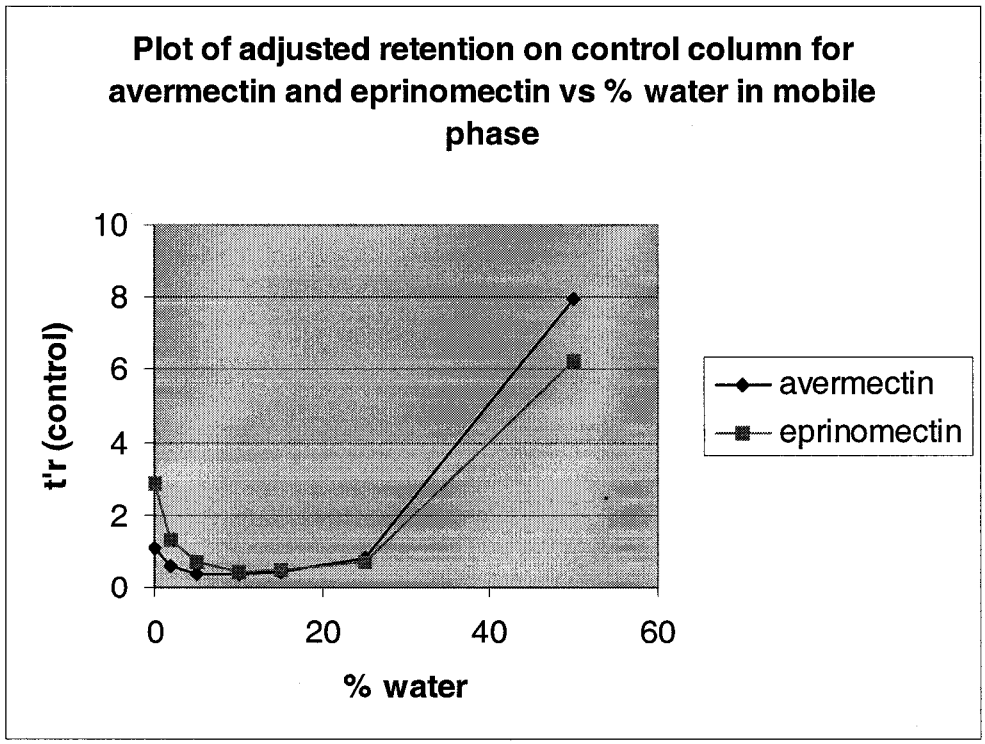


Figure 23. Plot of adjusted retention time [t'r = tr - t_0] versus % water in mobile phase for non-covalently prepared control column synthesized in chloroform.

The increase in retention is similar for both eprinomectin and avermectin, indicating that the hydrophobic interactions are relatively non-specific, and affect both the avermectin and eprinomectin similarly. Because avermectin is slightly less soluble in water than eprinomectin, the retention on both control and test columns increased slightly more for avermectin than for eprinomectin. Because the increase in retention was similar in both the control and test columns, the hydrophobic interactions do not provide any significant improvement in selectivity of the imprinted polymer over the control polymer. To compare the selectivity for avermectin and eprinomectin on the test column versus the control column, Figure 24 is a plot of the selectivity (α = k' (test)/ k' (control)) for avermectin and eprinomectin on the test versus control columns for increasing levels of aqueous buffer in the acetonitrile mobile phase.

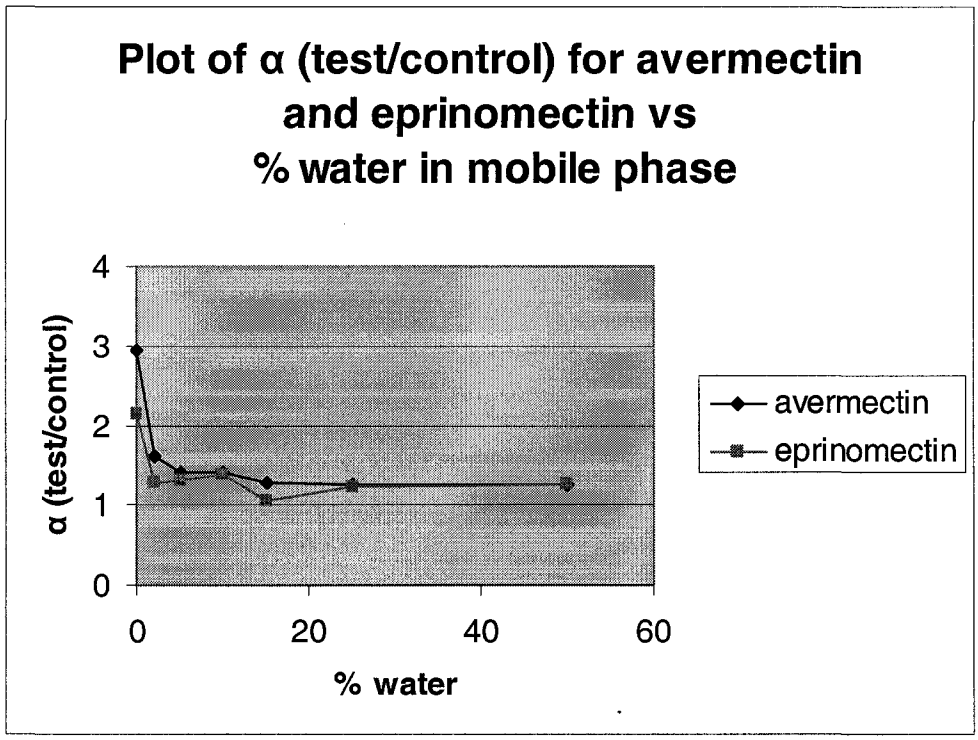


Figure 24. Plot of α values for avermectin (diamonds) and eprinomectin (squares) on the test versus control columns made in chloroform for increasing levels of water in acetonitrile mobile phase. (Avermectin and eprinomectin solutions were prepared in acetonitrile.)

The selectivity of the polymer for avermectin or eprinomectin decreases from 2.95 and 2.13, respectively, to 1.26 for both avermectin and eprinomectin as water is added to the mobile phase. This indicates that hydrophobic interactions are not effective at increasing the selectivity on the imprinted polymer for either compound in an aqueous medium, although a small amount of selectivity is maintained with the addition of water, indicated by the selectivity of 1.26, which is greater than a value of 1.0 which would indicate no selectivity.

Preparation of Polymer using Methacrylic Acid in Acetonitrile and Evaluation in Acetonitrile-Based Mobile Phase

As discussed earlier, because the microenvironment of the binding sites created during polymerization is affected by the solvent, the best selectivity, at least for non-aqueous applications, is obtained when rebinding is performed in the polymerization solvent.[19,63,64] This suggests that preparation of an MIP in acetonitrile may give better selectivity if the polymer is then used for binding in aqueous acetonitrile. To evaluate the potential to improve selectivity of the polymer, preparation of an imprinted polymer using methacrylic acid monomer and acetonitrile as the polymerization solvent was performed to determine if improvement in selectivity of the imprinted polymer for avermectin in an aqueous medium is possible.

The first HPLC evaluations (Figures 25 - 32) of this imprinted polymer were performed in acetonitrile mobile phase with various amounts of acetic acid (0%, 0.05% and 0.1%). The retention of avermectin on this imprinted polymer in a non-aqueous medium was compared with the retention on the previous imprinted polymer prepared in chloroform. In these mobile phases, both acetone and toluene were included in the solutions and co-eluted as the void markers (Figures 25 and 26).

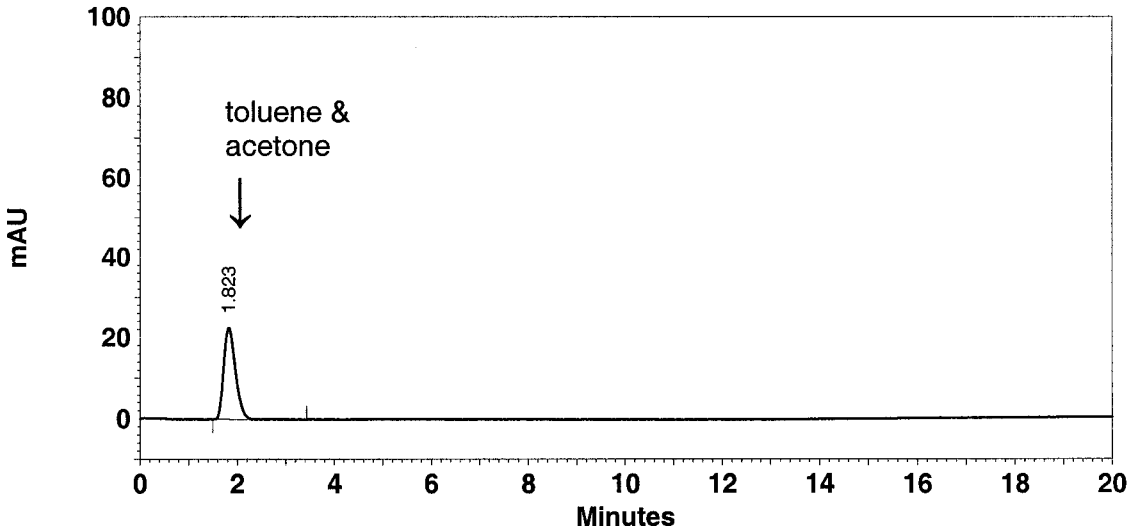


Figure 25. Chromatogram of solution of 0.1% acetone and 0.01% toluene prepared in acetonitrile in a mobile phase of 0.1% acetic acid in acetonitrile, evaluated on the control column.

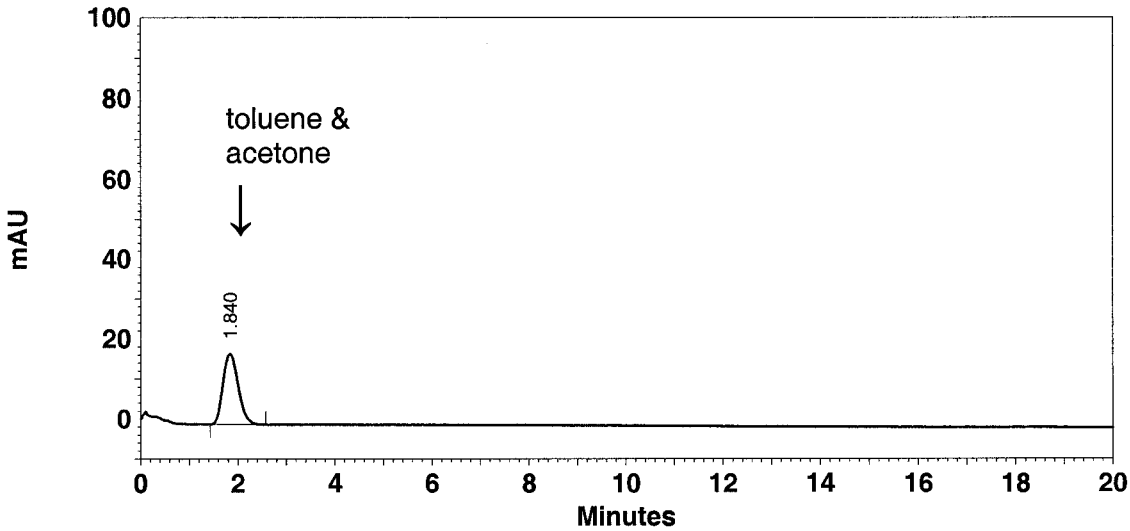


Figure 26. Chromatogram of solution of 0.1% acetone and 0.01% toluene prepared in acetonitrile in a mobile phase of 0.1% acetic acid in acetonitrile, evaluated on the test column.

Analysis of a solution of avermectin containing acetone and toluene in a mobile phase of 100% acetonitrile (Figures 27 and 28) shows that the test column retains avermectin

longer than the control column, indicating some selectivity of the imprinted polymer for avermectin.

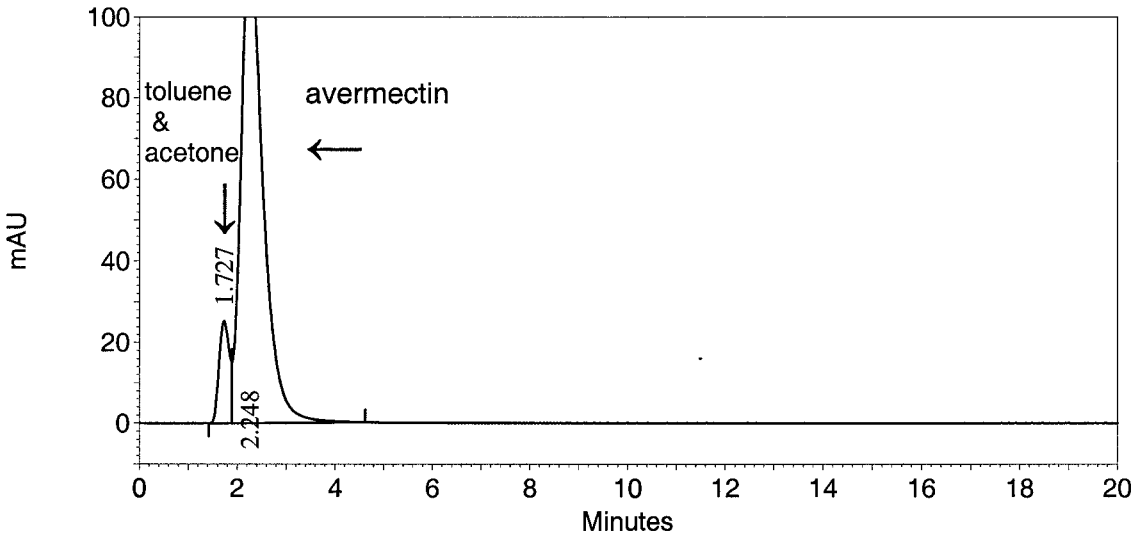


Figure 27. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in a mobile phase of 100% acetonitrile, evaluated on the control column.

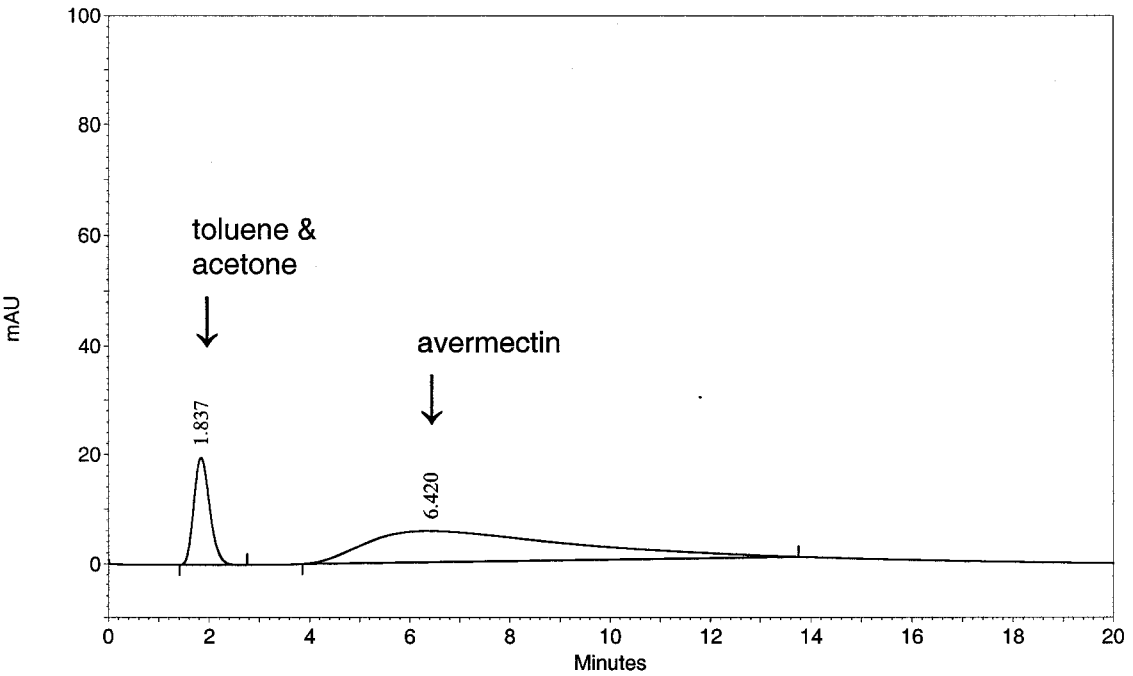


Figure 28. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in a mobile phase of 100% acetonitrile, evaluated on the test column.

Analysis of the same avermectin solution in a mobile phase of acetonitrile containing 0.05% acetic acid is shown in Figures 29 and 30, and in a mobile phase of 0.1% acetic acid is shown in Figures 31 and 32. The results of these studies are summarized in the top half of Table 7.

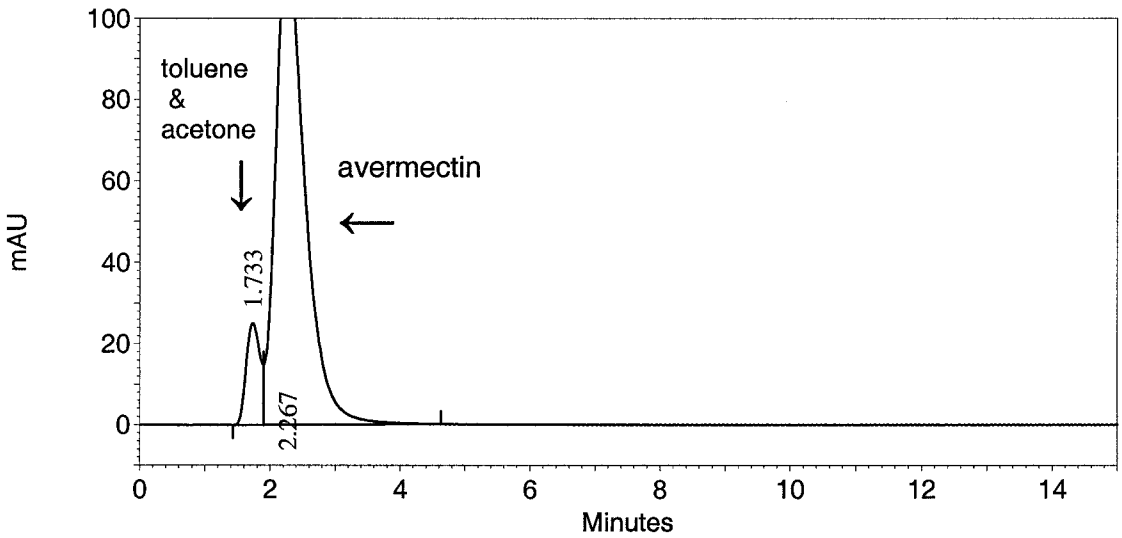


Figure 29. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in a mobile phase of 0.05% acetic acid in acetonitrile, evaluated on the control column.

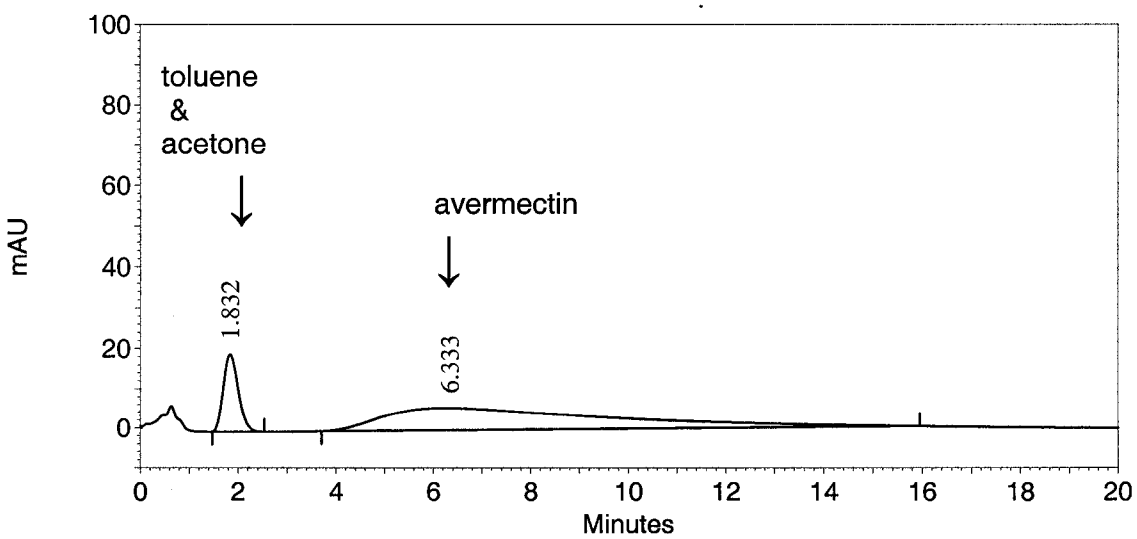


Figure 30. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in a mobile phase of 0.05% acetic acid in acetonitrile, evaluated on the test column.

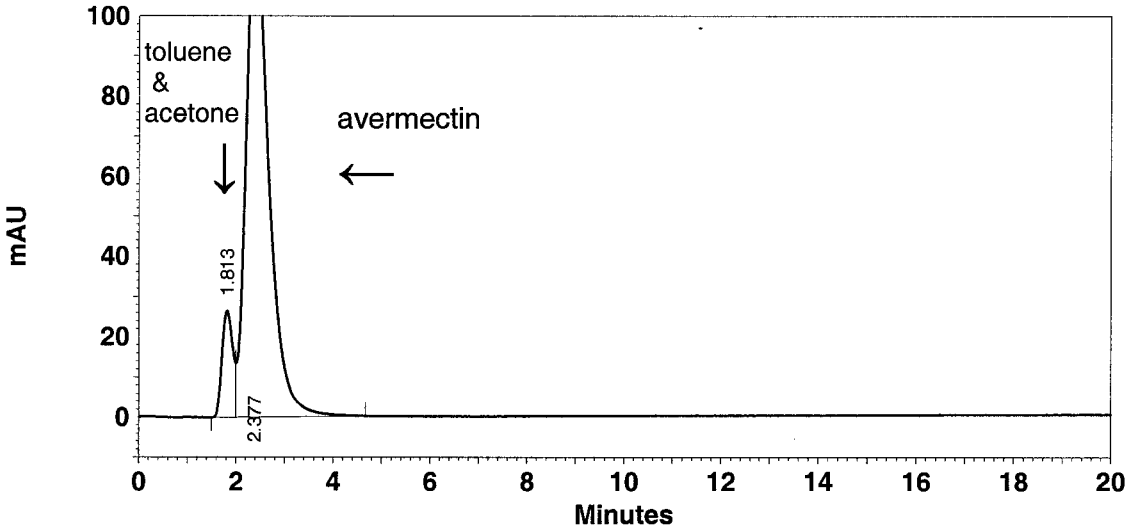


Figure 31. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in a mobile phase of 0.1% acetic acid in acetonitrile, evaluated on the control column.

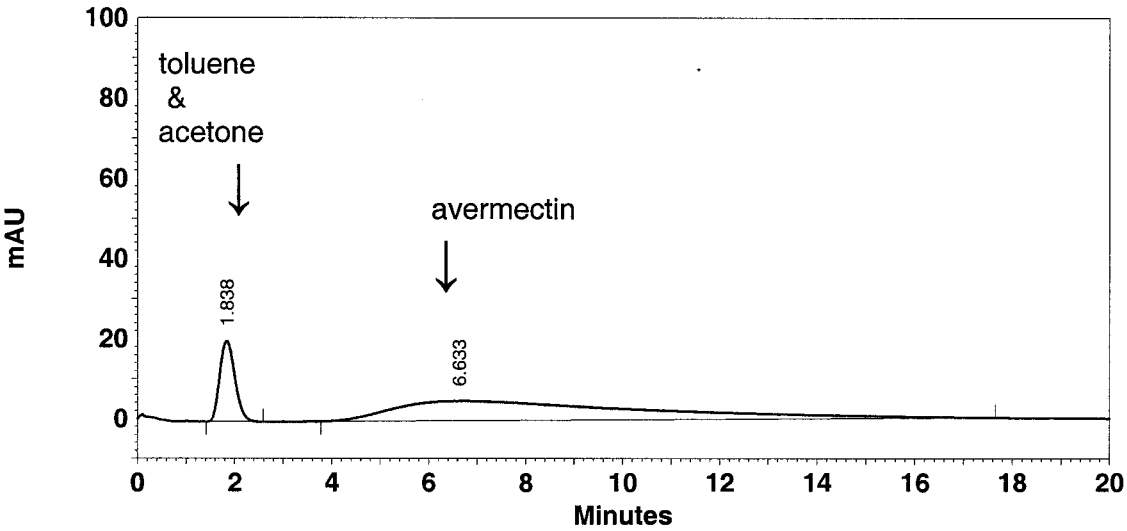


Figure 32. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in a mobile phase of 0.1% acetic acid in acetonitrile, evaluated on the test column.

Table 7. Comparison of retention of avermectin and eprinomectin solutions on the control and test columns made with MAA in acetonitrile using acetonitrile mobile phase with acetic acid.

Mobile Phase	t'r(avm) control	t'r(avm) test	k'(avm) control	k'(avm) test	α(avm) test/control
100% acetonitrile	0.52	4.56	0.30	2.48	8.27
0.05% acetic acid in acetonitrile	0.53	4.49	0.30	2.44	8.13
0.1% acetic acid in acetonitrile	0.56	4.79	0.31	2.60	8.39
	t'r(epr) control	**t'r(epr) test**	**k'(epr) control**	**k'(epr) test**	**α(epr) test/control**
100% acetonitrile	1.91	3.85	1.10	2.09	1.90
0.05% acetic acid in acetonitrile	1.83	3.58	1.05	1.95	1.86
0.1% acetic acid in acetonitrile	1.91	3.70	1.05	2.01	1.91

Results of these studies show that in acetonitrile mobile phase, the selectivity for avermectin (α = 8.13 in 0.05% acetic acid in acetonitrile) was much greater in the polymer prepared in acetonitrile than in the polymer prepared in chloroform (α = 2.43, Table 5). This indicates that the avermectin made a good imprint in the polymer in acetonitrile and that it is selectively retained even without optimization of the chromatographic conditions. This improvement in selectivity over the previous polymer when using acetonitrile mobile phase provides a better polymer for determining if selectivity can occur in a medium containing water.

When comparing the selectivity of the two imprinted polymers in the same mobile phase in which they were each prepared, the selectivity for the polymer prepared in acetonitrile when using 100% acetonitrile mobile phase (α = 8.27) is greater than the selectivity of the polymer prepared in chloroform when using 100% chloroform as the mobile phase (α = 3.93), using the same chromatographic conditions of flow, temperature, and injection volume (Table 8, and Figures 33, 34, 35, 36).

Table 8. Comparison of retention of avermectin on the control and test columns made with MAA in acetonitrile using acetonitrile mobile phases, and on columns made in chloroform using chloroform mobile phases. N/A indicates this mobile phase was not evaluated.

Mobile Phase	t'r(avm) control	t'r(avm) test	k'(avm) control	k'(avm) test	α(avm) test/control
MIP made using MAA and acetonitrile polymerization solvent					
100% acetonitrile	0.52	4.56	0.30	2.48	8.27
0.05% acetic acid in acetonitrile	0.53	4.49	0.30	2.44	8.13
0.1% acetic acid in acetonitrile	0.56	4.79	0.31	2.60	8.39
MIP made using MAA and chloroform polymerization solvent					
100% chloroform	2.04	6.82	1.22	4.80	3.93
0.05% acetic acid in chloroform	0.00	2.66	0.0	1.80	>18.0
0.1% acetic acid in chloroform	0.00	1.14	N/A	0.77	>7.7

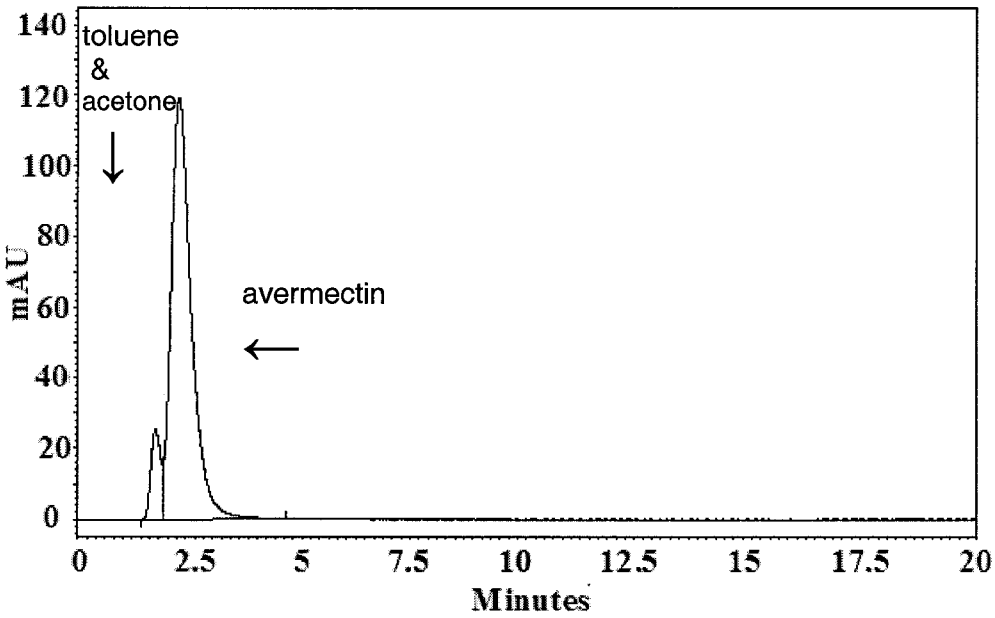


Figure 33. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in mobile phase of 100% acetonitrile, evaluated on the control column (prepared in acetonitrile).

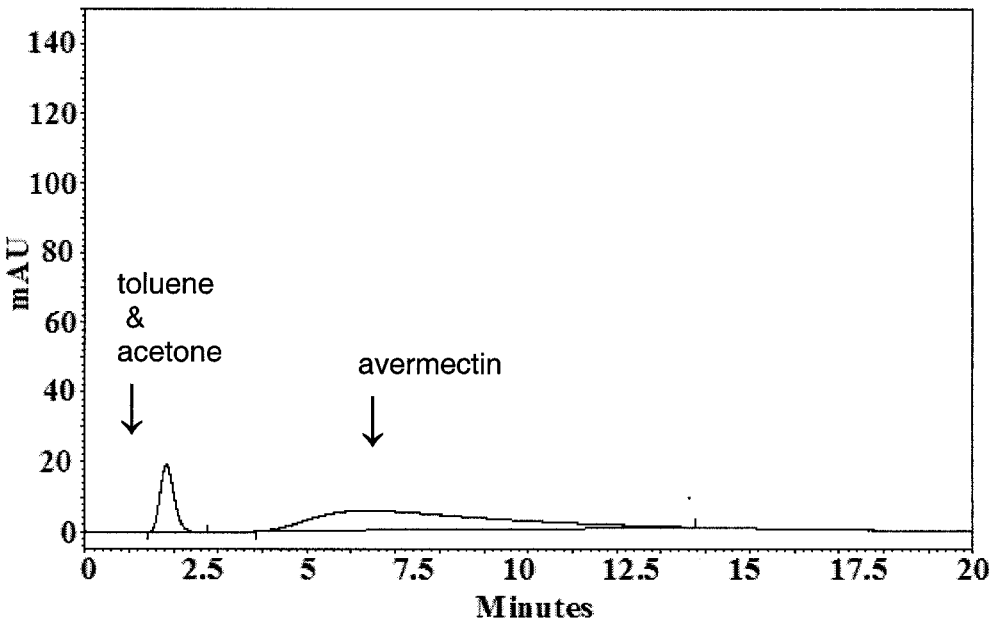


Figure 34. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in mobile phase of 100% acetonitrile, evaluated on the test column (prepared in acetonitrile).

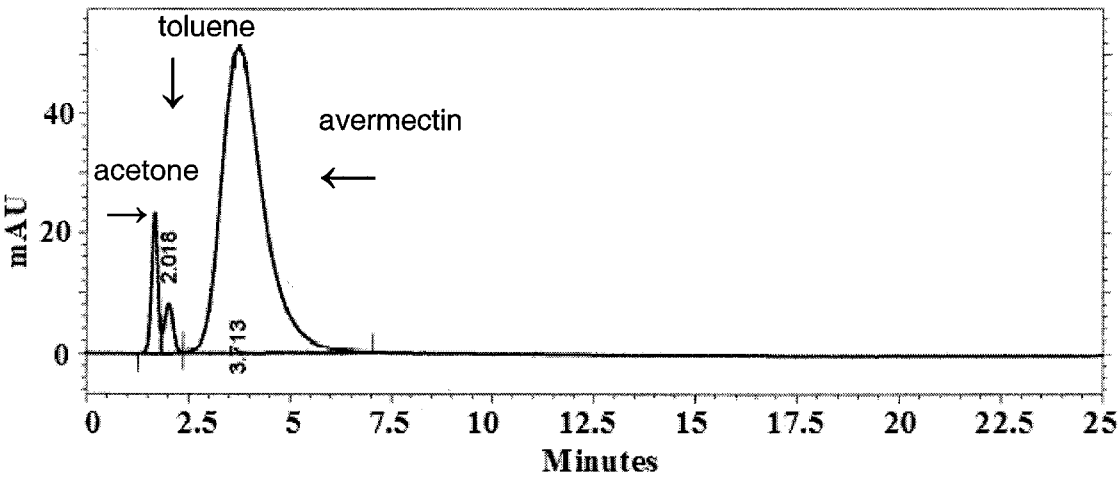


Figure 35. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in mobile phase of 100% chloroform, evaluated on the control column (prepared in chloroform).

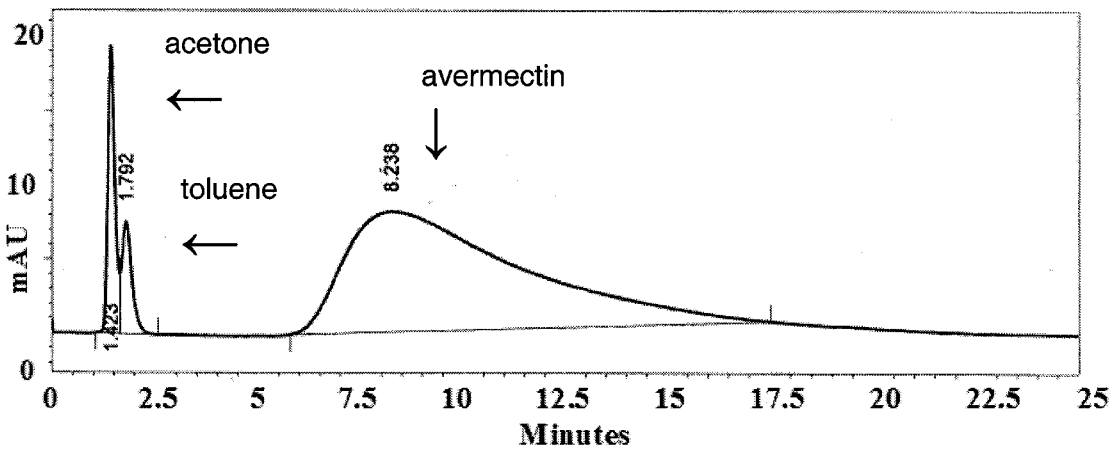


Figure 36. Chromatogram of a solution of 137 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in mobile phase of 100% chloroform, evaluated on the test column (prepared in chloroform).

This is not expected based on previous work, which generally showed better selectivity for chloroform over acetonitrile polymers when a mobile phase that is the same as polymerization solvent was used.[47,63] This is because chloroform is less polar than

acetonitrile, and the polar methacrylic acid monomer interacts with the template more strongly in chloroform than in acetonitrile during the polymerization process, leading to more selective imprinted sites in those polymers prepared in chloroform. In this study, the opposite occurs when comparing the selectivity in mobile phase that is the same as the polymerization solvent, but only when the mobile phase is 100% solvent with no acid added. The data in Table 8 indicate that, although the retention of avermectin was greatest in the test polymer prepared in chloroform and analyzed in 100% chloroform (t'r(avm) = 6.82), the avermectin was also retained somewhat in the control polymer (t'r(avm) = 2.04), indicating more non-selective interactions between the avermectin and the polymer in this mobile phase, reducing the selectivity of this polymer for avermectin. The selectivity in the set of polymers prepared in chloroform changes significantly when acetic acid is added to the mobile phase, increasing from 3.93 to >18.0 with the addition of 0.05% acetic acid. This enhanced selectivity with the addition of a polar modifier to the mobile phase has been noted in the literature.[71] The decrease in retention of avermectin on both the test and control columns with the addition of 0.05% acetic acid indicates that the addition of the acid reduces the non-selective interactions between the avermectin and the polymer. Although initially this may seem like an advantage, as more acid is added, it is likely that the retention of the avermectin on the test polymer may continue to decrease, which would result in an eventual decrease in the selectivity. This is based on the fact that the k' on the control column was reduced from 1.22 to 0.0 by the addition of 0.05% acid, which is a decrease of 1.22, eliminating the non-selective interaction between the avermectin and polymer. The reduction in k' on the test column from 4.80 to 1.80 was greater (3.00), which indicates some additional loss of selective recognition between the avermectin and the imprinted polymer. As more acid is added

(0.1%), the k' of the test column decreases further, indicating further displacement of the avermectin from the binding sites in the test polymer, resulting in loss of selectivity.

Thus, when using the polymer prepared in chloroform, the level of acid added to the chloroform mobile phase had a large effect on the selectivity of the polymer prepared in chloroform (see Table 8). The addition of a greater amount of acid reduced the selectivity of the polymer imprinted in chloroform, so optimization of the mobile phase conditions for evaluation of polymers prepared in chloroform is more important to provide the best selectivity.

For the polymer prepared in acetonitrile, the addition of acetic acid does not significantly change the selectivity of the polymer (see Table 8). The low k' value of 0.3 on the control column compared with 2.48 on the test column indicates that there is very little non-selective interaction contributing to the retention of avermectin in the test polymer, and the addition of an acid modifier that has the ability to hydrogen bond with the polymer does not reduce the selective recognition of the avermectin by the imprinted polymer. This suggests that the addition of water, which can also hydrogen bond with the polymer, to the mobile phase may not result in a loss of selectivity of the polymer for avermectin, which would be beneficial for using this polymer in an aqueous environment.

Before investigating the addition of aqueous buffer to the mobile phase, a solution of eprinomectin was evaluated on the polymers prepared in acetonitrile in acetonitrile mobile phases containing 0, 0.05% and 0.1% acetic acid. The selectivity information for the evaluation of eprinomectin compared with avermectin on this set of polymers is summarized in the bottom half of Table 7. Chromatograms of eprinomectin in a mobile phase of acetonitrile containing 0.1% acetic acid are shown in Figures 37 and 38.

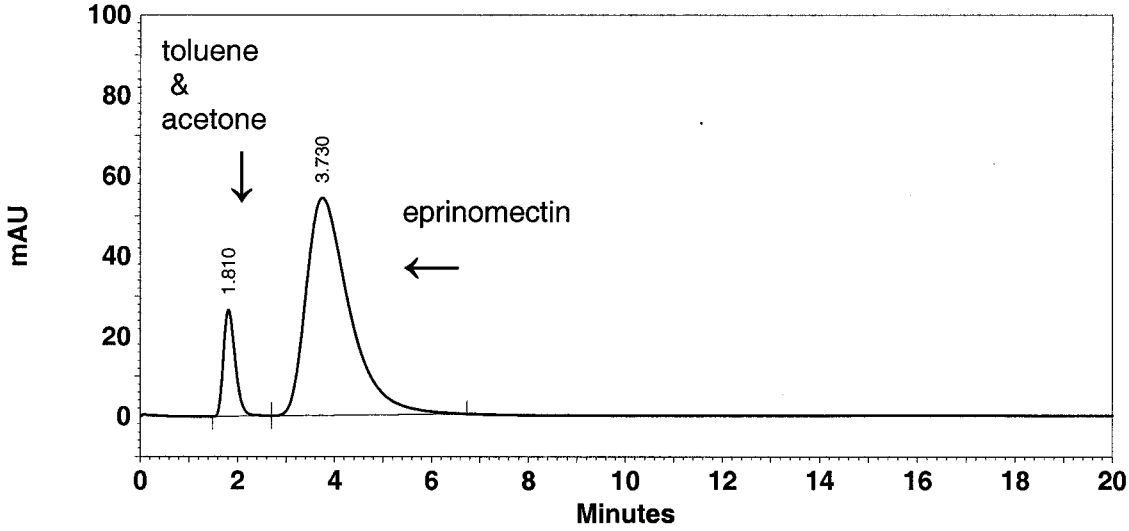


Figure 37. Chromatogram of a solution of 119 µg/mL eprinomectin, 0.1% acetone, and 0.01% toluene in mobile phase of 0.1% acetic acid in acetonitrile, evaluated on the control column.

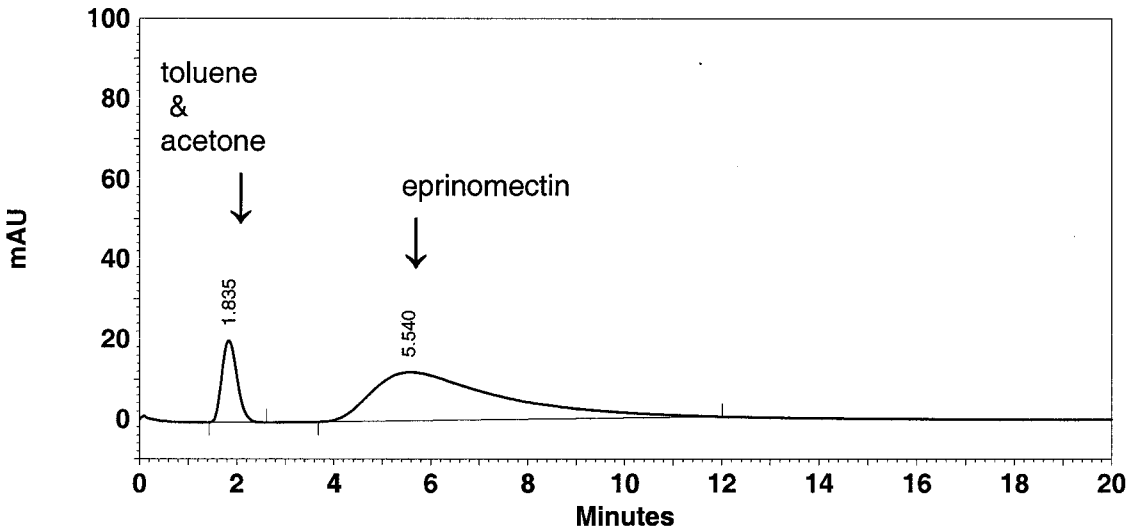


Figure 38. Chromatogram of a solution of 119 µg/mL eprinomectin, 0.1% acetone, and 0.01% toluene in mobile phase of 0.1% acetic acid in acetonitrile, evaluated on the test column.

As was seen in the polymer made in chloroform, eprinomectin is retained longer than avermectin on the control column due to non-specific interactions, which are stronger for

eprinomectin than for avermectin. However, on the test column, avermectin is retained longer than eprinomectin, which indicates that the imprinted phase is 4.4 times (8.39/1.91) more selective for avermectin than for eprinomectin.

Because the goal of the study is to develop an imprinted column that can recognize avermectin in an aqueous medium, retention of avermectin and eprinomectin was evaluated on the acetonitrile-prepared polymers in acetonitrile mobile phase containing increasing portions of 2 mM aqueous phosphate buffer. Figure 39 is a graph of the selectivity (α) for avermectin and eprinomectin on the test versus control columns in acetonitrile mobile phase containing 0%, 2%, 5%, 10%, 15%, 25%, and 50% v/v of 2 mM aqueous phosphate buffer.

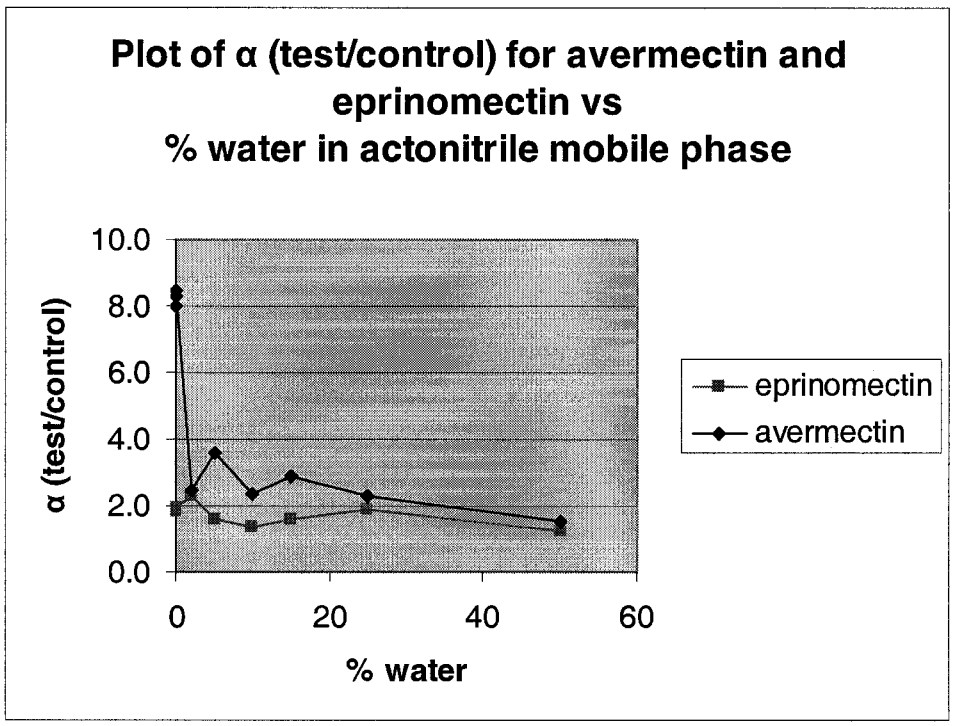


Figure 39. Plot of α values for avermectin (diamonds) and eprinomectin (squares) on the test and control columns made with methacrylic acid in acetonitrile with increasing levels of 2 mM aqueous phosphate buffer in acetonitrile mobile phase. (0% water data point was run in 0.1% acetic acid in acetonitrile).

Results show that as aqueous buffer is added to the mobile phase, the selectivity for avermectin decreases significantly. Based on these results, it may be concluded that preparation of the polymer in acetonitrile during polymerization did not significantly improve the usefulness of the imprinted polymer for use in an aqueous environment.

Preparation of Polymer using Acrylamide in Acetonitrile and Evaluation in Acetonitrile-Based Mobile Phase

Although MAA is the most commonly use hydrogen bonding functional group in molecular imprinting, the hydrogen bonding ability of this functional group is not very strong in polar solvents. The acrylamide monomer, which contains an amide functional group (Figure 4), is an alternative to MAA monomer that may improve the selectivity of an imprinted polymer in polar solvents.[72] Although in regards to imprinting solutions, no general agreement about the relative strength of amide and carboxylic acid hydrogen bonds has been reached, several clues from the literature suggest that the amide functional group may be capable of forming strong hydrogen bonds in polar solvents. Among these clues are: 1. previous results show that a polymer imprinted against a template having an amide group instead of an ester group normally gives much better enantiomeric resolution, and 2. the large differences between the dielectric constants and dipole moments of the amide group and the carboxyl group also suggest that the amide group may form stronger hydrogen bonds than the carboxyl group.[47]

To explore the potential of acrylamide to improve to selectivity of an imprinted polymer for avermectin, a set of polymers was prepared using acrylamide instead of MAA as the monomer, and acetonitrile as the polymerization solvent, with all other polymerization conditions identical to the previous studies. Avermectin and eprinomectin solutions were evaluated on this set of polymers using the same

acetonitrile mobile phases as in previous studies. Results summarized in Table 9 show that the α values for avermectin and eprinomectin for this polymer are lower than any of the previous polymers.

Table 9. Comparison of retention of avermectin and eprinomectin on the control and test columns made with acrylamide in acetonitrile, using acetonitrile mobile phase containing different amounts of acetic acid.

Mobile Phase	**t'r(avm) control**	**t'r(avm) test**	**k'(avm) control**	**k'(avm) test**	**α(avm) test/control**
100% acetonitrile	0.49	0.75	0.26	0.40	1.53
0.05% acetic acid in acetonitrile	0.45	0.71	0.24	0.38	1.58
0.1% acetic acid in acetonitrile	0.40	0.63	0.22.	0.34	1.55
	t'r(epr) control	**t'r(epr) test**	**k'(epr) control**	**k'(epr) test**	**α(epr) test/control**
100% acetonitrile	0.49	0.50	0.26	0.27	1.04
0.05% acetic acid in acetonitrile	0.47	0.53	0.25	0.28	1.12
0.1% acetic acid in acetonitrile	0.44	0.49	0.24	0.26	1.08

Retention of avermectin and eprinomectin was also evaluated in acetonitrile containing increasing portions of 2 mM aqueous phosphate buffer. Figure 40 is a graph of the selectivity (α) for avermectin and eprinomectin on the test versus control columns made in acetonitrile with acrylamide as the monomer.

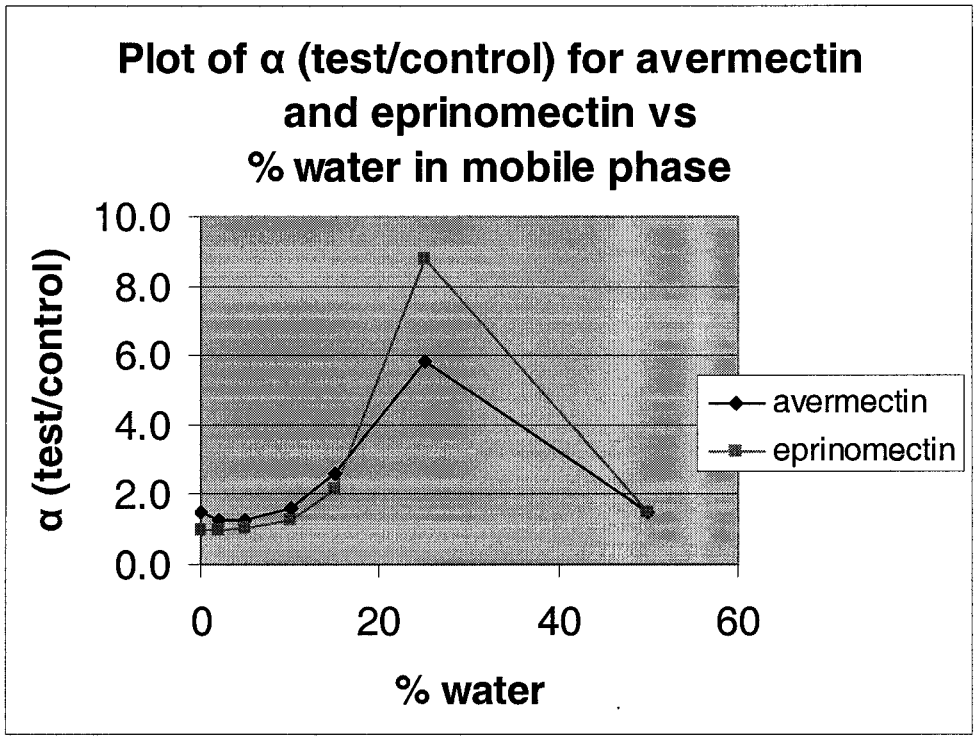


Figure 40. Plot of α values for avermectin (diamonds) and eprinomectin (squares) on the test versus control columns made with acrylamide in acetonitrile, using acetonitrile mobile phase with increasing levels of 2 mM aqueous phosphate buffer.

Although there appears to be an improvement in the selectivity for both avermectin and eprinomectin at 25% buffer, the graph is slightly misleading due to the very small capacity factor (k') values used for calculating the values for α for this polymer. The result of 5.8 seen at 25% is based upon k'(avm) values of ~0.11/~0.02 (test/control) = 5.8. These numbers are too small to be reliable, and any small change results in a very large change in the α value. Overall, based on the chromatography and the small size of the capacity factors for this polymer, it is not considered to be an improvement over the previous two imprinted polymers, and additional studies with this system were not pursued.

Comparison of Covalently Prepared Polymers

Preparation of First Covalent MIP using Avermectin-Methacrylate in Acetonitrile (1:40 Avermectin-Methacrylate:EGDMA)

Thus far it has been shown that the non-covalent technique for the preparation of an imprinted polymer for avermectin is very successful when used in a nonpolar medium. The selectivity of >18 for avermectin when compared with a non-imprinted polymer indicates a very successfully imprinted polymer. The imprinted polymer was also shown to be >6 times more selective for avermectin than for eprinomectin, which has a very similar structure. However, one of the main goals of the research is to prepare an imprinted polymer that can recognize avermectin in an aqueous medium.

In the preparation of non-covalent molecularly imprinted polymers, due to the somewhat random mixing of template, monomer, and crosslinker during polymerization, there is inherently less control over the exact nature of the binding cavities which are formed, resulting in a heterogeneous distribution of sites and binding affinities.[8] A more controlled arrangement of the functional groups on the cross-linked polymer network is more likely to provide better molecular recognition and less peak broadening.[36]

To improve the selectivity of a molecularly imprinted polymer for avermectin, two polymers were imprinted using the covalent approach. In this technique, the template molecule is covalently bonded to the monomer prior to polymerization. Once this template-monomer complex is synthesized, the polymer is then prepared by combining the complex with an excess of crosslinker and proceeding with polymerization as in the non-covalently synthesized polymers. Following polymerization, the template is

removed from the polymer by refluxing to disrupt the covalent bond between the template and polymer.

In the case of avermectin, a template-monomer complex could be prepared in which the methacrylic acid monomer could be covalently bonded to the avermectin at one of the hydroxyl groups on the avermectin molecule. Although the preparation of this avermectin-methacrylate template for polymerization would use a covalent bond, after removal of the template from the completed polymer, the eventual use of the polymer for avermectin recognition would rely on the same non-covalent interactions for avermectin rebinding that were also the basis of recognition in the non-covalently prepared polymer. The interaction responsible for the recognition of avermectin during rebinding is still the hydrogen bonding of the avermectin hydroxyl groups to the methacrylic acid residues on the polymer. That is why this technique is sometimes called "semi-covalent" polymer preparation, compared with true covalent binding, in which a functional group left on the polymer helps to rebind the molecule covalently. However, the covalent bonding of the monomer with the template during preparation of the polymer should provide better, more well-defined cavities to rebind the avermectin during use.

Preparation of a Pre-Assembled Avermectin-Methacrylate Template for Polymerization

To prepare the template-monomer complex in which the methacrylic acid is attached to the avermectin prior to polymerization, the avermectin was esterified at the 5 carbon hydroxyl with methacryloyl chloride, forming a methacrylate. To limit the esterification to only the most reactive location on the avermectin molecule, a molar ratio of 1:1.2 avermectin:methacrylic acid was used. The small amount of by-product in which the avermectin was esterified at two hydroxyl locations (the 5 carbon hydroxyl and

the 4" carbon hydroxyl) was removed from the product mixture by flash chromatography. A synthetic scheme is shown in Figure 41. Although the attachment of avermectin to the polymer at two locations, by preparing a monomer-template with two monomers attached to the avermectin, might provide a stronger anchor of the avermectin to the polymer in a more regular structure than would be possible with only one attachment, the rigid structure around the avermectin may prohibit the removal of the avermectin template, which would render the polymer useless.

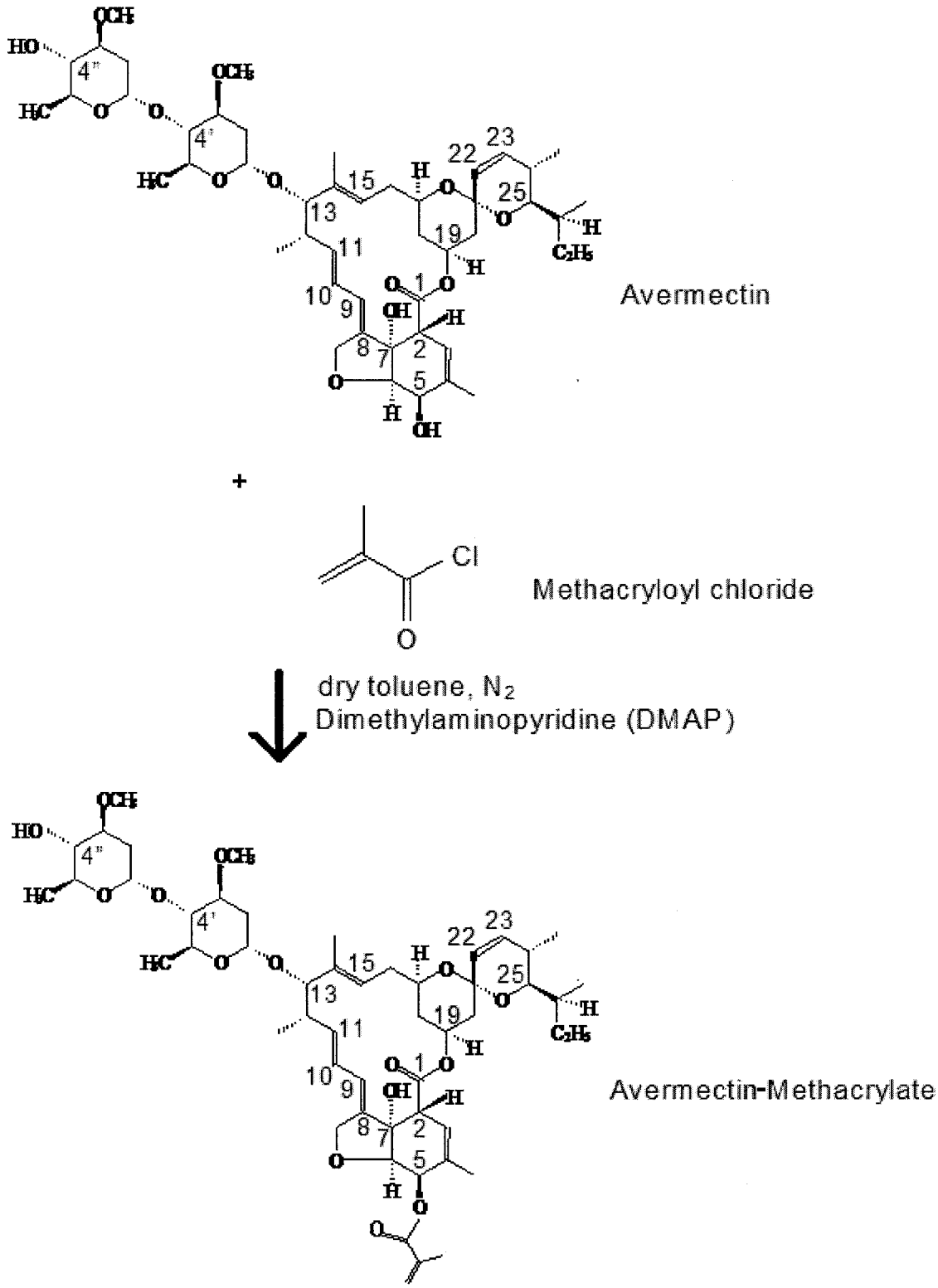


Figure 41. Synthetic scheme for the esterification of avermectin at the 5 carbon with methacryloyl chloride to form avermectin-methacrylate.

NMR Confirmation of the Structure of the Pre-Assembled Avermectin-Methacrylate Template for Polymerization

After synthesis of the avermectin-methacrylate, the identity of the structure was confirmed by NMR. Proton, carbon and DEPT NMR spectra of avermectin and of the product of the synthesis (avermectin methacrylated at the 5 carbon) were recorded. Figure 42 is the proton NMR spectrum of avermectin, and Figure 43 is the proton spectrum of avermectin-methacrylate (referred to as AM) with the avermectin esterified at the 5 carbon. Figure 44 is the structure of AM with the methacrylate portion of the structure labeled (see Figure 41 for labeling of the remainder of the AM structure). Table 10 lists the proton (^{1}H) NMR data for avermectin and for AM.

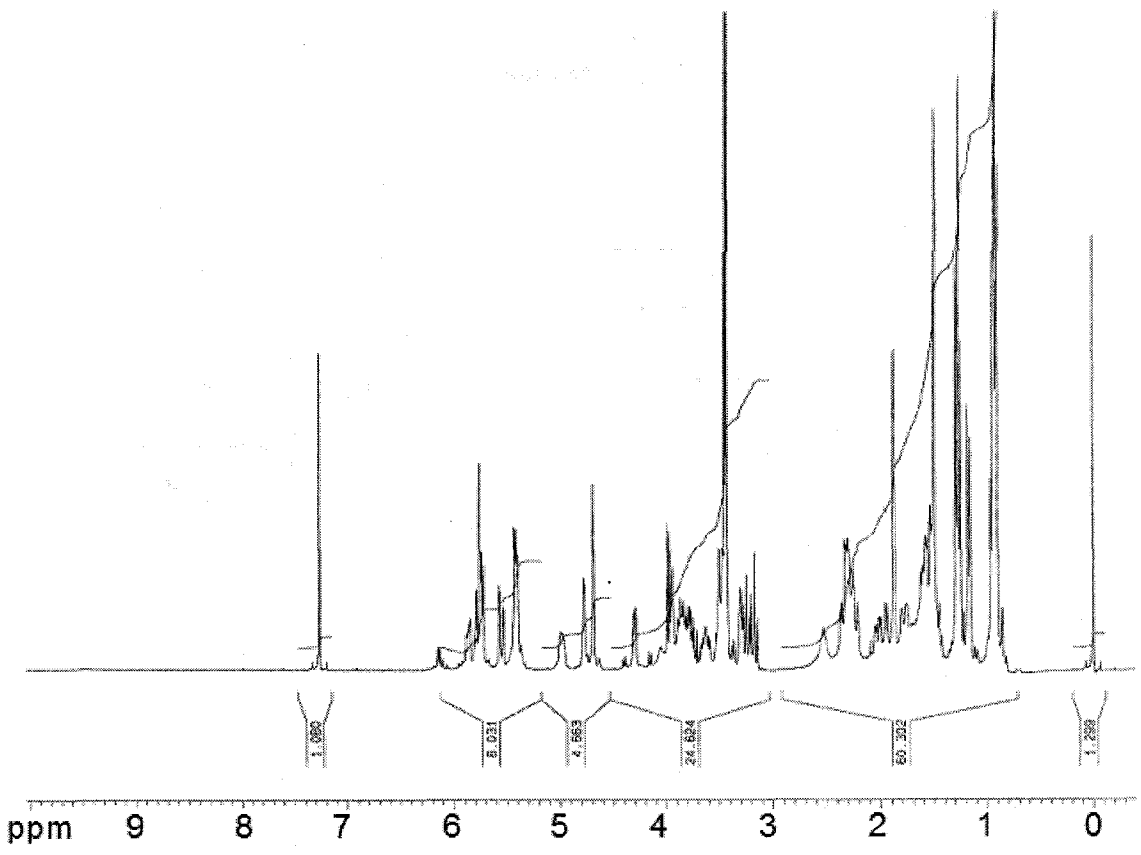


Figure 42. Proton NMR spectrum of avermectin.

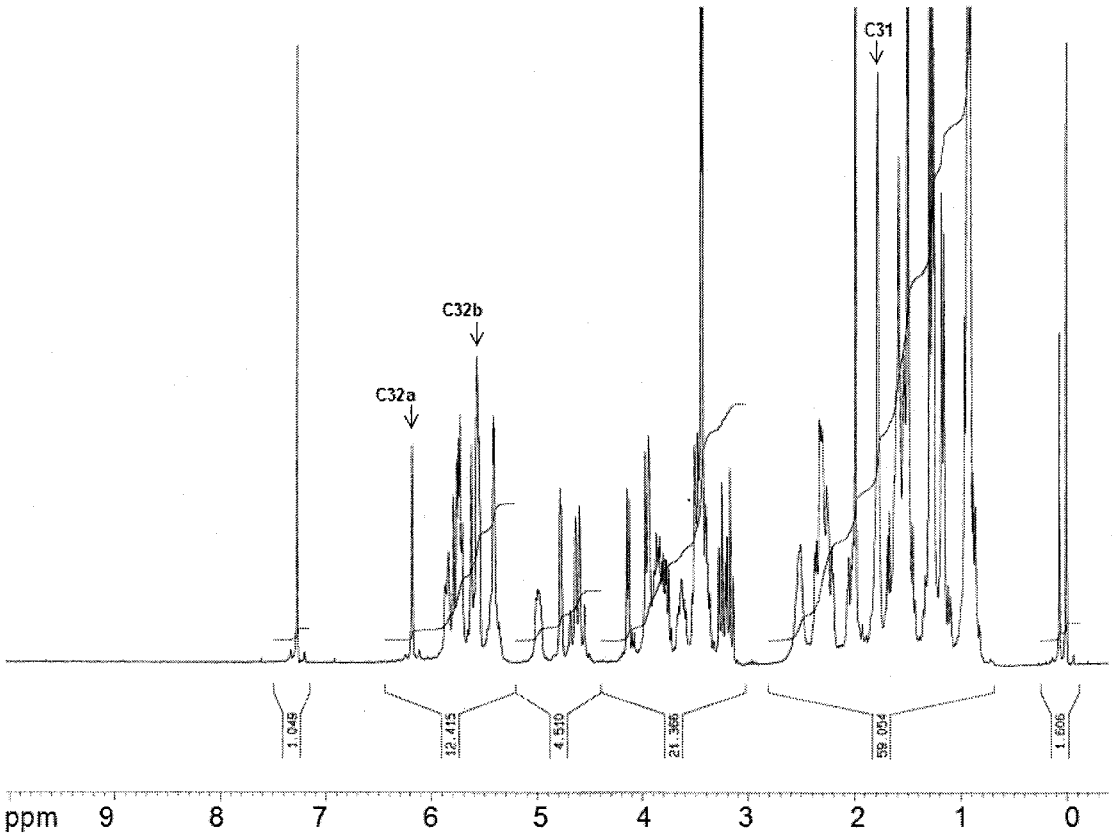


Figure 43. Proton NMR spectrum of avermectin-methacrylate with the avermectin esterified at the 5 carbon.

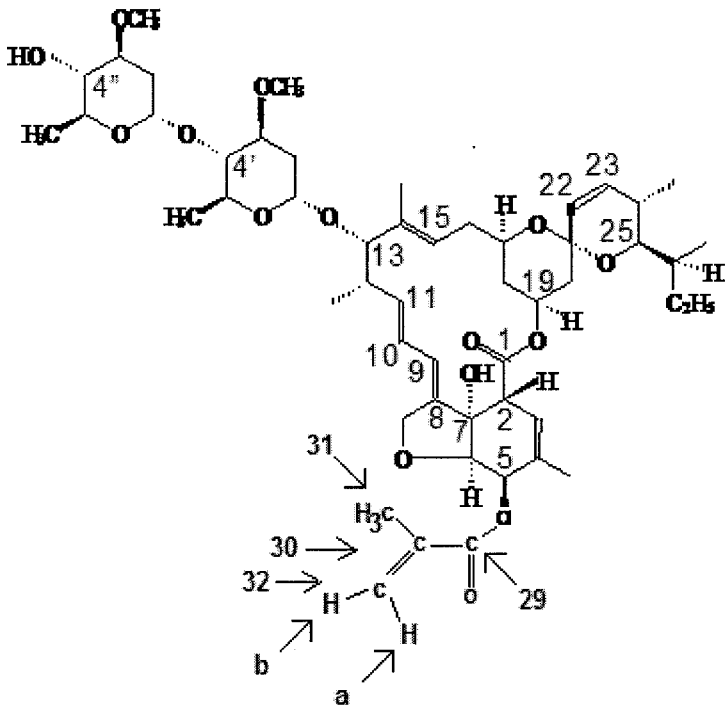


Figure 44. Avermectin-methacrylate labeled for NMR.

Table 10. Proton (^{1}H) NMR data for avermectin and avermectin-methacrylate. Only new or shifted assignments are listed for the avermectin-methacrylate.[18]

H	Avermectin (ppm)	Avermectin-Methacrylate (ppm)
C_{31}	---	1.7
C_{32} a (cis)	---	6.2
C_{32} b (trans)	---	5.6
C_2H	3.33	
C_3H	5.46	
C_4CH_3	1.90	
C_5OH	2.38	
C_5H	4.34 (t, 6.0)	4.15
C_6H	4.08 (d, 6.0)	
C_7OH	4.01	
$C_{8a}H_2$	4.74 (d,d 16.0, 2.0) 4.72 (d,d 16.0, 2.0)	
C_9H	5.91 (m)	
$C_{10}H$	5.78 (m)	
$C_{11}H$	5.78 (m)	
$C_{12}CH_3$	1.18 (d)	
$C_{12}H$	2.55	
$C_{13}H$	3.98	
$C_{14}CH_3$	1.50	
$C_{15}H$	5.03 (d,d, 10.0, 4.0)	
$C_{16}CH_2$	2.2-2.4	
$C_{17}H$	3.90 (m)	
$C_{18}H_2$	0.88 (q 12) 1.80 (br.d 12)	
$C_{19}H$	5.44 (m)	
$C_{20}H_2$	eq. 2.09 (dd 12, 4) ax 1.50-1.65	
$C_{22}H_2/C_{22}H$	5.82 (dd 10, 1.7)	
$C_{23}H_2/C_{23}H$	5.60 (dd 10, 2)	
$C_{24}H$	Unassigned	
$C_{24}CH_3$	0.93	
$C_{25}H$	3.52 (d, 9)	
$C_{26}H$	1.50-1.65	
$C_{26}CH_3$	0.92	
$C_{27}CH_2$	1.50-1.65	
$C_{28}CH_3$	0.95	

Table 10 (continued)

H	Avermectin (ppm)	Avermectin-Methacrylate (ppm)
C_1H	4.82 (d,3)	
$C_2'H_2$	2.2-2.4	
$C_3'H$	3.66 (ddd 12,7,4)	
$C_3'OCH_3$	3.46 or 3.44	
$C_4'H$	3.28 (t, 9.0)	
$C_5'H$	3.88 (d, q, 9, 6)	
$C_5'CH_3$	1.27	
$C_1"H$	5.44 (d, 3.5)	
$C_2"CH_2$	2.2-2.4	
$C_3"H$	3.52 (m)	
$C_3"CH_3$	3.46 or 3.44	
$C_4"OH$	2.52	
$C_4"H$	3.20 (t, 9.5)	
$C_5"H$	3.81 (d, q, 9.5, 6.5)	
$C_5"CH_3$	1.30	

In the structure of AM (Figure 44) compared with the spectrum of avermectin, three new types of protons are present: the methylene group cis hydrogen ($=CH_2$) that is spatially closest to the carbonyl (carbon 32"a"), the methylene group trans hydrogen ($=CH_2$) that is trans to the carbonyl (carbon 32"b") and the three methyl group hydrogens (carbon 31). These new peaks are labeled in Figure 44.

The typical chemical shift of a proton on a methyl carbon adjacent to a methylene group is 1.6.[73] In the NMR spectrum of AM, a singlet appears at ~1.7 ppm, corresponding to methyl group hydrogens (C_{31}) of the attached methacrylate. The estimated chemical shift of the cis olefinic proton (C_{32} a (cis)) is 6.14 ppm. A singlet appears in the spectrum at 6.2 ppm which is the cis H ("a") of the methylene group of the attached methacrylate group. The estimated chemical shift of the trans olefinic proton (C_{32}"b" (trans)) is 5.58 ppm.[73] A singlet appears in the AM spectrum at 5.6 ppm which is a result of the trans H ("b") of the methylene group of the attached methacrylate group. These three new signals are labeled on Figure 43.

It appears from this set of spectra that only one methacrylate group has attached to the avermectin because only one set of three signals appears in the spectrum of the synthesized product. In determining which of the three avermectin hydroxyl groups (5, 7 or 4" carbon) is bonded to the methacrylate group, it is known that the 5 carbon hydroxyl is the most reactive of the three, and the 4" carbon is the next most reactive.[18] Evaluating the spectra for the hydrogen atoms from the 5 carbon and 4" carbon hydroxyl groups does not provide much information because there is very little difference between the two spectra in this region. It is difficult to determine if there is any change in the 4" carbon hydrogen signal because this is a complex multiplet. However, the signal from the 5 carbon hydrogen has moved upfield in the AM spectra from about 4.3 to 4.15 ppm, indicating a change in the shielding at this carbon, probably due to the addition of the methacrylate group.

The carbon 13 spectra of avermectin and of the synthesized product are shown in Figures 45 and 46. Table 11 lists the carbon (^{13}C) NMR data for avermectin and for AM.

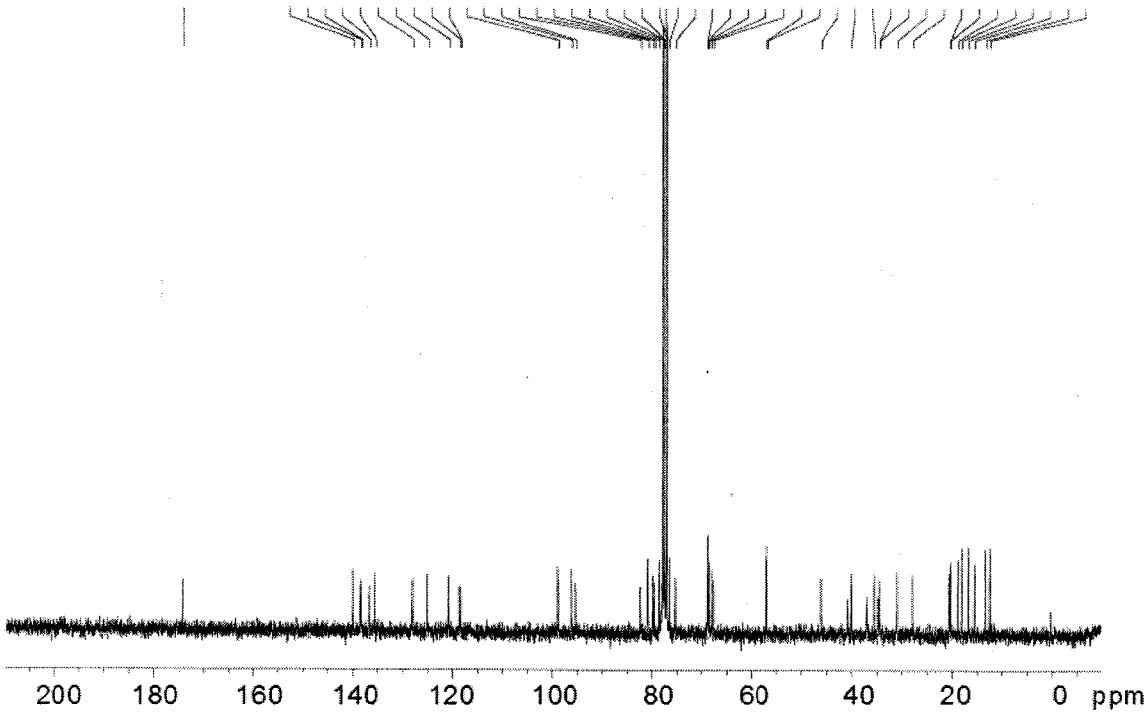


Figure 45. Carbon NMR spectrum of avermectin.

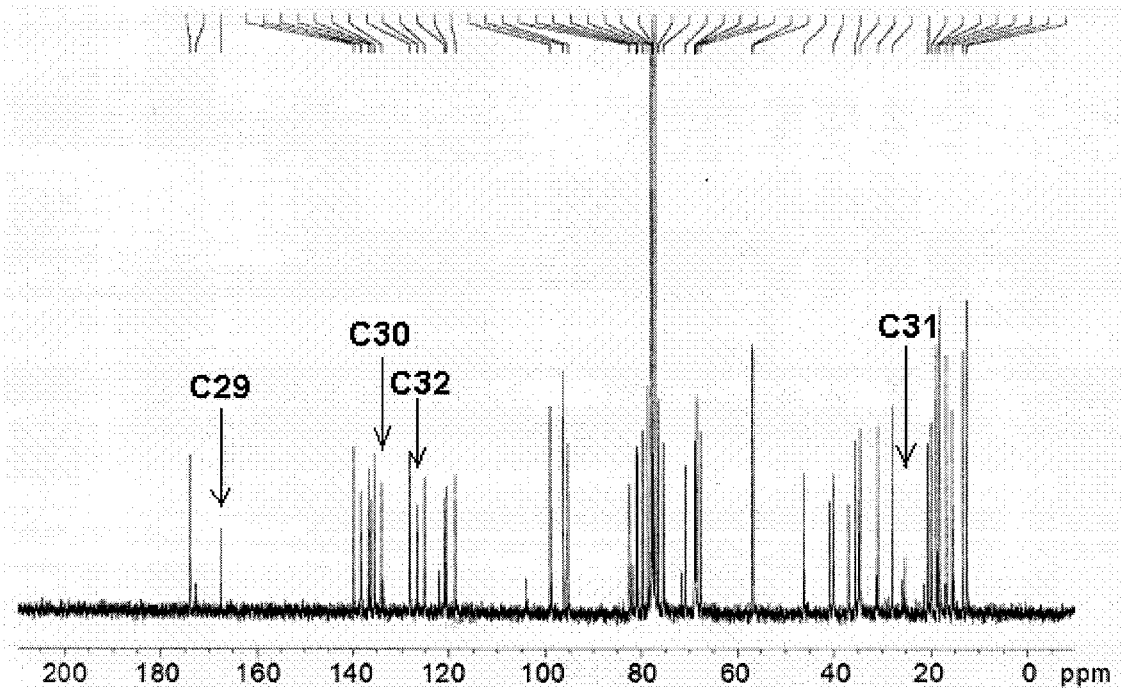


Figure 46. Carbon NMR spectrum of avermectin-methacrylate.

Table 11. Carbon (^{13}C) NMR data for avermectin and avermectin-methacrylate. Only new or shifted assignments are listed for the avermectin-methacrylate.[18]

Carbon (^{13}C)	Avermectin (ppm)	Avermectin-methacrylate (ppm)
32	---	127
31	---	26
30	---	134.7
29	---	167
28	12.0	
26a	12.9	
14a	15.1	
24a	16.4	
6'	17.7	
6"	18.4	
4a	19.9	
12a	20.2	
27	27.5	
24	30.6	
16	~34.3	
2'	~34.3	
2"	~34.3	
26	35.2	
18	36.6	
12	39.8	
20	40.5	
2	45.7	
3'a	56.4	
3"a	56.4	
5'	67.3	
5	67.8	71
19	~68.2	
8a	~68.2	
17	~68.4	
5"	~68.4	
25	75.0	
4"	76.1	
3"	78.3	
3'	79.4	
6	79.4	
4"	80.5	

Table 11 (continued)

Carbon (^{13}C)	Avermectin (ppm)	Avermectin-methacrylate (ppm)
7	80.5 (s)	
13	82.0	
1'	95.0	
21	95.8	
1"	98.5	
15	1118.1	
3	118.4	
9	120.4	
10	124.8	
23	127.9	
14	135.2	
22	136.2	
4	137.9	
11	138.0	
8	139.7	

In the carbon spectrum of the synthesized product (AM), a signal at 167 ppm which is not present in the spectrum of avermectin is due to the carboxyl carbon in AM (labeled C29 in Figure 44.) The terminal methylene group (C32) is expected to have a shift of ~129.9 ppm, but because a methyl group is added to the beta carbon, an upfield shift of ~2 ppm is expected (129.9 -2 = ~127.9 ppm).[73] Therefore, the signal that appears at ~127 ppm in the AM spectrum is consistent with the methylene carbon labeled C32. This is confirmed later in the DEPT spectrum. The internal carbon of the methylene group labeled C30 would be expected to have a shift of 128.7 ppm, but adding a methyl group to the carbon would shift the peak downfield about 6 ppm, which predicts a shift to ~134.7 ppm.[73] This supports the new peak assignment of C32 at ~137 ppm. The terminal methyl carbon labeled C31 appears at ~26 ppm.

The 5 carbon appears to shift downfield from 68 to 71 ppm, indicating that the methacrylate is adding at this location. A downfield shift of ~1 ppm is expected since a COR group is added to the beta position.

The DEPT spectra for avermectin and for AM are shown in Figures 47 and 48. For the DEPT spectra, the direction of the signals from the CH_2 carbons is down, the signals from the CH_3 and CH carbons are up, and tertiary carbons do not appear. Therefore, in the spectrum for AM, the carbons C29 and C30 do not appear, and carbon 32 which is a CH_2, appears down at ~127 ppm, thereby confirming the tentative assignment based on the calculated chemical shift in the carbon 13 spectrum. The terminal methyl group C31 appears up at ~27 ppm, also confirming the carbon 13 assignment. A new peak also appears at ~69 ppm, which could be the 5 carbon peak which has shifted due to the addition of the methacrylate group at this location and appears up because it is a CH.

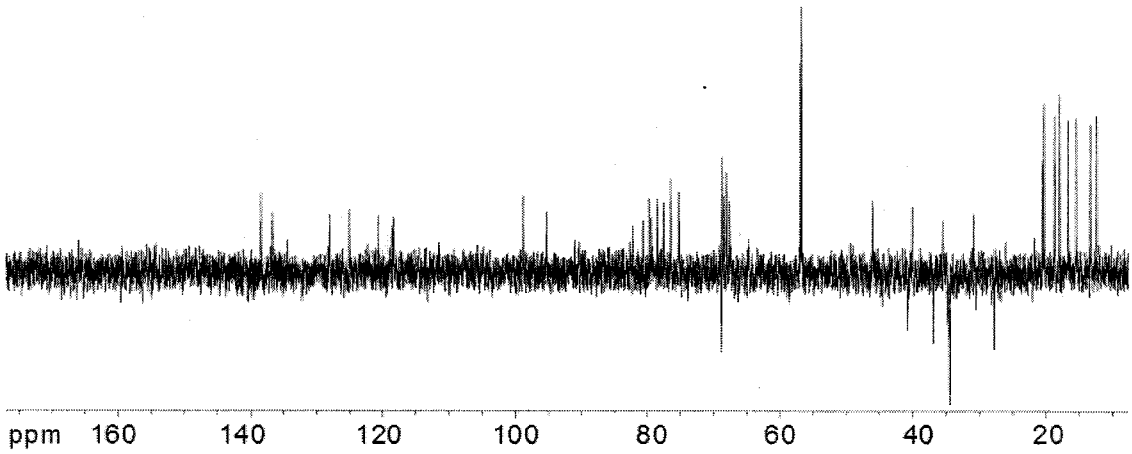


Figure 47. DEPT spectrum of avermectin (CH_2 signals are down, CH and CH_3 signals are up, C does not appear).

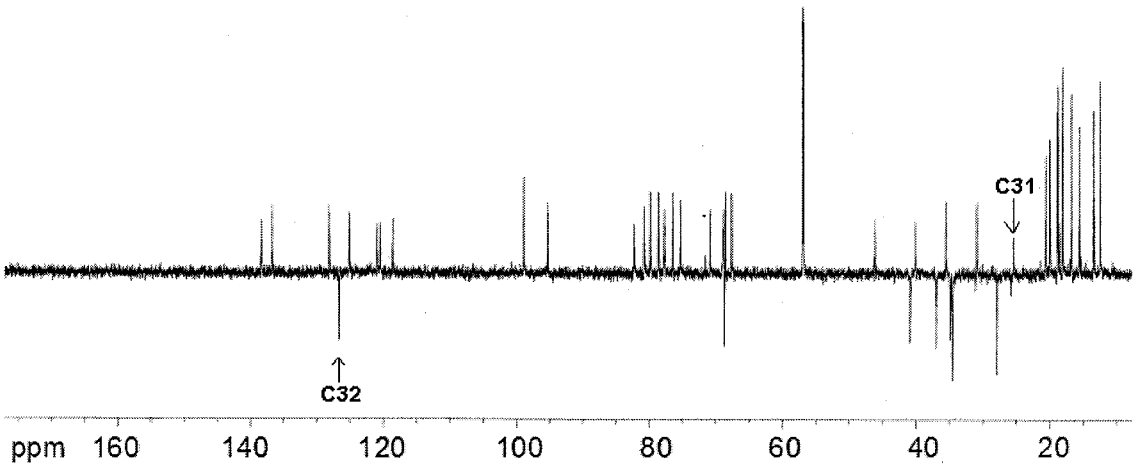


Figure 48. DEPT spectrum of avermectin-methacrylate (CH_2 signals are down, CH and CH_3 signals are up, C does not appear).

Synthesis of the First Covalently Prepared Imprinted Polymer for Avermectin

For the synthesis of the first of two covalently prepared imprinted polymers, the avermectin-methacrylate was combined with excess crosslinker in acetonitrile in a ratio of 2:40. No "free" MAA was added to prevent random MAA monomers from being

incorporated into the polymer network, which would increase the chance of heterogeneous binding sites. 2,2"-Azobisisobutyronitrile initiator was added and polymerization was completed as in the non-covalently prepared polymers. A control polymer was prepared in the same way, but by combining MAA and crosslinker in a molar ratio of 2:40.

After polymerization, the polymers were ground and the imprinted polymer was refluxed in 50/50 methanol/2M aqueous sodium hydroxide to remove avermectin. The ground polymer before and after refluxing was analyzed by FTIR to determine the extent of avermectin removal from the polymer.

Fourier Transform Infrared (FTIR) for Confirmation of Template Removal from Polymer

Fourier Transform Infrared was used to determine the extent of removal of the avermectin from the polymer by refluxing. An FTIR spectrum of avermectin was collected first to determine that the band with the highest absorbance is due to the secondary alcohol at 983 cm^{-1}. FTIR spectra of the imprinted (test) polymer before and after reflux were collected, and the spectra from 1010 to 960 cm^{-1} are shown in Figure 49. To determine the percentage of the avermectin remaining in the test polymer after refluxing, the control polymer was spiked with increasing amounts of avermectin (0, 20, 40, 60, 80 and 100% of the molar ratio of avermectin-methacrylate in the polymerization mixture), and spectra of each were collected.

Comparison of the spectra in Figure 49 shows that about 50% of the avermectin used to prepare the test polymer was removed by refluxing. At 983 cm^{-1}, one of the top two spectra is the test polymer before reflux, which contains the avermectin in the matrix from preparation. The other spectrum which contains a band of the same height, as

expected, is the control polymer spiked with 100% of the amount of avermectin used in the test polymer preparation. The next lower spectrum at 983 cm^{-1} is the control polymer spiked with 80% of the avermectin used in the preparation of the test polymer, and the next spectrum is of the control polymer spiked with 60% of the starting avermectin. The next spectrum is the test polymer after refluxing for 12 hours. The next two lower spectra are of the control polymer spiked with 40% and 20% of the avermectin used in the preparation, respectively. The bottom spectrum is the control polymer, which contains no avermectin, and does not have a band at 983 cm^{-1}, as expected. This study indicates that about 50% of the avermectin originally added to the polymerization mixture remains in the test polymer matrix after refluxing. The FTIR study was repeated after the test polymer was refluxed a second time for an additional 24 hours, and no change in the spectrum was observed. These results indicate that because only 50% of the avermectin that was incorporated into the polymer matrix was removed by refluxing, only 50% of the expected sites are available to bind avermectin during rebinding studies, because 50% of the sites are occupied by avermectin that could not be removed from the matrix.

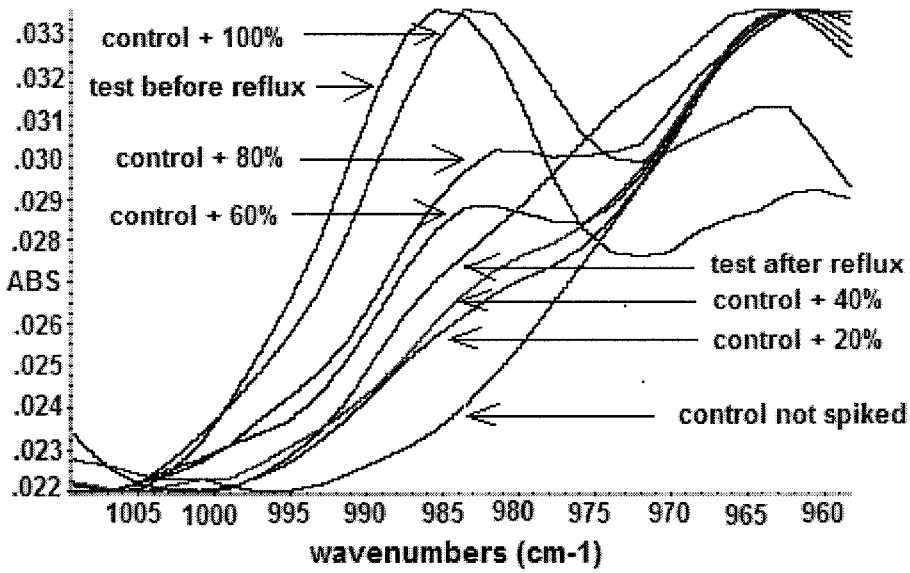


Figure 49. At 983 cm^{-1}, from top to bottom: control + 100% (control polymer spiked with avermectin at 100% of the mole ratio of avermectin-methacrylate used to make the test polymer), test polymer before reflux, control + 80%, control + 60%, test polymer after reflux, control + 40%, control + 20%, control polymer not spiked.

Because the original ratio of avermectin-methacrylate template to EGDMA crosslinker in the polymer preparation was 2:40 (essentially 2:2:40 avermectin:monomer:EGDMA), and only 50% of the avermectin was removed by refluxing, the effective ratio of available binding sites to monomer to crosslinker is 1:1:40, compared with the ratio of 1:15:40 used in the non-covalently prepared polymer syntheses.

Chromatographic Evaluation of the First Covalently Prepared Polymer

After washing the test and control polymers and packing them into HPLC columns, evaluation of avermectin and eprinomectin was performed in the same mobile phases that were used for the non-covalently prepared polymer evaluations. Table 12

summarizes the selectivity information for the evaluation of avermectin and eprinomectin on these polymers in acetonitrile containing different amounts of acetic acid (0, 0.05% and 0.1%).

Table 12. Comparison of retention of avermectin and eprinomectin on the control and test polymers covalently prepared with no extra MAA added during polymerization.

Mobile Phase	**t'r(avm) control**	**t'r(avm) test**	**k'(avm) control**	**k'(avm) test**	**α(avm) test/control**
100% acetonitrile	0.20	0.43	0.12	0.23	1.92
0.05% acetic acid in acetonitrile	0.21	0.35	0.12	0.18	1.50
0.1% acetic acid in acetonitrile	0.25	0.72	0.15	0.40	2.67
	t'r(epr) control	**t'r(epr) test**	**k'(epr) control**	**k'(epr) test**	**α(epr) test/control**
100% acetonitrile	0.27	0.72	0.15	0.39	2.60
0.05% acetic acid in acetonitrile	0.29	0.63	0.18	0.33	1.83
0.1% acetic acid in acetonitrile	0.36	1.44	0.21	0.79	3.76

Results show that selectivity for avermectin in the test column versus control was best in acetonitrile containing 0.1% acetic acid, which is similar to the result seen for the non-covalently prepared polymers. Compared with the non-covalently prepared polymers in the same or similar mobile phase, the selectivity of this covalently-prepared polymer for avermectin ($\alpha = 2.67$) is better than two of the non-covalently prepared polymers, one prepared with MAA in chloroform ($\alpha = 2.43$, Table 5 - 0.05% acetic acid in acetonitrile (evaluation in 0.1% was not performed)) and one prepared with acrylamide in acetonitrile ($\alpha = 1.55$, Table 9 - 0.1% acetic acid in acetonitrile). However, the selectivity is not as good as the non-covalently prepared polymer made with MAA in acetonitrile ($\alpha = 8.39$, Table 7). Figures 50 and 51 show the chromatograms of the solution of avermectin with acetone and toluene in a mobile phase of 0.1% acetic acid

on both the control and test columns, respectively. Only slight retention of avermectin can be seen in the test column compared with the non-retained acetone.

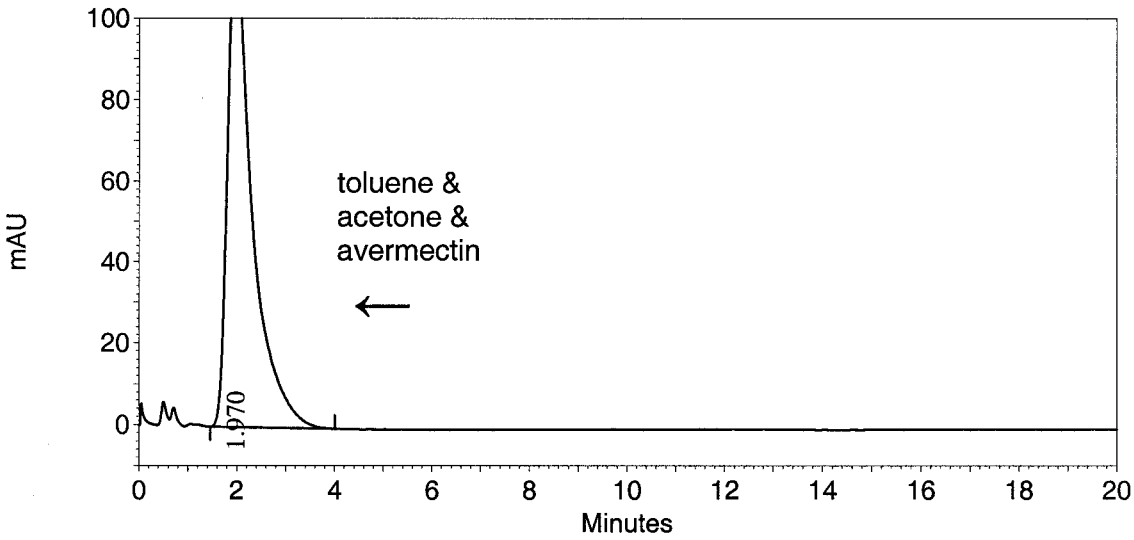


Figure 50. Chromatogram of a solution of 125 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in mobile phase of 0.1% acetic acid in acetonitrile, evaluated on the control column.

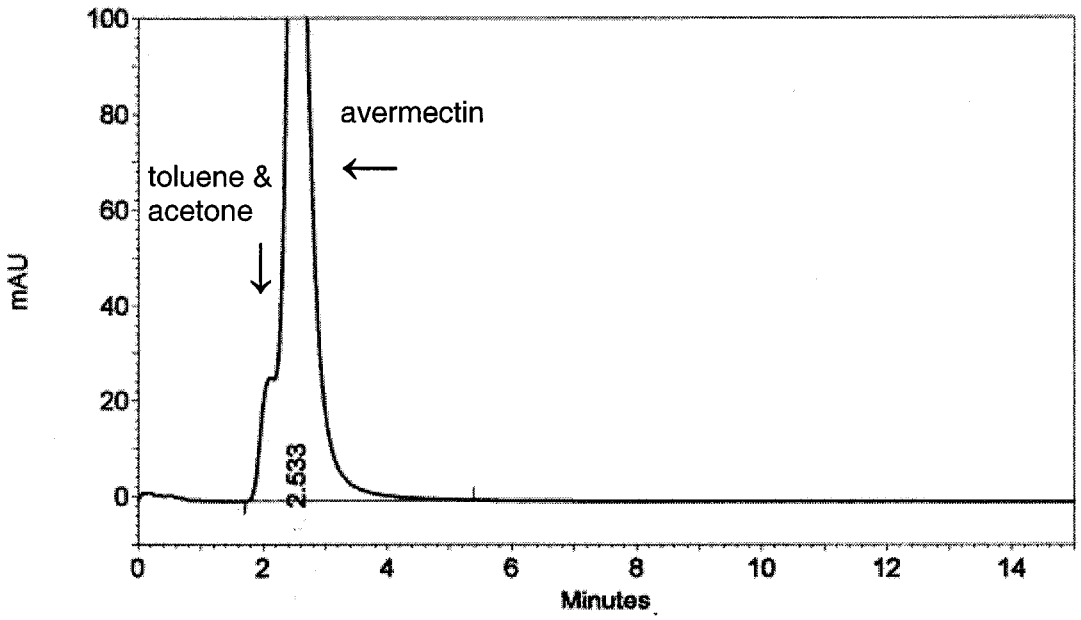


Figure 51. Chromatogram of a solution of 125 µg/mL avermectin, 0.1% acetone, and 0.01% toluene in mobile phase of 0.1% acetic acid in acetonitrile, evaluated on the test column.

Although the separation of avermectin on this covalently prepared polymer is not as good as on the best non-covalently prepared polymer, by comparing Figure 51 and Figure 17, it can be seen that the avermectin peak in the covalently prepared polymer appears to be less broad and has less tailing (tailing factor = 1.2) than the avermectin peak on the non-covalent test polymer with approximately the same selectivity (Figure 17, tailing factor = 4.2). The use of covalent imprinting to reduce peak broadening and tailing has been reported in the literature.[74] The greater peak tailing seen in the non-covalent polymer is most likely due to more than one binding possibility due to heterogeneous binding sites as a result of random polymerization. The covalently prepared polymer, on the other hand, is believed to have more homogenous cavities for binding the avermectin, resulting in more homogenous movement of the avermectin through the column, reducing the peak tailing that is observed when using the non-covalent polymer.

In the covalently-prepared polymer, the selectivity for eprinomectin was slightly greater than for avermectin, which was not observed in the previous studies, although longer retention of eprinomectin than avermectin on the non-covalent test polymers was common. This indicates that eprinomectin fits into the cavities formed by the avermectin during polymerization, and the hydrogen bonding interaction that causes retention is slightly stronger in eprinomectin than in avermectin.

Retention of avermectin and eprinomectin was also evaluated in acetonitrile containing increasing portions of 2 mM aqueous phosphate buffer. Figure 52 is a graph of the selectivities (α) for avermectin and eprinomectin versus the amount of water in the mobile phase.

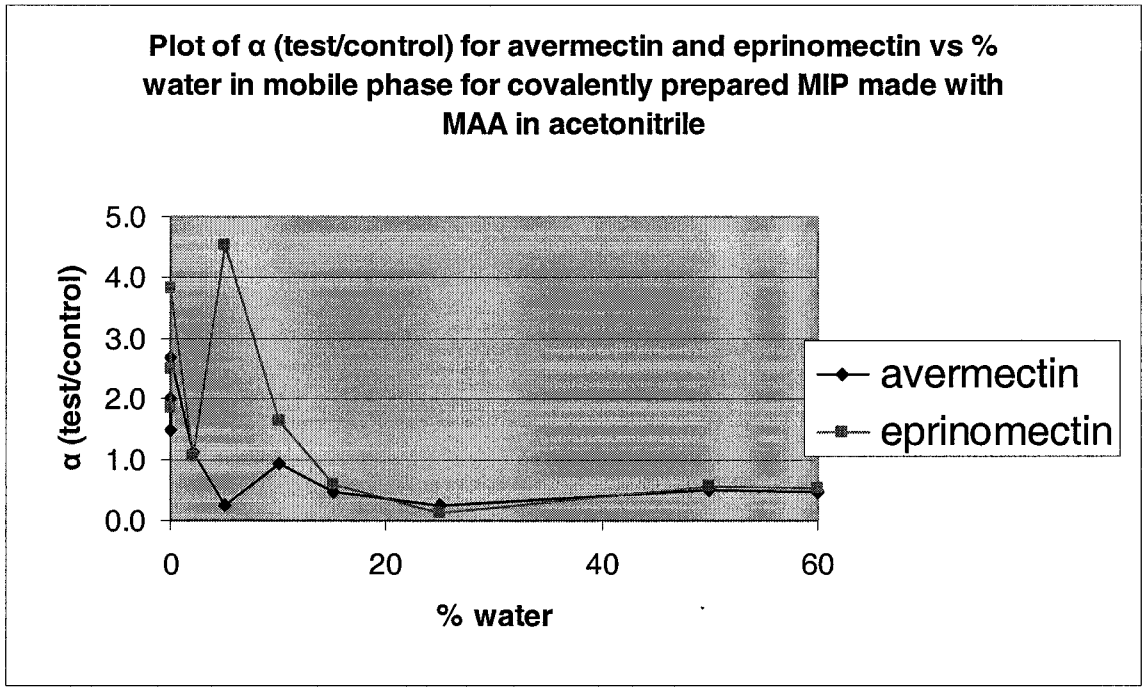


Figure 52. Plot of α values for avermectin (diamonds) and eprinomectin (squares) on the test versus control columns covalently prepared with avermectin-methacrylate with no excess MAA added during polymerization, for increasing levels of 2 mM aqueous phosphate buffer in acetonitrile mobile phase. (0% water data point was run in 0.1% acetic acid in acetonitrile).

Results show that other than a slight increase in the selectivity for eprinomectin at a water content of 5%, the selectivity of the polymer for avermectin did not improve as water was added to the mobile phase Although there appears to be an improvement in the selectivity for eprinomectin at 5% water, the graph is misleading due to the very small capacity factor (k') values used for calculating the values for α for this polymer. The result of 4.5 seen at 5% is based upon k'(epr) values of ~0.15/~0.033 (test/control) = 4.5. These numbers are too small to be reliable, and any small change results in a large change in the α value. Overall, results indicate that a hydrophobic effect is not significant and cannot be used to improve the performance of this polymer for recognition of avermectin.

Preparation of Second Covalently Prepared Imprinted Polymer, using Excess MAA

Because the first covalently prepared polymer did not exhibit selectivity as good as the non-covalently prepared polymer synthesized with MAA in acetonitrile, a second covalently prepared polymer was synthesized. In the most selective non-covalently prepared polymer, the ratio of template to monomer to crosslinker was 1:15:40. In the first covalently prepared polymer, the ratio of avermectin:monomer:crosslinker was effectively 1:1:40 because only half of the avermectin was removed from the polymer during reflux, and half remained associated with the monomer in the polymer matrix. To improve the selectivity of the polymer, a second covalently-prepared polymer was synthesized using the same avermectin-methacrylate template that was used during the first preparation, but with additional methacrylic acid monomer added to the polymerization mix. Although a potential disadvantage of adding extra MAA during polymerization may be a less precise fit of the avermectin in the more "open" polymer, in theory, the presence of extra MAA monomer in the polymer network could potentially improve recognition by forming hydrogen bonds with other functional groups on the avermectin, such as the ether groups, during polymerization. The position of these extra MAA groups in the polymer around the cavity after the removal of the avermectin by reflux could assist in rebinding the molecule during later recognition. Another potential benefit of adding extra MAA in the polymer is the possibility of improving the removal of avermectin from the polymer during refluxing due to a less crosslinked, more "open" polymer network.

To evaluate this potential improvement in selectivity, fourteen equivalents of "free" MAA were added during polymerization to obtain an effective molar ratio of 1:15:40 avermectin:monomer:crosslinker, which is similar to the ratio in the preparation

of the most selective non-covalently prepared polymer. The control polymer was prepared in the same ratio with one equivalent of MAA added in place of the avermectin-methacrylate during polymerization.

FTIR for Confirmation of Template Removal from Polymer

After preparation, grinding and refluxing of the second covalently prepared polymer, the removal of the avermectin was evaluated by FTIR to determine the percent removal of avermectin from the test polymer. Figure 53 shows the FTIR spectra from 1002 to 969 cm^{-1} of the test polymer after refluxing and of the control polymer spiked with increasing amounts of avermectin. The spectra show that about 15% of the avermectin used to prepare the test polymer remained after refluxing, leaving 85% of the sites in the test polymer matrix accessible for rebinding. This improvement in avermectin removal essentially results in a polymer prepared at an effective ratio close to 2:16:40, because most of the avermectin template was removed compared with 50% removal in the first covalently prepared polymer. The additional MAA resulted in a more "open" polymer that allowed better removal of the template compared with the polymer with no additional monomer. Because the crosslinker is essentially diluted by added free MAA, the polymer network may be less compact, making the avermectin more accessible for degradation during refluxing. This resulted in better removal of avermectin.

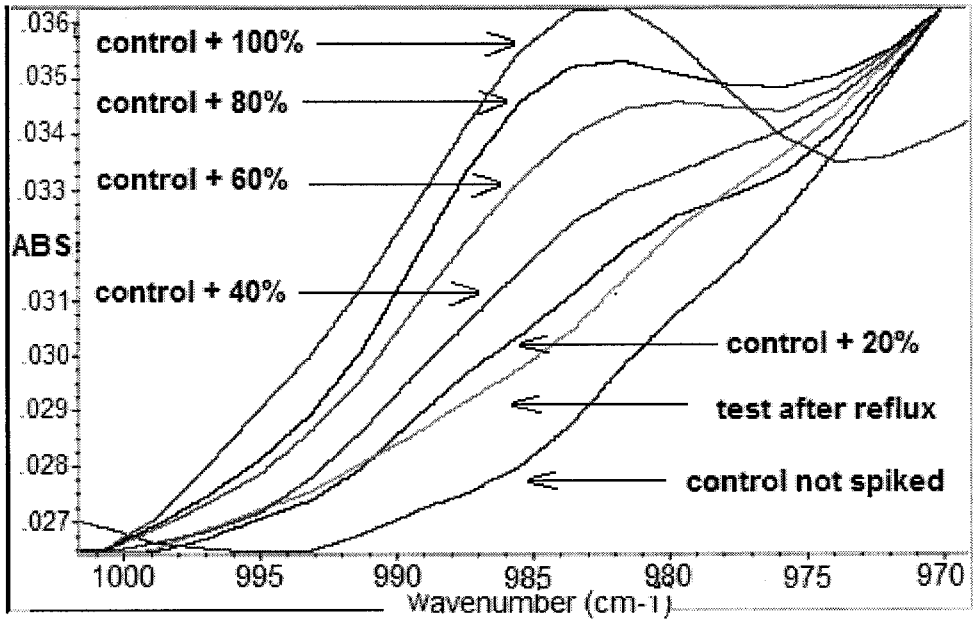


Figure 53. At 983 cm^{-1}, from top to bottom: control + 100% (control polymer spiked with avermectin at 100% of the mole ratio of avermectin-methacrylate used to make the test polymer), control + 80%, control + 60%, control + 40%, control + 20%, test polymer after reflux, control polymer not spiked.

Chromatographic Evaluation of the Second Covalently Prepared Polymer

Evaluation of the selectivity of the second covalently prepared polymer was performed using a mobile phase of 100% acetonitrile and acetonitrile containing 0.1% acetic acid. Table 13 summarizes the selectivity data for evaluation of avermectin and eprinomectin on this set of covalently prepared polymers.

Table 13. Comparison of retention of avermectin and eprinomectin on the control and test polymers covalently prepared with an effective ratio of 1:16:40 avermectin-methacrylate:MAA:EGDMA.

Mobile Phase	t'r(avm) control	t'r(avm) test	k'(avm) control	k'(avm) test	α(avm) test/control
100% acetonitrile	0.67	1.44	0.37	0.69	1.86
0.1% acetic acid in acetonitrile	0.63	1.44	0.35	0.70	2.00
	t'r(epr) control	**t'r(epr) test**	**k'(epr) control**	**k'(epr) test**	**α(epr) test/control**
100% acetonitrile	2.33	4.93	1.29	2.39	1.85
0.1% acetic acid in acetonitrile	2.13	4.51	1.18	2.19	1.86

Although expected to demonstrate better selectivity than the first covalently prepared polymer due to the potential increase in available binding sites, selectivity of the polymer for avermectin was slightly less (α = 2.00 in 0.1% acetic acid in acetonitrile) than the first covalently prepared polymer (α = 2.67 in 0.1% acetic acid in acetonitrile). The extra MAA in the polymer did increase the retention of avermectin in the second covalently prepared polymer, but retention was increased in both the control and test polymers, which does not improve the selectivity of the imprinted polymer for avermectin.

Compared with the non-covalently prepared polymer using MAA in acetonitrile (Table 7), although the k' of the control polymers for avermectin are essentially the same, the selectivity of the covalently-prepared polymer is much less than the selectivity of the non-covalently prepared polymer. This indicates that the nonselective interactions that cause avermectin to be retained slightly on the control polymer are similar in the two types of preparations, but the selective interaction that causes retention of avermectin on the imprinted polymer is much better when the polymer is prepared non-covalently.

This is supported by the k' values for eprinomectin evaluated on the second covalently prepared test and control polymers, which are essentially the same on both

the non-covalently (Table 7) and covalently (Table 13) prepared polymers. Because retention of eprinomectin is due primarily to nonselective interactions on both columns, and because these interactions are similar on both covalently and non-covalently prepared columns, the α values for eprinomectin on both types of imprinted polymers are very similar.

Retention of avermectin and eprinomectin were evaluated in acetonitrile containing increasing portions of 2 mM aqueous phosphate buffer. Figure 54 is a graph of the selectivity (α) for avermectin and eprinomectin on the test versus control columns made from this covalently prepared polymer. Results show a decline in selectivity of the polymer as the aqueous portion was increased. The selectivity for this polymer declines further than the decline that is seen in the non-covalently prepared polymer (Figure 39) as water content of the mobile phase is increased. This indicates that even though the arrangement of the avermectin and monomer during polymerization is more rigid and controlled than in the non-covalent preparation, because the recognition of the imprinted polymer for avermectin relies on hydrogen bonding of the avermectin to the polymer in both cases, the presence of water overwhelms the hydrogen bonding capability of the imprinted polymers, preventing selective recognition of both types of polymers for avermectin based on hydrogen bonding alone. As a result, the covalently prepared imprinted polymers do not provide any improvement for recognition of avermectin in an aqueous environment compared with the non-covalently prepared polymers evaluated thus far.

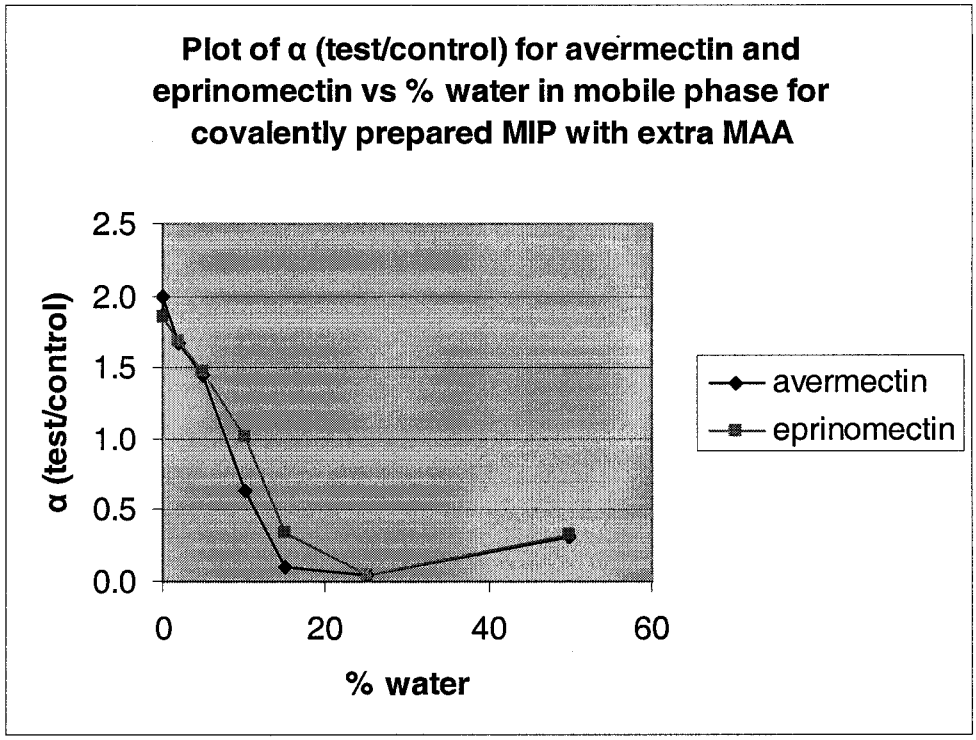


Figure 54. Plot of α values for avermectin (diamonds) and eprinomectin (squares) on the test versus control columns made covalently with avermectin-methacrylate template:MAA:crosslinker in a ratio of 1:15:40, for increasing levels of 2 mM aqueous phosphate buffer in acetonitrile mobile phase. (0% water data point was run in 0.1% acetic acid in acetonitrile).

Solid Phase Extraction Studies of Non-Covalently Prepared Polymer Synthesized with Methacrylic Acid Monomer in Acetonitrile

Thus far it has been shown that MIPs for avermectin can be prepared either non-covalently using only weak hydrogen bonding in the imprinting process, or covalently, using covalent bonding during imprinting, and hydrogen bonding during recognition. The best imprint prepared for avermectin was the polymer prepared non-covalently with MAA monomer. The most selectivity ($\alpha > 18.0$) was observed when the polymer was prepared in chloroform and evaluated in 0.05% acetic acid in chloroform. However, to use the polymer in an aqueous medium, mobile phase containing aqueous

buffer was required, and the polymer prepared in acetonitrile and evaluated in acetonitrile (α = 8.3) performed better than the polymer prepared in chloroform and evaluated in acetonitrile (α = 2.4).

The final goal of this study was to use the most selective polymer in an aqueous medium for development of a solid phase extraction (SPE) method to determine its potential as a practical method for evaluation of avermectin in aqueous samples. Solid Phase Extraction is routinely used to preconcentrate analytes of interest at low concentrations in complex matrices and often to change the solvent from aqueous to organic before chromatographic analysis.[75,76] The use of an MIP in this application would provide a very practical method for the analysis of avermectin in aqueous samples. To evaluate this potential, the imprinted polymer which gave the best selectivity for avermectin in a partially aqueous mobile phase was the polymer prepared non-covalently using methacrylic acid as the monomer and acetonitrile as the solvent. The polymers (imprinted and control) were packed into SPE cartridges to evaluate the ability to selectively bind avermectin for the separation and concentration from an aqueous sample. In general, the procedure used was to load the avermectin onto the cartridges, where it would be selectivity retained on the imprinted polymer. The remainder of the sample was washed off the polymer, and the avermectin was then eluted and collected for analysis by HPLC.

Six attempts were made to develop a loading, washing and eluting scheme to selectively isolate and concentrate avermectin from a sample containing water using only an MIP with no additional extraction step. During the first evaluation, both the test and control cartridges were first conditioned with acetonitrile, followed by elution with a solution of 10% 2 mM aqueous phosphate buffer in acetonitrile, followed by 100% acetonitrile. A solution of avermectin (12.0 µg) in 25% acetonitrile in water was then

loaded onto the control and test cartridges. The cartridges were washed with 100% water, which according to theory would elute any polar impurities in the sample while allowing the avermectin to be retained on the test cartridge.[77] The sample was eluted with 10% 2 mM aqueous phosphate buffer in acetonitrile, because this is the solution which eluted avermectin quickly during the HPLC studies. The final wash was 100% acetonitrile. Fractions were collected during each step of the process and analyzed for avermectin (Table 14 and Trial #1 in Table 16).

Table 14. Results from the first trial to develop a method to use SPE to retain and concentrate avermectin from an aqueous sample. Water is used as the initial wash solvent.

Step	**Control (µg avermectin collected)**	**Test (µg avermectin collected)**
Condition	0.0	0.6
Load	0.0	0.0
Initial Wash	0.0	0.0
Elute	10.8	17.9
Final Wash	0.0	1.4
Total % Recovered	90.0 %	160.5 %

Results show that because avermectin was found in the conditioning and final wash steps from the test cartridge, but not from the control, the test polymer was not sufficiently washed before loading the avermectin, and avermectin remaining from the preparation step was eluting. This is supported by an avermectin recovery of 160.5% from the test column, compared with only 90.0% from the control column. The test cartridge was thoroughly washed until no avermectin was detected before it was used for more studies.

Results also showed that washing the cartridges with 100% water did selectively retain avermectin on the imprinted polymer column. This is not surprising based on the earlier chromatographic studies in which the retention of avermectin on both the test and

control polymers increased as the water in the mobile phase increased (graphs of these results are not shown, but are similar to Figures 22 and 23 for the polymer prepared in chloroform). Because the retention was similar in both the control and test polymer columns, the retention was non-selective, probably due to hydrophobic interactions. It was also determined based on the chromatography studies that elution with 10% 2 mM aqueous phosphate buffer in acetonitrile removed all the avermectin from both cartridges.

A second evaluation was performed to determine if a different wash solution would improve the selectivity of the imprinted polymer. To decrease the retention of avermectin on the control column due to non-selective interactions, while continuing to retain avermectin on the imprinted polymer, the use of a solvent with high elution strength to wash the cartridges after sample loading was investigated. In this SPE study, 20.1 µg of avermectin was loaded onto the control and test cartridges in 1% acetonitrile in water to more closely resemble the matrix of a typical sample to be analyzed. Theoretically, this should promote binding of the avermectin to the MIP particles and 'stick' the avermectin to both control and test columns non-selectively by hydrophobic interactions, since the avermectin is not water soluble. The initial wash was changed to several column volumes of 100% acetonitrile to elute the avermectin from the control cartridge and to remove any non-specific binding of unwanted compounds, while allowing the avermectin to be selectively retained on the test cartridge. Elution was again performed with 10% 2 mM aqueous phosphate buffer in acetonitrile because this solution is strong enough to break the non-covalent bonds that are responsible for binding the avermectin to the polymer and to elute the avermectin as it did in the HPLC studies. The final wash was 100% acetonitrile. Results of analyses of the fractions

collected during each step of the process are shown in Table 15 (and Trial #2 in Table 16).

Table 15. Results from the second trial to develop a method to use SPE to retain and concentrate avermectin from an aqueous sample. Acetonitrile is used as the initial wash solvent.

Step	Control (µg avermectin collected)	Test (µg avermectin collected)
Condition	0.0	0.0
Load	0.0	0.0
Initial Wash	14.0	12.8
Elute	0.1	5.7
Final Wash	0.0	0.0
Total % Recovered	69.9 %	91.8 %

Although no avermectin passed though either column during the loading step, as anticipated, most of the avermectin was eluted from both columns during the initial wash with 100% acetonitrile, which should have only washed the avermectin from the control column. Only a small amount of avermectin remained on the test column for removal during the elution step. This indicates that the acetonitrile wash solution was too strong and overcame the hydrogen bonds holding the avermectin to the imprinted polymer.

Because the loading step was successful at allowing non-selective hydrophobic interactions to retain the avermectin on both columns, this step was repeated in the following trials, but the wash was changed to a less polar solvent (THF) to help selective retention by increasing the more polar interactions, namely the hydrogen bonding of avermectin to the test MIP but not to the control polymer. Elution was repeated with 10% aqueous buffer in acetonitrile. Results (Trial #3 in Table 16) showed that although recoveries were low (40.3% control, 63.3% test), no avermectin was detected in any step other than the initial wash step, indicating that the wash solution removed everything from both columns, preventing selective non-covalent bonding of the

avermectin to the imprinted polymer, which is similar to what was observed when acetonitrile was used as the wash solvent.

Because washing the loaded cartridges with 100% water did not remove any avermectin from either column, and 100% acetonitrile removed all the avermectin from both columns, a wash solvent of acetonitrile containing just enough water to retain the avermectin should be sufficient as the wash solvent. When a mixture of 25% water in acetonitrile was used for the initial wash, all avermectin was eluted from both cartridges during the initial wash step (Trial #4, Table 16; recoveries were 62.9% control and 61.4% test). This is similar to the results when 100% acetonitrile or 100% THF were used for the initial washes.

Because this combination of acetonitrile and water was still too strong and disrupted the non-covalent interactions that should hold the avermectin in the imprinted polymer, a study using a mixture of 75% water in acetonitrile was used for the initial wash to find a wash solution that was sufficient to promote selective binding of avermectin to the imprinted polymer but not to the control. Results (Trial #5 Table 16) indicate that no avermectin was removed from either the control or test cartridge during the initial wash step, but all the avermectin was eluted from both columns during the elution step with 10% 2 mM aqueous phosphate buffer in acetonitrile (recoveries were 69.9% control and 83.3% test). This indicates that non-selective binding of avermectin to both polymers occurred in the wash solution, probably due to overwhelming hydrophobic interactions. This was also observed when 100% water was used as the wash solution (Trial #1).

Instead of continuing with trial and error studies to determine the combination of acetonitrile and water that might be sufficient for a wash solution, an evaluation was performed to find a more suitable solvent mixture for the wash step. A solution of 50%

toluene in acetonitrile was used for the wash step, and the sample was eluted with 100% acetonitrile to prevent immiscibility with the toluene. Results (Trial #6 Table 16) show that all avermectin was washed from both columns during the wash step, indicating that the wash solution was again too strong to maintain the selective non-covalent interactions to selectively bond the avermectin to the test polymer (recoveries were 80.7% control and ≥68.4% test).

Table 16. Summary of SPE studies performed on control and test polymer prepared with MAA in acetonitrile. Elution condition was 10% aqueous 2 mM phosphate buffer in acetonitrile for all trials.

Trial #	Loading Condition	Washing Condition	Step in which avermectin was collected (Control)	Step in which avermectin was collected (Test)
1	25% acetonitrile in water	100% water	100% elution	100% elution
2	1% acetonitrile in water	100% acetonitrile	99.3% wash 0.7% elution	69.2% wash 30.8% elution
3	1% acetonitrile in water	100% THF	100% wash	100% wash
4	1% acetonitrile in water	25% water in acetonitrile	100% wash	100% wash
5	1% acetonitrile in water	75% water in acetonitrile	100% elution	100% elution
6	1% acetonitrile in water	50% toluene in acetonitrile	100% wash	100% wash

In summary, loading the avermectin in 1% acetonitrile in water is sufficient to bond avermectin non-selectively to both columns. Washing the columns with either 75% water (Trial #5) or 100% water (Trial #1, polarity index of water is 10.2) did not remove avermectin from either column during the wash step, indicating that the hydrophobic interactions were too strong to allow removal of the avermectin from the control column during washing. Washing with less polar acetonitrile (Trial #2, polarity index of acetonitrile is 5.8) improved the use of the SPE cartridges, causing most of the avermectin to be washed from the control column in the wash step, but only 69.2% of

the avermectin to be washed from the test column, indicating that acetonitrile provides some selective retention of avermectin in the test polymer indicated by the small amount of analyte still remaining after the wash and eluted during the elution step. However, results suggest that 100% acetonitrile is too polar and disrupts some of the hydrogen bonding of avermectin to the imprinted polymer. This suggests that washing with a less polar solvent should improve the selective retention of avermectin on the test column. However, when washing with less polar THF (Trial #3, polarity index of THF is 4.0), all avermectin was removed from both columns with the wash and no specific binding of the avermectin to the test column is indicated. These results appear to be the reverse of what was observed during chromatography, in which a less polar solvent increased retention of avermectin and the addition of water decreased retention. In the SPE studies, washing with a less polar solvent decreased retention and washing with a more polar water solution increased retention.

The difference in the two types of studies was in the environment of the polymers in the HPLC columns versus the SPE cartridges. In the HPLC evaluations, the flow of the eluent was continuous over the polymer, and the sample was injected into this environment which was continually refreshed. Therefore, in non-polar solvent, the binding sites were available to hydrogen bond with the sample as it passed over the test polymer, and this binding was stronger in non-polar solvents than in polar solvents for reasons explained previously. In the test polymer used in the SPE studies, the environment of the binding sites when the avermectin was introduced to the polymer was quite different. The final wash of the polymers before loading the sample was with acetonitrile, but the sample was loaded in a 99% water matrix containing 1% acetonitrile. This sample matrix was chosen to resemble a practical sample that would be evaluated using this imprinted polymer. In this situation, the environment of the test polymer

binding sites is saturated with water at the same time as the much lower level of avermectin is introduced to the polymer. The presence of water significantly lowers the effectiveness of the hydrogen bonding between the analyte and polymer receptor site due to competition for hydrogen bond donor and acceptor sites. As a result, in Trials #2, #3, #4, and #6, in which the sample is loaded in 99% water, the retention of avermectin is very small, and it eluted with the wash due to very little interaction with the imprinted sites, although there was a small amount of retention in the test column of Trial #2. In Trials #1 and #5, although the sample is loaded in an aqueous matrix (75% for Trial #1, and 99% for Trial #5), the higher level of water in the wash solution did result in retention of the avermectin on the polymer until eluted with 10% 2 mM aqueous phosphate buffer in acetonitrile. As in the HPLC studies, the increase in water concentration to levels well above 25% resulted in increased retention of the avermectin by both the control and test polymers due to hydrophobic interactions (see Figures 22 and 23).

Although more research could be pursued based upon the trial which showed partial success (Table 15), further trial and error studies to develop a method for direct extraction of avermectin from aqueous samples were not carried out. However, results from the chromatographic studies indicate that MIP could successfully be used for SPE of avermectin if the avermectin were first extracted from the aqueous sample with chloroform. Although this would provide a successful application of the imprinted polymer to SPE of avermectin in aqueous samples, an extra extraction step using chloroform would be required.

Improvement in the selectivity of either a non-covalently or covalently prepared polymer for avermectin in an aqueous medium would improve the development of an SPE method for the direct binding of avermectin in an aqueous medium. Evaluation of

different monomers or monomer-combinations, crosslinkers, and polymerization solvents for the synthesis of a non-covalently imprinted polymer with improved selectivity would make the application of this technique more practical. Synthesis of a covalently prepared polymer with the avermectin esterified at the 4" carbon, or both the 4" and 5 carbons, may also improve the selectivity of a polymer for avermectin which could then be used in an aqueous medium.

Conclusions

A very successful non-covalently imprinted polymer was prepared for avermectin using methacrylic acid monomer, ethylene glycol dimethacrylate crosslinker, 2,2"-azobisisobutyronitrile initiator, chloroform as the solvent for polymerization, and chloroform containing 0.05% acetic acid for chromatographic analysis. The imprinted polymer exhibited at least 18 times more selectivity for avermectin than a non-imprinted polymer. Selectivity of this imprinted polymer for eprinomectin, a compound with a similar structure, was only 3.0 compared with the non-imprinted polymer. Selectivity of the polymer for avermectin decreased to 2.4 when using acetonitrile with 0.05% acetic acid as the mobile phase. However, preparing a polymer using acetonitrile as the solvent for polymerization and acetonitrile containing 0.1% acetic acid for chromatographic analysis, demonstrated selectivity for avermectin of 8.4 (versus 1.9 for eprinomectin). Preparation of a polymer using acrylamide monomer resulted in a polymer with less selectivity for avermectin than the polymers prepared with MAA. Addition of aqueous buffer to the mobile phase further decreased selectivity. Preparation of two covalently prepared polymers, one with no extra MAA added during polymerization, and one with excess MAA added during polymerization resulted in

imprinted polymers with less selectivity for avermectin than the most successful non-covalently prepared polymer. Using the most selective polymer prepared in a water-compatible solvent (acetonitrile), method development for a solid phase extraction for the isolation of avermectin from an aqueous sample was evaluated. A successful method for the selective isolation of avermectin from an aqueous sample on the imprinted polymer was not fully developed.

Results of the research show that a very successful imprinted polymer for a large, complex molecule can be synthesized. Polymerization in a non-polar solvent (chloroform) and analysis in the same solvent containing a small amount of acetic acid provides the best selectivity for the large water-insoluble analyte. In chloroform mobile phase, the amount of acetic acid added to the mobile phase has a large effect on the selectivity of the polymer. For use in an aqueous medium, preparation of the imprinted polymer in a more polar polymerization solvent (acetonitrile) provides better selectivity than a polymer prepared in a non-polar solvent. In acetonitrile mobile phase, the amount of acetic acid added to the mobile phase has very little effect on the selectivity of the polymer. The addition of a small amount of water (2% to 5%) to the solvent for chromatographic analysis rapidly reduces the selectivity of the imprinted polymers which rely on hydrogen bonding for recognition of the analyte. When the water content of the mobile phase is greater than 25%, hydrophobic interactions increase the retention of the water insoluble analyte, but retention is increased on both control and imprinted polymers, which does not improve the selectivity of the imprinted polymer for the analyte.

In MIPs prepared covalently, followed by non-covalent recognition of the analyte, peak shape during chromatographic analysis improves. However, covalent preparation of an imprinted polymer for a large water-insoluble analyte does not improve the

selectivity of the imprinted polymer for the analyte when recognition is based on hydrogen bonding between the analyte and the imprinted polymer.

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Vita

Lou Ann Tom (née Miller) was born to William Kenneth Miller and Margaret Louise Miller (née Peters). After graduating from Montoursville High School, she obtained a Bachelor's of Arts degree from Lycoming College, in Williamsport PA, in 1987, with a double major in Chemistry and Biology. She graduated Summa Cum Laude with Honors, and received the following Honors: Outstanding Senior Chemistry Major of the Year, The John C. McCune Memorial Prize in Chemistry, The Byron C. Brunstetter Science Award, Certificate of Achievement in recognition of successful completion of requirements for Lycoming Scholars, Certificate of Achievement in recognition of outstanding academic achievement during senior year and Dean's List, Phi Kappa Phi National Honor Society, and achieved the Dean's list every semester. Other activities included Lycoming Scholars Program, Beta Beta Beta National Biology Honor Society (Treasurer 1987), Chemistry Advisory Committee, Phi Sigma Tau National Philosophy Honor Society, and Gold Key Freshman Scholastic Honor Society.

Following graduation, Lou Ann began working as a Laboratory Technician at Merck & Co., Inc in Danville, PA in 1987, and continued in positions of increasing responsibility to the current position of Senior Scientist within the Technical Operations Department.

While working full-time at Merck, Lou Ann began graduate studies at Bucknell University and received a Master's of Science Degree in 1994, with a major in Chemistry (Masters Thesis: Solvent Isotope Effects on the Reduction of Lipoxygenase by Alkylhydroxylamines).

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