INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior de Tecnologia e Gestão

Molecular Imprinting and Grafting of N-acetylneuraminic Acid in Synthetic Polymers for Virus Targeting

VERÔNICA TEIXEIRA NORONHA

Final Report of the Work Project presented to

Escola Superior de Tecnologia e Gestão

Instituto Politécnico de Bragança

To obtain the Master's Degree in

Chemical Engineering

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Supervisor

Prof. Dr. Rolando Carlos Pereira Simões Dias

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“Tu deviens responsable pour
toujours de ce que tu as apprivoisé.”
Antoine de Saint-Exupéry

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Abstract

In this work, the synthesis of Molecularly Imprinted Polymers (MIPs) was executed using different solvents, N,N-dimethylformamide (DMF), Acetonitrile (ACN), and a mixture of both reagents, two different monomers, 4-vinylpiridine (4VP) and 4-Vinylphenilboronic acid (4VBA), and different concentrations of solutions for the template, N-acetylneuraminic acid, in order to evaluate which conditions would lead to a final synthetic material with higher retentions of N-acetylneuraminic acid.

The characterization of the synthesised MIPs was performed beginning with batch adsorption, allowing the material to react and adsorb the target molecule until reaching an equilibrium. After two methods were chosen to quantify the N-acetylneuraminic acid, to evaluate the retention of this molecules for each of the MIPs.

The methods used were derivatisation with thiobarbituric acid (TBA) followed by ultraviolet (UV) spectroscopy and high-performance liquid chromatography (HPLC). MIPs were evaluated under different N-acetylneuraminic acid concentrations and different pH conditions.

It was concluded that high-performance liquid chromatography (HPLC) was the most reliable and efficient method to quantify N-acetylneuraminic acid, once derivatization with thiobarbituric acid approach has various steps that could be error prone. In addition, MIPs produced with 4-vinylpiridine showed a higher N-acetylneuraminic acid adsorption in the analysed conditions. Furthermore, MIP synthesised using only N,N-dimethylformamide as solvent, and N-acetylneuraminic acid in a concentration of 0.06 mol/L as a template, exhibited a higher retention of this compound, specially at acid pHs.

Keywords: *Molecularly Imprinted Polymers; N-acetylneuraminic acid; Molecular Recognition; Thiobarbituric Acid Method.*

Resumo

No presente trabalho, a síntese de Polímeros Molecularmente Impressos foi realizada usando diferentes solventes, nomeadamente N,N-dimetillformamida (DMF), acetonitrila (ACN) e uma mistura de ambos reagentes, dois monômeros distintos, 4-vinilpiridina (4VP) e ácido 4-Vinilfenilboronico (4VBA), e soluções de diferentes concentrações do "template", ácido N-acetilneuraminico, com o objetivo de avaliar quais condições levariam a um material sintético final com retenções de ácido N-acetilneuraminico mais elevadas.

A caracterização dos MIPs sintetizados foi executada iniciando com a adsorção em batch, de modo a permitir que o material reagisse e adsorvesse a molécula alvo até atingir o equilíbrio. Posteriormente, dois métodos foram escolhidos para quantificar o ácido N-acetilneuraminico, para avaliar a retenção destas moléculas por cada um dos MIPs.

Os métodos usados foram a derivatização com ácido tiobarbitúrico (TBA) seguida de espectroscopia de ultravioleta (UV) e cromatografia líquida de alta eficiência (CLAE). Os MIPs foram avaliados sob diferentes concentrações de ácido N-acetilneuraminico e diferentes condições de pH.

Foi concluído que a cromatografia de alta eficiência (CLAE), foi o método mais confiável e eficiente para quantificar o ácido N-acetilneuraminico, uma vez que a derivatização com ácido tiobarbitúrico possui muitas etapas, que podem ser passíveis de erro. Os MIPs produzidos com 4-vinilpiridina apresentaram uma mais elevada adsorção de ácido siálico nas condições analisadas. Ademais, os MIPs sintetizados usando apenas N,N-dimetilformamida como solvente, e ácido N-acetilneuraminico com concentração de 0.06 mol/L como template, demonstrou uma maior retenção deste composto, especialmente em pHs ácidos.

Palavras-chave: *Polímeros Molecularmente Impressos; Ácido N-acetilneuraminico; Reconhecimento Molecular; Método de Ácido Tiobarbitúrico.*

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LIST OF ABBREVIATIONS AND SYMBOLS

4VBA	4-vinylphenylboronic acid
2-VP	2-vinylpyridine
4-VP	4-vinylpyridine
AA	Acrylic acid
A	Artitrary unit
ACID	4,40-azo(4-cyanovaleric acid)
ACN	Acetonitrile
ADVN	Azobisdimethylvaleronitrile
AIBN	Azobisisobutyronitrile
BDK	Dimethylacetal of benzyl
BPO	Benzoylperoxide
DVB	Divinylbenzene
DMF	N,N-dimethylformamide
EGDMA	Ethylene glycol dimethacrylate
FRP	Free Radical Polymerisation
FTIR	Fourier-Transform Infrared Spectroscopy
HEMA	2-hydroxyethyl methacrylate
HIV	Human immunodeficiency viruses
M	Monomer
MAA	Methacrylic acid
MBAA	N-N-methylenebisacrylamide
MIP/MIPs	Molecularly Imprinted Polymer/Molecularly Imprinted Polymers
Neu5Ac	N-acetylneuraminic acid
R	Radical
RNA	Ribonucleic acid
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SBSE	Stir Bas Sorption Extraction
TEM	Transmission Electron Microscopy

TRIM	Trimethylolpropane trimethacrylate
VIPs	Virus-Imprinted Particles

CHAPTER I

1. INTRODUCTION

Over the last decades, molecular imprinting have been a widely technique used to construct polymer networks for the recognition of molecules [1], [2]. The principle behind this technique is to form polymers with specific chemical structures specifically designed to interact with the template through covalent or noncovalent bonds. These polymers, known as Molecular Imprinted Polymers (MIPs) are capable to recognize specific molecules with a binding and/or delivery behaviour [1].

As a consequence of its stability and low cost of preparation, MIPs have been seen with a wide range of applications, such as catalysis, separation and purifications, drug delivery and detection [2]–[5]. MIPs can also be an alternative for virus targeting as they can be designed to bond specifically with some parts of a molecule, removing it from the system [5].

1.1 Objectives

The two main objectives of this work were to produce and characterize particles of molecularly imprinted polymers and to graft N-acetylneuraminic acid in synthetic polymers. In order to achieve such objectives, it was made a molecular imprinting N-acetylneuraminic acid in crosslinked polymer particles through radical polymerisation and the reactions with hydroxyl and carboxylic functional groups will be considered for grafting N-acetylneuraminic acid in polymers.

CHAPTER II

2. BIBLIOGRAPHIC REVIEW

2.1 History of Molecular Imprinting

The first report of Molecular Imprinted was back in the 1930s when M. V. Polyakov synthesized silica gel and observed that when the silica gel was prepared in the presence of a solvent additive, the molecules produced showed a preferential binding capacity for the same solvent [6]. Later, in 1949, Frank Dickey, a student of Linus Pauling, published a paper showing the results of an experiment made by the production of silica gel in the presence of dyes. This experiment observed that, after the removal of the dye, the produced silica would rebind the same dye used rather than others [7].

More than 20 years later, Wulff reported that he and his group of researchers had prepared a molecularly imprinted organic polymer, using what today is called “covalent approach”, capable of recognizing the enantiomers of glyceric acid. These discoveries resulted in a big step change in the molecular imprinting [8].

Another big step change concerning MIPs was in 1981, when Mosbach and Arshasy reported a production of these molecules using then the “non-covalent approach”, that was shown as a simpler methodology, causing an explosion in the research about Molecular Imprinting in the 1990s [9].

An important study object was the comparison between the covalent and the non-covalent approach and their pros and cons. However, in 1995, Whitcombe et al. reported an approach that combined the two previous approaches, taking the advantages of each of them. Briefly, this new proposal used covalent interactions in the polymerisation and non-covalent interactions in the rebinding. Another innovation of Whitcombe’s approach was the adding of a spacer group, to be lost when the template is removed, in order to improve the binding geometry [10].

2.2 Molecularly Imprinted Molecules

Molecularly Imprinted Molecules (MIPs) are molecules made by the Molecular Imprinting method, in which the polymerisation occurs in the presence of a template in order to create sites with selective recognition in those synthetic polymers [11]. The main aim of MIPs is to create materials with a high selectivity for specific target molecules,

generated in the polymerisation steps, where the chemical interactions made with the matrix and functional groups of the target are memorized and activated after the process. [12].

The scheme of Figure 1 shows the traditional procedure to produce MIPs. This procedure consists in several steps. First, the complexation of functional monomers and target templates is carried, followed by cross-linking of the monomers and finishing with the removal of the templates. After the removal, it is possible to identify adsorption sites that are complementary with shape and size of the templates [13]. The same type of recognition produced in MIPs can be found in biological systems, such as immune responses, where the enzymes recognize foreigner antigen, and as its response, the antibodies are generated [14].

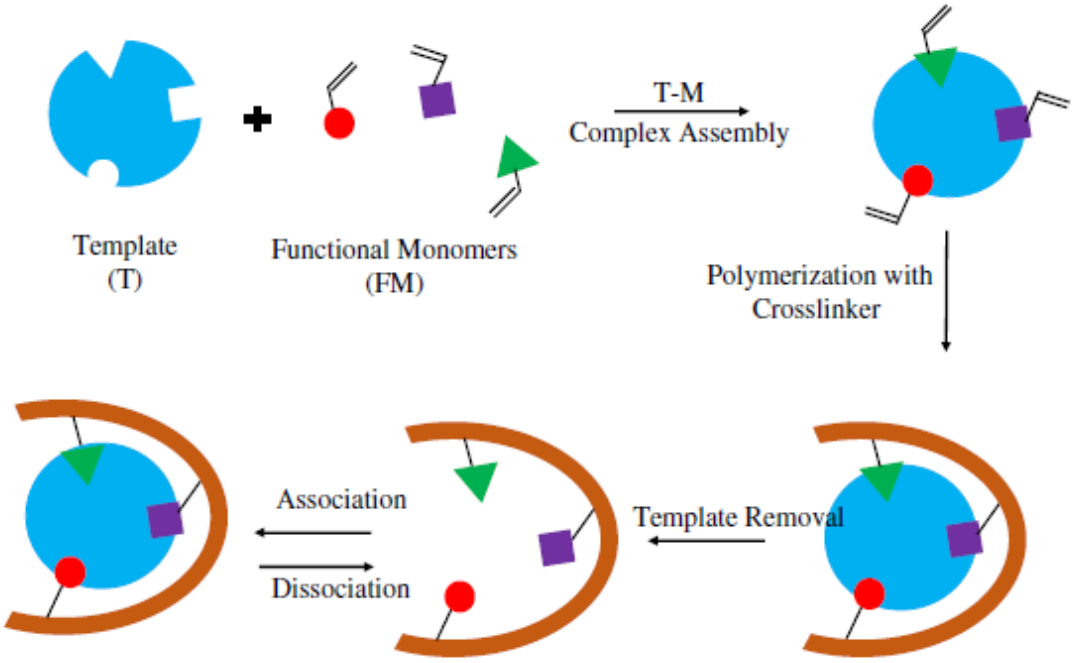


Figure 1 - Graphical illustration of molecular imprinting process.[15]

Molecularly Imprinted Polymers have many characteristics, namely low cost of production, easy synthesis, high stability to harsh chemical and physical conditions and good reusability [16], that lead them to be applied in different fields, for instance catalysis [17]–[19], separation process [20], solid phase extraction [21]-[17], drug delivery systems [23]–[25], binding assays and sensors [26]–[34].

The synthesis of MIPs is a complex process due to the quantity of variables involved in it, specifically the nature and levels of templates, functional monomers, cross-

linkers, solvents and initiators used, as well as the initiation method and the duration time of the polymerisation reaction [35].

When MIPs are evaluated, the called NIPs (Non-Imprinted Polymers) are also produced and taken into account for comparison reasons. For each MIPs synthesized, a NIP is produced with the same reagents following the same steps, but without the presence of the template molecule. The objective is to understand if the MIP has more affinity to bond with the template rather than the NIP.

2.2.1 Components of molecular imprinting

The components used in a classic molecular imprinting process are template, functional monomer, cross-linker, polymerisation initiator and solvent. The choice of the cited elements varies according to the properties wanted in the final product [36].

2.2.1.1 Template

The eventual and greatest objective of the molecular imprinting process is to be able to produce MIPs that have specificity and affinity to a certain type of molecule. In order to achieve those properties, the templates used in the process should contain functional groups that will bond to the functional monomers and form complexes. They also must be chemically stables during the polymerisation reaction and contain in their structures, functional groups that do not prevent the occurrence of the polymerisation reaction [16], [37].

A large variety of molecules has been used as target templates, such as ions (e.g. Pb(II); Sr(II); Hg(II); Cd(II); among others), organic molecules, biomacromolecules, cells and viruses (e.g. tobacco mosaic virus; bovine leukemia virus; dengue virus; gut-homing T) [16], [36], [38].

2.2.1.2 Functional Monomer

Functional monomers have as a role to form a pre-polymerisation complex with the templates, which bind with the functional groups provided by the monomers. For that reason, it is important to choose a functional monomer capable to interact with the template in order to arrange specific donor-receptor (similar to antigen body) preceding to the polymerisation [36]. As a result, the affinity of the MIPs is determined by the strength of the interactions made between the template and the monomer [39], [40].

The suitable selection of the functional monomer is crucial, considering that stronger interactions between the monomer and the template leads to a more stable complex, which results in a higher binding capacity of the produced MIP [16]. To achieve that, many trial-and-error tests are performed and the stability of functional monomer and template complex, analysis such as nuclear magnetic resonance [41]–[44], UV-vis [45], [46], Fourier-transform infrared spectroscopy [47], [48], computer simulation [49] and isothermal titration calorimetry [50] have been performed.

Characteristics as affinity and imprinting efficiency of MIPs are affected by the molar ration between template and monomer during the synthesis process, where lower molar ratios lead to less binding sites in polymers and high molar ratios induce higher non-specific binding capacity [16].

Functional monomers frequently used for molecular imprinting involve methacrylic acid (MAA), acrylic acid (AA), 2-vinylpyridine (2-VP), 4-vinylpyridine (4-VP), acrylamide, trifluoromethacrylic acid and 2-hydroxyethyl methacrylate (HEMA) [51].

2.2.1.3 Cross-linkers

Cross-linkers in MIPs have three main roles. The first is to control the morphology of the polymer matrix, that could be a gel-type, microporous or a microgel power. In second place, cross-linkers stabilise the imprinted binding side and, the third role function is to provide mechanical stability to the polymer matrix [37].

Generally, small quantities of cross-linkers produce MIPs with unstable mechanical properties. On the other hand, higher amounts of cross-linkers result in a diminished number of recognition sites per unit mass of MIPs [36].

The most commonly used cross-linkers for producing MIPs are ethylene glycol dimethacrylate (EGDMA), trimethylolpropane trimethacrylate (TRIM), N-N-methylenebisacrylamide (MBAA) and divinylbenzene (DVB) [16].

2.2.1.4 Solvent (Porogen)

The main function of the solvent is to bring into one phase all the components used in the polymerisation process (i.e. template, functional monomers, cross linker and initiator). Another, but not less important, function is to be responsible for creating the pores in the microporous polymers [37]. Solvents also make an influence on the bonding

strength among functional monomers and templates, as well as on the properties and morphology of the polymer [16].

The interaction between the functional monomer and template can be affected by the polarity of the solvent. In order to obtain good imprinting efficiency, in non-covalent imprinting, for instance, are often used non-polar and less polar organic solvents (i.e. toluene, acetonitrile and chloroform) [36] as they stabilise hydrogen bonds [37]. Still, water can be chosen as solvent if hydrophobic forces are being used to carry the complexation of the reagents [37].

2.2.1.5 Initiator

MIPs are majority produced by free radical polymerisation (FRP), photopolymerisations and electro polymerisation. Free radical polymerisation can be thermally or photochemically initiated by a large variety of functional groups and template structures. The most used initiators are peroxy and azo compounds, such as azobisisobutyronitrile (AIBN), azobisdimethylvaleronitrile (ADVN), 4,40-azo(4-cyanovaleric acid) (ACID), benzoylperoxide (BPO), dimethylacetal of benzyl (BDK) and potassium persulfate [52]. When working at decomposition temperatures (50-70°C), AIBN is the most used initiator [36].

2.2.2 MIP Preparative Approaches

There are three different approaches that can be used to synthetize MIPs, namely covalent, non-covalent and semi-covalent approaches.

2.2.2.1 Covalent approach

The first model of molecular imprinting of polymers introduced by Wulff et al. was based in a covalent approach [8]. In this approach, during the polymerisation process, it is established a covalent approach between the functional monomers and the template. Posteriorly, in the molecular recognition, a covalent bond is again established between the template and the polymer [23].

One of the main advantages of this approach is that is possible to have a known stoichiometry between the functional monomer and the template. In addition, it is also viable to achieve a high density of well-defined sites on the MIPs, once that the template can be recovered in hight yields. On the other hand, there are some disadvantages of this process, especially because of the limited number of covalent linkages satisfying these

criteria, and the slow kinetics observed for rebinding by reformation of the covalent bond. [23], [53].

2.2.2.2 Non-covalent approach

The non-covalent approach is the most used method for preparing MIPs [54]. This approach is based on non-covalent self-assembly between the template and the functional monomer preceding the polymerisation, followed by a free radical polymerisation with cross-linkers and templated extraction, and succeeded by rebinding through non covalent interactions [55].

The major advantages of the non-covalent approach are not only the facility to prepare the template/monomer complex, but also to remove the templates from the polymers. Further, the fast binding of templates to MIPs and its potential application to a wide variety of target molecules contributes for the large choice of this approach [56].

2.2.2.3 Semi-covalent approach

The semi-covalent approach can be considered as a hybrid version that sums the covalent and the non-covalent approach. Following this logic, such approach relies on covalent bons to form the template/monomer complex and is based on a non-covalent interaction for the subsequent rebinding to the polymer [10], [56].

For those reasons, semi-covalent approach associates the advantages of covalent and non-covalent approaches, as it is possible to obtain a stable stoichiometric complex and a fast guest binding [53].

2.3 Polymer synthesis

2.3.1 Free radical Polymerisation

Free radical polymerisation is the most used and important polymerisation method, mainly because it can be performed under mild conditions, in bulk or in solution and it also shows a very high tolerance for functional groups in the monomers and impurities in the system. In addition, free radical polymerisation allows a wide variety of molecules to be polymerized and cross-linked and, for these reasons, is commonly chosen to prepare MIPs [37], [57].

The kinetic scheme for free radical polymerisation is defined by three stages: initiation, propagation and termination. In order to improve the process, additional reactions can be added, such as chain transfer [58].

2.3.1.1 Initiation

The initiation reaction holds the decomposition of initiator molecules A to form very active primary radicals R*, as represented by Equation 1. These primary radicals are capable of initiating new polymer chains, adding themselves to the molecule of monomer M, as shown by Equation 2 [58].

$$A \rightarrow 2R^* \quad (1)$$

$$R^* \xrightarrow{M} RM^* \quad (2)$$

2.3.1.2 Propagation

The propagation stage involves the growth of the polymer chain by a series of fast sequential addition of monomer to the active centre, represented by the Equation 3 [58].

$$RM^* \xrightarrow{nM} RM^*_{n+1} \quad (3)$$

2.3.1.3 Termination

In the termination stage, the active centre is deactivated and the propagation finishes, originating the polymer molecule. The termination can be associated with different reactions, generally involving combination (Equation 4) and disproportionation processes (Equation 5) [58].

$$RM^*_m + RM^*_{n+1} \rightarrow RM_{n+m+1}R \quad (4)$$

and/or

$$RM^*_m + RM^*_{n+1} \rightarrow RM^*_m + RM_{n+1}H \quad (5)$$

2.3.2 Cross-linked polymers

Cross-linked polymers are polymers formed by more two or more polymeric chains, that are attached by a cross-link, bond that can be either ionic or covalent, being the last one much stronger [59].

Due to the strong links between the polymer chains, cross-linked polymers show an improved mechanical behaviour. These materials can be classified as branched macromolecules, macroscopic network and microgels [37], [60].

In order to prepare cross-linked polymer networks, it is possible to use different strategies, and the most commonly used is the Free Radical Polymerisation, that enable to set the properties of the final network, by changing the relative amount of monomers and cross-linkers used in the polymerisation reactions [57].

2.4 Formats of MIPs

MIPs obtained by free radical polymerisation can be prepared in a variety of physical forms, using different techniques, such as bulk, suspension, emulsion, precipitation, or multi-step polymerisation. The format of the MIPs can make an influence on their capability of binding and also on the accessibility by the template to the binding sites. Another characteristic that can be related to the format of the MIPs is the response time when the materials are appraised as molecular recognition components [61], [62].

2.4.1 Bulk Polymerisation

Bulk Polymerisation is considered a simple method once it is only uses the template, functional monomer, cross-linker, initiator and porogen to synthesize the MIPs.

This method is carried on by a series of steps, that starts with the polymerisation and is followed by grinding and sieving [62]–[64].

Through the bulk polymerisation, it is obtained particles with irregular size and shape and generally are not used for chromatographic applications [62].

2.4.2 Suspension Polymerisation

Suspension Polymerisation is a one step polymerisation executed in water medium and continuous phase, that can be perfluorocarbon liquids and mineral oils [62].

The product obtained though Suspension Polymerisation is in spherical shape wig big size (in the range from micrometres to millimetres), and the MIPs obtained have, in general, a low recognition as a result of the influence of the dispersing medium [65]–[67].

2.4.3 Emulsion Polymerisation

Emulsion Polymerisation is a method in which the liquid monomer is dispersed in an insoluble liquid, resulting in an emulsion. Through this type of polymerisation, it is

obtained monodispersed polymer particles with the size in a range of tens to hundred nanometres [68].

Such method, however, is not to attractive to produce MIPs because it was observed that the water and surfactant can influence the efficiency of the process, once it affects the stability of the interactions between the template and the functional monomers [62], [69], [70].

2.4.4 Precipitation Polymerisation

Precipitation Polymerisation is carried on by a single preparative step, without the presence of surfactant and concerns the polymerisation of monomers in dilute solutions leading to polymer particles precipitating from the solution [71]–[73].

The particles obtained by this method are spherical and uniform, generally with diameters lower than 1 μm [62].

2.4.5 Inverse Suspension Polymerisation

Inverse Suspension Polymerisation occurs in a dispersion of water-soluble monomer in a continuous matrix. The characteristics of the particles obtained depends upon the stirring speed, volume ratio of water to monomer, concentration, and type of the stabilizer and the viscosities of both phases [74].

2.5 N-acetylneuraminic acid

N-acetylneuraminic acid (Neu5Ac) is the most abundant sialic acid in the nature and in eukaryotic organisms. For that reason, many researches have been carried out in order to develop effective synthesis of it and to investigate possible application in processes, such as cell-to-cell recognition, cell-adhesion, neural cell development, tumour metastasis and biosensors[75]–[79]. In addition, N-acetylneuraminic acid is usually recognized by viruses, bacteria and parasites, which implies on the study of using it on the treatment of some diseases, such as Influenza [75], [77].

Figure 2 shows the chemical structure of N-acetylneuraminic acid, which was first isolated by Gottschalk et al. [80]. The biological functions of sialic acids come from their physical properties and from their chemical structure, that have as terminal residue on ell-surface glycoconjugates, which can act directly in three areas, namely mediation of interactions based on their charge, masking of underlying glycoconjugate structures and being receptors for binding and adhesion [81].

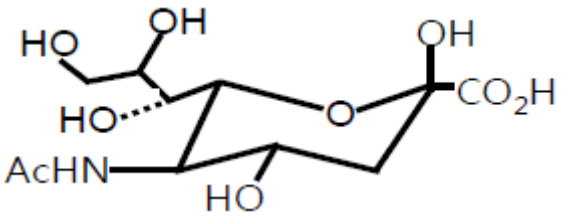


Figure 2 - Chemical Structure of N-acetylneuraminic acid (Neu5Ac) [82].

2.5.1 N-acetylneuraminic acid and virus recognition

N-acetylneuraminic acid is associated with several diseases, caused by different types of pathogens (virus, bacteria and protozoans) [83] and, as a consequence, it has been studied as consequence of its potential to be used in treatments for those diseases.

N-acetylneuraminic acid is present in immunological responses to foreign bodies. Specifically regarding to virus, once it goes into the bloodstream and attacks the cells, two main viral enzymes, hemagglutinin and neuraminidase, are involved in the infection process and recognize residual sialic acid existent in the cells, and induce the fusion and incorporation of the virus envelope into the cell. Inside the cell, the virus introduces its RNA (ribonucleic acid) and, as a consequence, viruses are copied inside the cells and go to the bloodstream to infect other cells [84].

Understanding the mechanism of the immunological system and how it is related with the sialic acid has been attracting interest of the scientific community due to the wide possibility to develop medicines, alternative treatment and diagnosis for viral diseases, such as Influenza and HIV infection [75], [85], [86].

2.5.2 N-acetylneuraminic acid and MIPs

Due to the strong relation between Neu5Ac and the recognition of pathogens, as it mediates a large variety of physiological and pathological processes [87], many studies using this molecule as a template to produce MIPs have been carried out, with the aim to develop novel approaches for diagnosis and treatment for diseases [88]–[94].

The evaluation of the recognition capacity of sialic acid by the MIPs was firstly demonstrated as positive. Kugimiya et.al. assessed the binding ability of polymer selective for sialic acid, concluding that it showed pH-dependant characteristics to bind to sialic acid [93]. In a second study, it was found by the same author that a similar approach was capable to produce polymers with excellent binding performance in aqueous media, opening new possibilities for using such MIPs in different fields related

with life sciences [92]. In parallel, Piletsky et. al. prepared MIPs to act as a receptor system for sialic acid using allylamine, vinylphenylboronic acid and ethylene glycol dimethacrylate. Such polymer showed a rapid detention of sialic acid [88].

Kugimiya et. al. also studied a new non-covalent system to produce Neu5Ac imprints, using 4-vinylpyridine and N,N,N-trimethylaminoethyl methacrylate chloride as functional monomers. Those evaluated molecules demonstrated recognition ability for sialic acid [95].

Recent researches have demonstrated that sialic acid targeted materials can reach a facile detection to sialic acid-bearing glycoconjugates as a consequence of their properties, specially optical, electrochemical and hydrophilic-hydrophobic, allowing them to perform as bio detectors, biosensors, controllable drug delivery and release devices and bio-separators [89]. It was shown possible to use sialic acid imprinted polymers for cancer cell imaging [96]–[100], anticancer drug delivery for treatment of tumours [101]. Furthermore, sialic acid-responsive bio-inspired interface materials presented high potential for the separation or enrichment of sialic acid derivatives [89].

Mavliutova et. al. studied the development of MIPs with high adsorption of sialic acid and their contribution for an early diagnosis of cancer. In this investigation, three functional monomers were combined to target different functionalities on the template, and this combination improved both imprinting factors and selectivity. The authors also found that the use of tetrabutylammonium salt of sialic acid enhanced the imprinting [102].

2.6 Applications of MIPs

Due to the good properties shown by MIPs already described previously, they are widely used in many applications, that can be divided in three main groups: pre-treatment techniques, chromatography and sensors [36], [56].

Pre-treatment techniques include Solid Phase Extraction (SPE), where the chosen MIPs should have good adsorption capacity, high selectivity and stability, Solid Phase Microextraction (SPME), as fiber coatings with high selectivity, sensitivity and reproducibility, and Stir Bas Sorption Extraction (SBSE) using MIP coated stir bars [36].

For chromatography techniques, MIPs are used in stationary phases considering their high affinity and selectivity to the target analytes. In such context, MIPs can be applied as packing materials and monolithic column materials [36].

Inside the biosensor's field, MIPs can substitute biological molecules, allowing the development of high stable sensors, able to be used in harsh environments [103]. This

range associates drug agent detection, food analysis, medical diagnosis and environment monitoring [103]–[105].

As a consequence of the operation principle of MIPs, they can be used also as artificial antibodies, once they both bind target molecules selectively and reversibly [106], [107]. Another featured application for MIPs in biomedical area is in drug delivery systems, that has been forcefully expanded over the last decade [23], [24], [108], [109].

2.6.1 MIPs for Biomolecular Recognition

The use of MIPs for big biomolecular entities, such as virus and bacteria, recognition has been arousing great interest, once it can be applied for purification, diagnosis and therapy [110]. However, there are some difficulties involving large biomolecules and their access to biding sites within the produced MIPs [110], [111]. Specifically for molecular imprinting of protein templates, besides the large size of the molecule, the chemical and structural complexity and the environmental instability of proteins were shown as obstacles to be overcome [112].

Cumbo et. al. studied what they called virus-imprinted particles (VIPs), an artificial organic-inorganic nanoparticulate material with its surface imprinted with virus. The research showed that there is an relation between the thickness of the recognition layer and the affinity of the material for its template [110].

Another challenge for using biomolecules as templates for MIPs happens when live cells are used for imprinting, as is the case of bacteria. For this process, it is necessary to consider the most suitable conditions to ensure the integrity of the cells, once they are fragile. In addition, the capturing selectivity can be influenced by secreting chemicals produced by the live cells when they are adapting to the environment. [113], [114].

Studies carried out by Ren et. al. proposed mechanisms using inactivated bacteria in order to overcome those mentioned obstacles for the use of live cells. The research demonstrated that such approach increased both selectivity and biosafety [113].

Hayden and Dickert studied microorganism detection with cell surface imprinted polymers using non-covalent molecular imprinting approach. In this research, surface imprinting of polyurethane with yeasts shown good selectivity and highly sensitive enrichment of microorganisms [115].

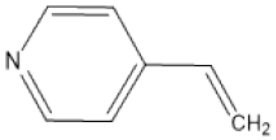
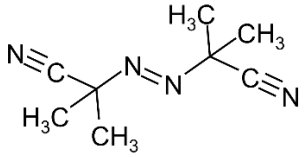
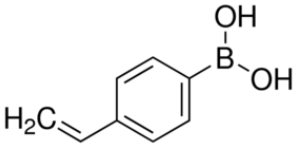
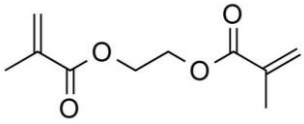
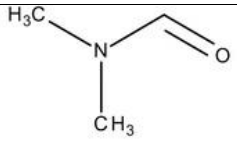
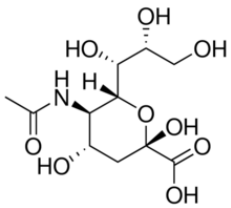
CHAPTER III

3. MATERIALS AND METHODS

3.1 Reagents

The chemical structures and properties of different reagents used for the synthesis of MIPs and NIPs, as well as the ones used for their analysis are summarized in Table 3.1 and Table 3.2.

Table 3.1 - Reagents used for MIPs e NIPs synthesis.

Reagents	Chemical Structure	Molar Mass (g/mol)	Density (g/mL)	Producer
4-vinylpyridine (4VP)		105.14	1.051	
2,2'-azo-bis-isso-butironitrile (AIBN)		164.21	1.1	Fluka
4-vinylphenylboronic acid (4VPBA)				Acros Organics
Ethylene glycol dimethylacrylate (EGDMA)		198.22	1.051	Sigma Aldrich
N,N-dimethylformamide (DMF)		73.09	0.944	Sigma-Aldrich
N-acetylneuraminic acid (Neu5Ac)		345.30		Jennewein Biotechnologie

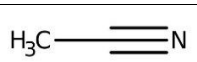
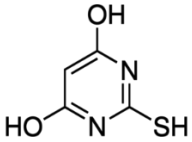
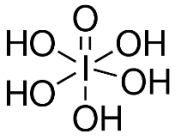
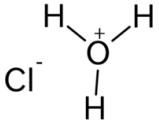
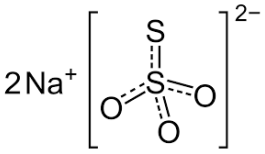
Acetonitrile (ACN)		55.06	0.781	Fisher Chemical

Table 3.2 - Reagents used for Neu5Ac derivatisation.

Reagents	Chemical Structure	Molar Mass (g/mol)	Density (g/mL)	Producer
2-thiobarbituric acid (TBA)		144.15		Sigma-Aldrich
Periodic acid		227.94	1.4	Sigma-Aldrich
Hydrochloric acid (HCl)		36.46		PanReac
Sodium thiosulfate		158.11	1.67	Panreac/Fisher Chemical
Sodium hydroxide (NaOH)		39.98	2.13	Vencilab

3.2 Equipment

In Table 3.3 is presented the equipment used in the experimental procedure performed in this work.

Table 3.3 - Equipment used in the experimental procedure.

Equipment	Model	Company
Analytical Balance	AS/220/C/2	RADWAG
Lab Centrifuge	RADWAG	VWR Advanced Series
Ultrasonic Bath	SW 1	SONO SWISS
pH meter	inoLab	WTW
Heating Plate	VMS – C7	VWR Advanced Serie

Vacuum Oven	22	Vacucell
Steering Plate	S03 series	LBX instruments
HPLC	MD-4010	Jasco
UV Spectrophotometer	vis V-530	Jasco

3.3 Synthesis of MIPs and NIPs procedures

MIPs and NIPs were synthesized using two different molecules as functional monomers: 4-vinylpyridine (4VP) and 4-vinylphenylboronic acid (4VBA). In addition, as solvent, it was used N,N-dimethylformamide (DMF), acetonitrile (ACN) and the combination of them in different proportions. For all the MIPs, Neu5Ac was used as a template and for both MIPs and NIPs, EGDMA was the crosslinking agent.

The procedure to obtain MIPs and NIPs were the same for each pair of materials, but for the NIPs, the procedure was carried out without Neu5Ac. Into the solvent, the template (Neu5Ac) and the functional monomer (4VP or 4VPBA) were dissolved, then the crosslinking agent (EGDMA) was added. The polymerization was initiated by the addition of AIBN under UV light radiation for 12h at 4°C. The polymers obtained were ground and sieved through a filter. The template molecule was extracted by washing with 0.01 M hydrochloric acid/methanol (1:1,v/v) for 3 times.

The quantities used to produce each of the polymers are summarized in Table 3.4.

Table 3.4 - Quantities of each reagent used to produce all different MIPs and NIPs.

Experiment	Solvent (v/v)	Functional Monomer		AIBN		EGDMA		Neu5Ac	
		Mass (mg)	Concentration (mol/L)	Mass (mg)	Concentration (mol/L)	Mass (mg)	Concentration (mol/L)	Mass (mg)	Concentration (mol/L)
MIP 1 (4VP)	DMF 100	29.805	0.060	6.941	0.009	1587.440	1.697	29.805	0.060
								NIP 1	
MIP 2 (4VP)	DMF 100	105.140	0.116	24.632	0.017	5589.804	3.278	345.300	0.116
								NIP 2	
MIP 3 (4VP)	DMF 100	103.975	0.285	6.941	0.012	196.025	0.285	341.446	0.285
								NIP 3	
MIP 4 (4VP)	ACN/DMF 18/82	103.952	0.080	25.000	0.012	195.981	0.080	341.400	0.080
								NIP 4	
MIP 5 (4VP)	ACN/DMF 42/58	126.519	0.116	20.405	0.012	0.116	1.051	69.252	0.019
								NIP 5	
MIP 6 (4VP)	ACN/DMF 42/58	204.961	0.225	34.189	0.012	386.412	0.225	56.094	0.019
								NIP 6	
MIP 7 (4VP)	DMF 100	555.000	1.117	10.000	0.013	1036.000	1.106	303.440	0.186
								NIP 7	
MIP 8 (4VP)	ACN/DMF 60/40	100.000	0.112	16.000	0.012	188.530	0.112	20.500	0.007

									NIP 8
MIP 9 (4VP)	ACN/DMF 60/40	130.000	0.145	21.000	0.015	245.088	0.145	20.500	0.007 NIP 9
MIP 10 (4VP)	ACN/DMF 60/40	114.000	0.128	18.000	0.013	214.924	0.128	20.500	0.007 NIP 10
MIP 11 (4VP)	ACN/Water 80/20	130.000	0.139	21.000	0.014	245.088	0.139	20.500	0.007 NIP 11
MIP 1 (4VBA)	DMF100	200.000	0.958	11.098	0.048	267.919	0.958	77.786	0.160 NIP 1 (4VPBA)
MIP 2 (4VBA)	DMF 100	380.000	2.172	11.098	0.057	27.000	0.115	NIP 2 (4VPBA)	
MIP 3 (4VBA)	DMF 100	400.000	2.337	11.098	0.058	0.000	0.000	NIP 3 (4VPBA)	
MIP 4 (4VBA)	DMF/Water 95/5	400.000	2.337	11.098	0.058	0.000	0.000	NIP 4 (4VPBA)	

3.4 MIP and NIP analysis

3.4.1 Batch Adsorption

Batch Adsorption test was performed in order to analyse the adsorption capacity of the produced MIPs and NIPs regarding Neu5Ac. Solutions with different concentrations of Neu5Ac were prepared. In those solutions, 20mg of NIP or MIP were added, and the samples were mixed for 24 hours at room temperature in an orbital shaker at 80 rpm, as represented in Figure 3.

Figure 3 - Batch adsorption in an orbital shaker at room temperature.

After reaching the equilibrium, samples of those solutions were taken. After, it was performed the determination of sialic acid to evaluate the adsorption capacity of MIPs through Derivatisation with TBA and HPLC Analysis.

3.4.2 Sialic Acid Determination – Derivatisation with TBA

UV Spectroscopy is one method widely used to determine analyte interactions with MIPs. Such approach considers the change in absorbance as a function of wavelength [116]. However, the Neu5Ac molecule is not possible to be read through UV Spectroscopy due to its double bonds, what makes necessary to make some changes in its molecular structure in order to enable this analysis [117].

Derivatisation is the use of derivatives in order to obtain a better result of an analysis, changing physicochemical properties to more favourable ones. This derivative is generally obtained by a simple reaction that leads to a little change of chemical structure. [118]

Warren have reported a thiobarbituric acid (TBA) assay to quantify sialic acids through UV analysis. The principle behind this method is based on the quantification of red chromophore with maximum absorption at 549 nm. To achieve that, periodate oxidation of Neu5Ac is carried out, which results in the formation of β-formylpyruvic

acid. Thus, this acid is coupled with TBA, resulting in the formation of a red chromophore [117]. Figure 4 shows the reaction involved in this procedure.

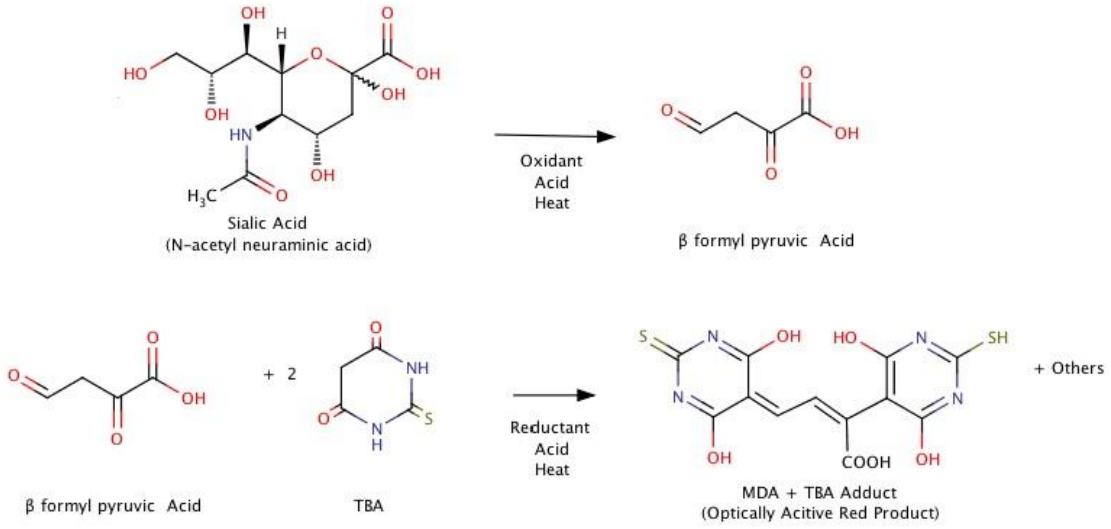


Figure 4 - Chemical reactions involved in the derivatization of Neu5Ac through the thiobarbituric acid method.

The first step of the experiment consisted in the analysis of solutions with known concentrations of Neu5Ac, in order to build a calibration curve relating each response intensity of UV with its concentration.

To carry on this procedure, initially the oxidation of sialic acid was performed. Solutions with different concentrations of Neu5Ac were prepared and 300 µL of sialic acid of each concentration was oxidized by the addition of 25 µL of 50 mmol/L periodic acid and 40 µL of 0.22 mol/L HCl. The solutions were shaken and placed in water bath at 37°C for 40 minutes. Then, it was added 60 µL of 2% sodium thiosulfate to these solutions and they were shaken until the yellow-brown colour appeared.

In sequence, to the oxidised sample, it was added 250 µL of 0.1 mol/L TBA prepared in 70 mmol/L NaOH. The solution was shaken and heated in oil bath at 100°C for 8 minutes. Then, the solution was cooled in ice bath for 5 minutes.

Posteriorly, the UV analysis in a spectrophotometer Jasco UV-vis V-530 was performed, where the maximum wavelength selected was λ_{max}=545 nm. With these results, a calibration curve was obtained.

After, the same procedure was made with MIPs and NIPs and, with the calibration curve, it was possible to obtain the absorbed quantity of Neu5Ac to evaluate the behaviour of the materials.

3.4.3 High-Performance Liquid Chromatography Analysis

High-Performance Liquid Chromatography (HPLC) is a technique used to separate and identify compounds in a sample that can be dissolved in a liquid in small concentrations. HPLC is used in a wide range of applications, such as pharmaceutical, environmental, chemicals and forensic analysis.

HPLC has as a main principle a column with chromatographic packing material (stationary phase) in which is pumped a sample mixture or analyte in a solvent (mobile phase) at high pressure. The sample is carried by a moving carrier gas stream of helium or nitrogen. As the sample passes through the column it interacts between the two phases at different rate, primarily due to different polarities in the analytes. Analytes that have the least intensity of interaction with the stationary phase or the most amount of interaction with the mobile phase will exit the column faster.

In this experiment, the equipment used was HPLC Jasco MD-4010. The analysis was carried out for all the samples taken from the bath adsorption test, in order to evaluate the amount of Neu5Ac present in each of them. The samples were injected in the equipment, to pass through the column type C18, with a flow rate of 1mL/min. The mobile phase was composed by 90% of H_2O (pH=3) and 10% of ACN, and the analysis time for each sample was 6 minutes.

For each material analysed, a respective blank solution was used to calculate the concentrations needed. Those concentrations were calculated by the area of the sialic acid peak obtained by the equipment, using the software Origin for the calculations.

CHAPTER IV

4. RESULTS AND DISCUSSION

4.1 Characterization of Molecularly Imprinted Polymers

4.1.1. Derivatisation with TBA

4.1.1.1. Calibration Curve

Derivatisation with TBA reactions were performed with sialic acid solutions with concentrations of 2.5mM, 1.5mM, 1.0mM, 0.5mM, 0.25mM, 0.1mM, 0.04mM, 0.03mM and 0.02mM and with a blank solution to obtain a calibration curve for the derivatisation method. Those samples were read through visible UV analysis with maximum wavelength λ_{max}=545nm. The results were synthesized in Table 4.1.

Table 4.1 - Experimental UV response for different concentrations of sialic acid solution after derivatisation with TBA.

C (mM)	Experimental UV absorbance
1.00	1.337
0.50	0.79
0.25	0.378
0.10	0.189
0.04	0.069
0.03	0.057
0.02	0.041
0	0.005

From the results of table 4.1, the calibration curve, along with its linearization equation were obtained, as shown in Figure 5.

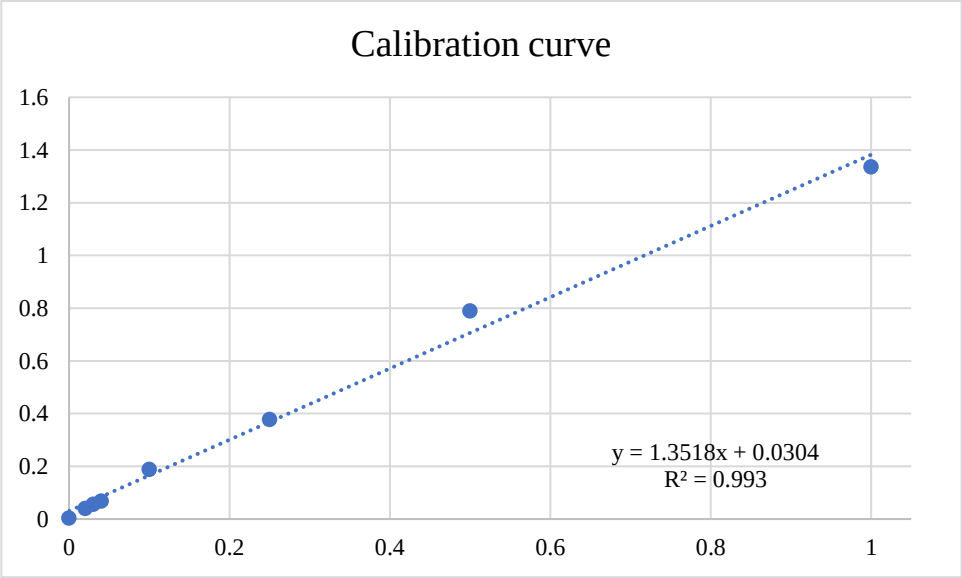


Figure 5 - Calibration Curve for Sialic Acid quantification from Derivatisation with TBA.

4.1.1.2. 1st Batch

The first batch was carried out to analyse MIP7 and NIP7 and it was proceed following the procedure described at the topic 3.4.1, with sialic acid solutions with concentrations 0.03mM, 0.04mM, 0.05mM, 0.1mM and 0.2mM. The samples taken after reaching the equilibrium were analysed by derivatisation method, in order to compare the results, validate and understand the reliability of this method. From each sample, small separate volumes were taken to perform two analyses with TBA solution with concentration of 0.1mM, one with TBA concentration of 0.07mM and one with TBA concentration of 0.04mM.

The results obtained from the UV analysis after the derivatisation reactions are shown in Table 4.2. For the analysis using TBA concentration of 1.0mM, the sialic acid equilibrium concentration was calculated by the equation obtained through the calibration curve. On the other hand, for TBA concentration 0.07mM and 0.04mM, a blank sample for each sialic acid concentration was derivatised and read by UV to obtain the equilibrium concentration with a more precision. For all the results, the retention was calculated by Equation 1.

$$Q_e = \frac{(C_i - C_e) * Vol}{Mass} * 1000 \quad (1)$$

Where:

Q_e = Sialic Acid adsorbed by the material (µmol/g)

C_i = Initial concentration of Sialic Acid (mmol/g)

C_e = Equilibrium concentration of Sialic Acid (mmol/g)

Vol = Batch volume (ml)

$Mass$ = Mass of the material analysed through Batch (mg)

Table 4.2 - Values for Sialic Acid Retention for MIP 7 and NIP 7 through Derivatization with TBA and UV analysis.

		Sialic Acid Retention (µmol/g)			
Material	**Sialic Acid Concentration (mM)**	**TBA (1.0mM) (1)**	**TBA (1.0mM) (2)**	**TBA (0.07mM)**	**TBA (0.04mM)**
MIP 7	0.03	3.420	1.255	-2.246	-1.521
	0.04	-0.461	-1.007	-3.432	-1.218
	0.05	0.372	-0.562	-102.017	1.317
	0.10	-0.632	-4.331	-1.182	-1.182
	0.20	15.052	18.260	20.261	17.130
NIP 7	0.03	1.850	0.921	0.672	N/A
	0.04	2.318	-1.399	-4.178	-3.275
	0.05	2.512	-2.336	-1.266	-2.457
	0.10	4.615	8.370	7.031	5.316
	0.20	-1.509	0.688	0.179	1.430

For a better visualisation of the results, Figure 6 shows a graph comparing the Sialic Acid initial concentrations and the Sialic Acid retention for MIP 7 and NIP 7, after the derivatization using TBA with concentration of 0.1 mmol/L.

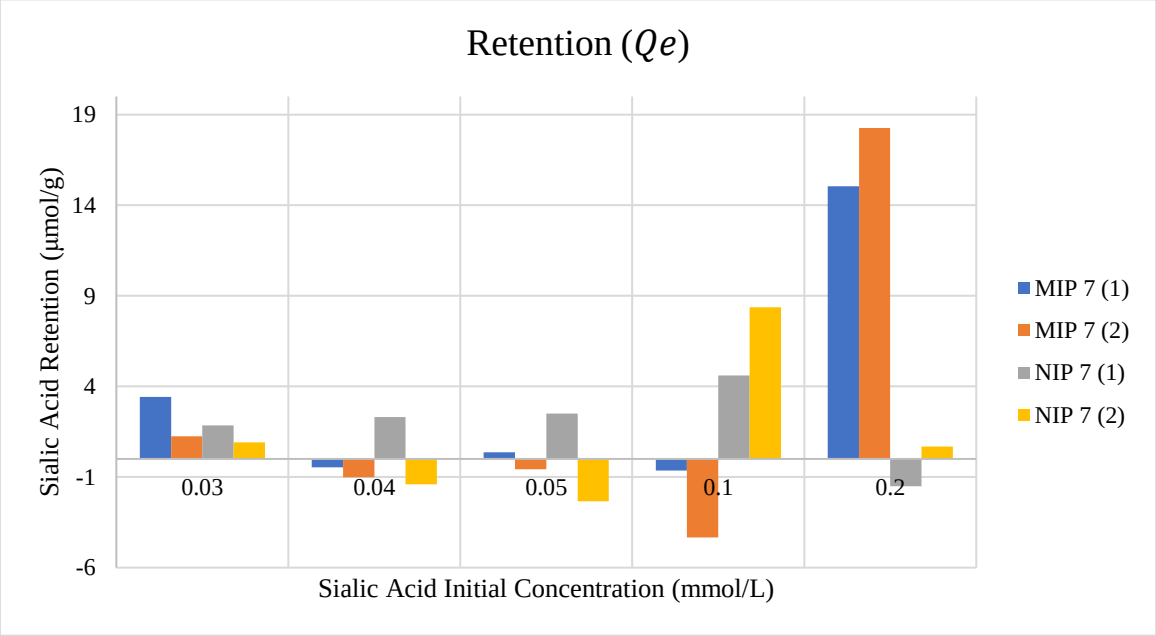


Figure 6 - Retention (Qe) x Sialic Acid Initial Concentration for MIP 7 and NIP 7 using TBA concentration 0.1 mmol/L for Derivatisation.

It is possible to observe that even for the same materials and having the same conditions for the experiments, the results had a large difference, what can lead us to induce that the derivatisation method could not be the best one to quantify the sialic acid on MIPs and NIPs on those concentrations, once it has a lot of steps that can carry some experimental errors. On the other hand, it was also possible to initially conclude that a higher concentration of sialic acid (0.2 mM) lead to a higher retention rather than lower concentrations.

In addition, it was noticed a behaviour out of what was expected, as for both MIP 7 and NIP 7, it was found negative retentions, which corroborates to the idea that the many steps of the derivatisation method and also the initial step of washing the materials after their synthetisation, could implicate in errors during the procedure and results.

Figure 7 brings the same comparison as Figure 6, but for derivatisation using TBA with concentrations of 0.07 mmol/L and 0.04 mmol/L.

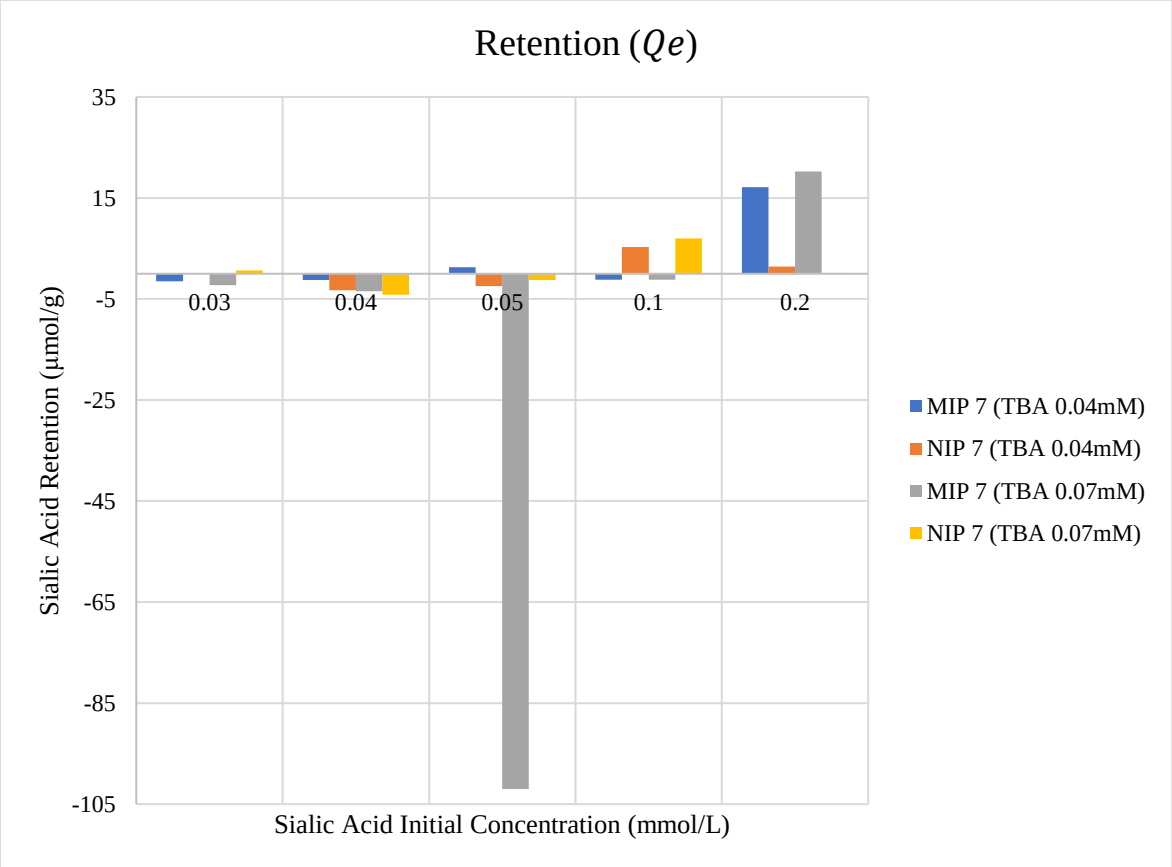


Figure 7 - Retention (Qe) x Sialic Acid Initial Concentration for MIP 7 and NIP 7 using TBA concentration 0.07 mmol/L and 0.04 mmol/L for Derivatisation

Changing the concentrations of TBA for the derivatisation method, some behaviours were again observed. MIP 7 and NIP 7 showed a negative retention for some sialic acid concentrations, especially MIP 7 for sialic acid concentration of 0.05 mM.

Comparing the results for TBA with those new concentrations, it was observed a difference between the results, but without a pattern that could be explained.

These results corroborate to an initial conclusion that the procedure could carry some experimental errors, impactions the results.

4.1.1.3. 2nd Batch

The second batch was performed also to analyse MIP 7, NIP 7 and synthetic materials, namely Reillex 402 and Reillex 425, and it was carried out as described at the topic 3.4.1, but with sialic acid solutions with concentrations 1.5 mM, 1.0 mM, 0.5 mM and 0.2 mM. The samples were analysed through UV analysis after derivatisation with TBA (1.0 mM) and HPLC analysis. From each sample, small separate volumes were taken to perform those analysis.

Table 4.3 compiles the results obtained. Retention was equally calculated by Equation 1 and, to make these calculations, the equilibrium concentration of sialic acid was obtained using a blank reference solution of sialic acid analysed through UV analysis in the derivatisation with TBA, and in HPLC analysis.

Table 4.3 - Values for Sialic Acid Retention for MIP 7, NIP 7 and Reillex 402 and 425 through Derivatization with TBA/ UV Analysis and HPLC Analysis.

		Sialic Acid Retention (µmol/g)	
Material	**Sialic Acid Concentration (mM)**	**Derivatisation TBA (1.0 mM)**	**HPLC Analysis**
MIP 7	1.5	-305.805	5.348
	1.0	161.353	22.769
	0.5	17.088	6.558
	0.2	5.772	24.767
NIP 7	1.5	-355.433	8.341
	1.0	96.216	37.200
	0.5	25.822	31.733
	0.2	6.327	34.430
Reillex 425	1.5	130.562	3.020
	1.0	49.667	12.937
	0.5	39.301	42.952
	0.2	9.183	28.677
Reillex 402	1.5	-192.276	-9.818
	1.0	0.000	10.954
	0.5	5.607	34.460
	0.2	10.172	55.026

Figure 8 shows the retention of Sialic Acid for the materials analysed through derivatization with TBA.

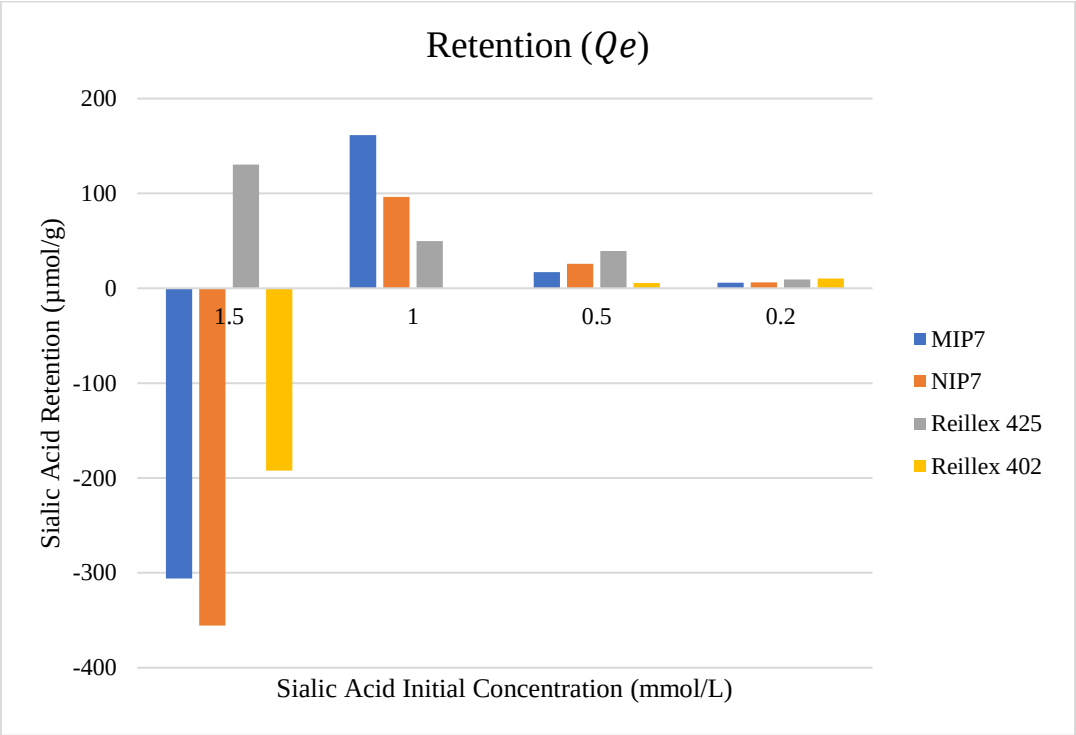


Figure 8 - Retention (Qe) x Sialic Acid Initial Concentration for MIP 7, NIP 7 and Reillex 402 and 425 obtained through Derivatisation with TBA.

Analysing the results, it is possible to identify that once again, MIP 7 and NIP 7 presented negative values for retention, now for sialic acid concentration of 1.5 mM, but regarding the other concentrations, the higher retentions were observed for the highest sialic acid concentration (1.0 mM). In addition, for sialic acid concentrations of 1.0 mM and 0.2 mM, MIP 7 retention was higher than for NIP 7, showing that the molecular imprinting has worked as expected.

Accordingly, once the derivatization with TBA method to quantify sialic acid showed some unreliable results, the samples taken from batch adsorption were analysed through UV analysis.

Figure 9 illustrates the sialic acid retention for the same materials, that were after analysed using HPLC analysis.

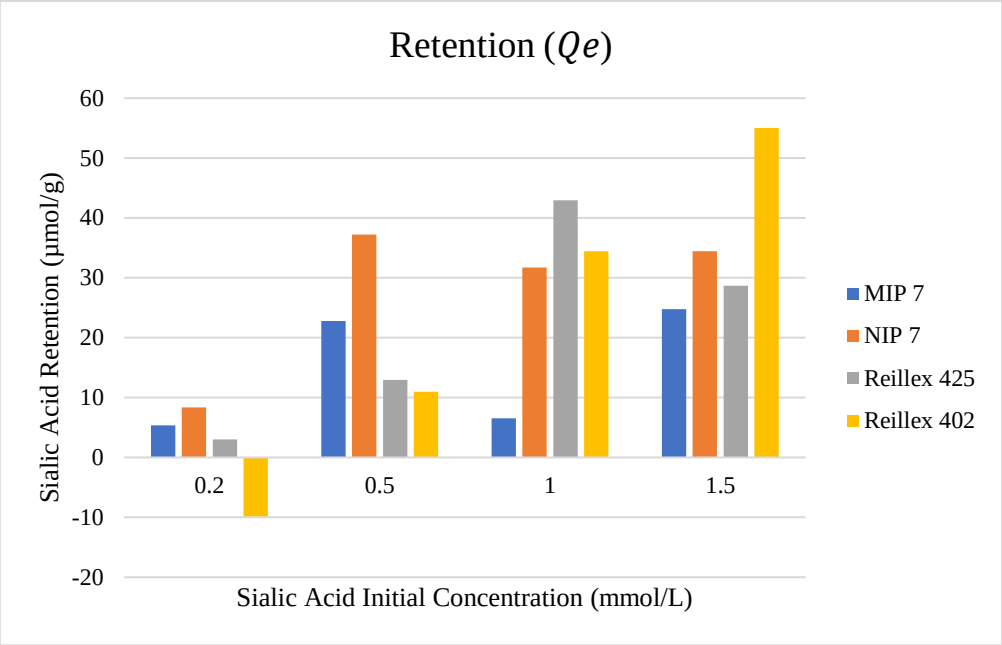


Figure 9 - Retention (Qe) x Sialic Acid Initial Concentration for MIP 7, NIP 7 and Reillex 402 and 425 obtained through HPLC Analysis for 2nd Batch.

The results for HPCL analysis showed a higher retention for NIP 7 instead of MIP 7, what leads to the conclusion that those materials, once they were used before for the previous experiments, could not have been well washed, impacting the results. On the other hand, only for Reillex 402 with sialic acid concentration of 0.2 mM, it was observed negative retentions, contributing for the idea that HPLC analysis to quantify sialic acid can be more reliable for these materials.

4.1.2. HPLC Analysis

4.1.2.1. 3rd Batch

The third batch was performed in the same way as second batch, to evaluate MIP7, NIP7 and synthetic materials, namely Reillex 402 and Reillex 425, with sialic acid solutions with concentrations 1.5 mM, 1.0 mM, 0.5 mM and 0.2 mM. The samples were analysed through HPLC analysis. From each sample, a small separate volume was taken to perform those analysis. Table 4.4 brings a compilation of the results obtained in second and third batch, to make an easier comparison between those results.

Table 4.4 - Values for Sialic Acid Retention for MIP 7, NIP 7 and Reillex 402 and 425 through HPLC Analysis obtained for 2nd and 3rd Batch.

		Sialic Acid Retention (µmol/g)	
Material	**Sialic Acid Concentration (mM)**	**HPLC Analysis 2nd Batch**	**HPLC Analysis 3rd Batch**
MIP 7	1.5	5.348	-7.628E-06

	1.0	22.769	6.725
	0.5	6.558	11.424
	0.2	24.767	22.968
NIP 7	1.5	8.341	-31.251
	1.0	37.200	1.941
	0.5	31.733	26.494
	0.2	34.430	48.439
Reillex 425	1.5	3.020	-3.614
	1.0	12.937	20.310
	0.5	42.952	17.459
	0.2	28.677	45.405
Reillex 402	1.5	-9.818	-5.674
	1.0	10.954	11.799
	0.5	34.460	21.423
	0.2	55.026	33.787

As expected, comparing the retentions obtained for the same materials and conditions, there was a difference on the results that can be explained by the washing step before the batch adsorption. However, it was still detected a higher retention for NIP 7 instead of MIP 7 in sialic acid concentrations of 1.0 mM and 1.5 mM.

The bar chart of Figure 10 illustrates the Retention of Sialic Acid obtained through HPLC Analysis for the 3rd Batch.

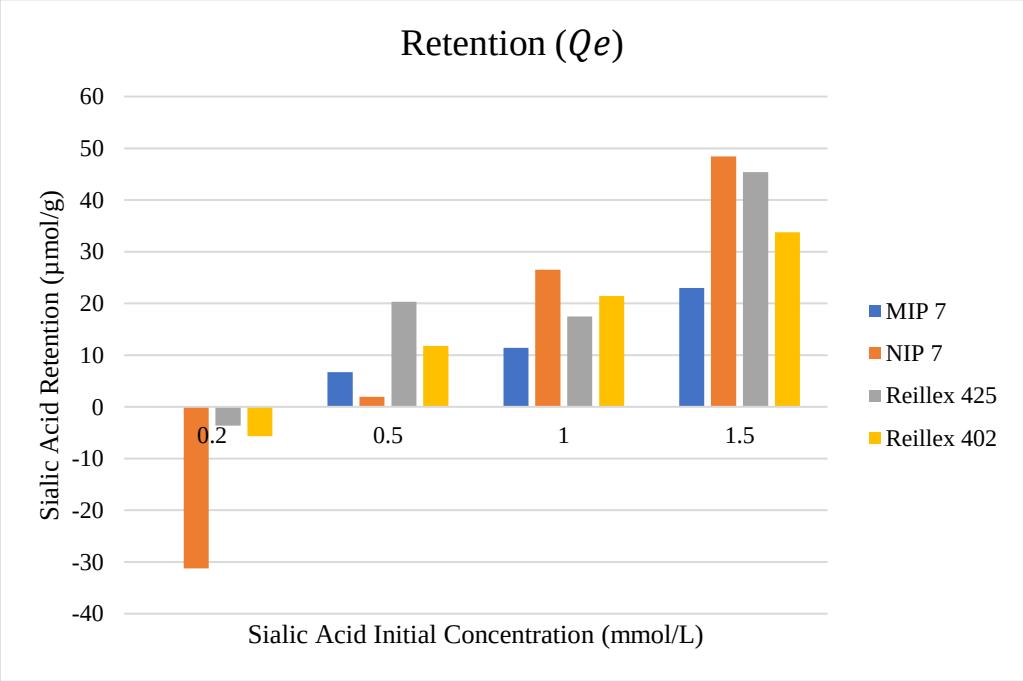


Figure 10 - Retention (Qe) x Sialic Acid Initial Concentration for MIP 7, NIP 7 and Reillex 402 and 425 obtained through HPLC Analysis for 3rd Batch.

As stated in Table 4.4, the retention for MIP 7 was different of what was expected, lower than for NIP 7 in some concentrations. In addition, for NIP 7, it was again obtained a negative retention for sialic concentration of 0.2 mM, and one possible reason for that was an incorrect preparation or analysis of the blank solution of sialic acid, impacting directly in these calculations.

Once again, another possible reason for the unexpected results could have been an insufficient washing before the analysis. For the next Batch Adsorption procedures, a higher care was taken in order to avoid those impacts.

4.1.2.2. 4th Batch

The fourth batch was performed to make a different evaluation from the previous ones, and it was carried out testing MIP 1, NIP 1, MIP 2, NIP 2, MIP 3, NIP 3 and synthetic materials Reillex 402 and 425, but now with only one concentration of Sialic Acid (0.1M) in three different pHs: acid, neutral and basic. The main objective was to evaluate the behaviour of the synthesized MIPs in different conditions, and HPLC was used for the analysis.

The Sialic Acid solutions with different pHs were obtained from a buffer solution containing Na_2HPO_4 (0.1M) and NaH_2PO_4 (0.1M). From this neutral buffer solution, three volumes were taken, in which one remained neutral, but with pH = 4.71 after the addition of Sialic Acid. For the other two volumes, in one it was added NaOH until achieving pH = 8, and in the other HCl was added until achieving pH = 2.

Table 4.5 summarizes the results of sialic acid retention for the materials tested in the fourth batch.

Table 4.5 - Sialic Acid Retention for MIP 1 NIP 1, MIP 2, NIP 2, MIP 3, NIP 3, Reillex 402 and 425 tested with Sialic Acid (0.1M) with pH=8, pH=4.71 and pH=2.

	Sialic Acid Retention (µmol/g)		
Material	**pH=8**	**pH=4.71**	**pH=2**
MIP 1	7557.576	9107.664	6110.17
NIP 1	5651.479	3201.441	7558.816
MIP 2	6946.685	6763.725	5619.258
NIP 2	2470.033	6945.424	7699.651
MIP 3	7032.078	4953.764	6725.947
NIP 3	7225.765	5704.661	6330.977
Reillex 402	2201.242	7871.793	7308.727
Reillex 425	6951.862	6938.393	6865.519

A better visualization of these results is shown in Figure 11.

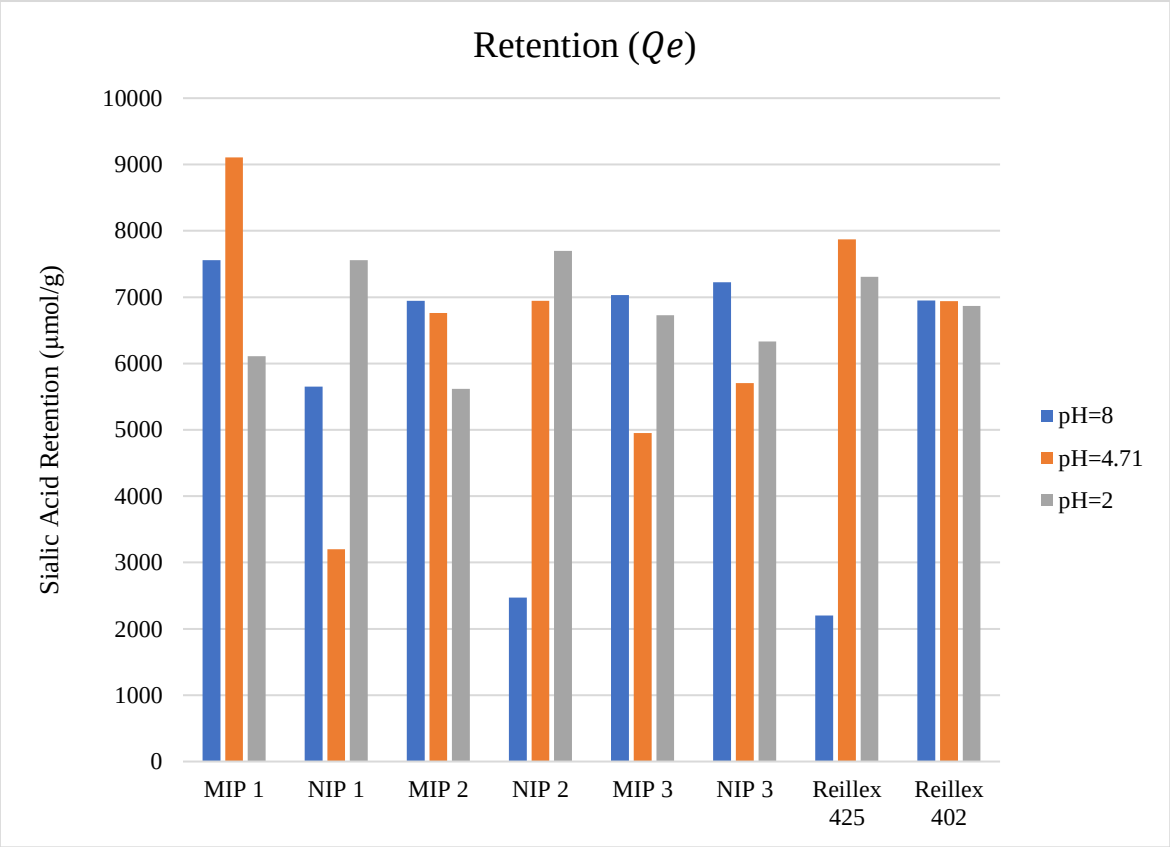


Figure 11 - Sialic Acid Retention MIP1, NIP1, MIP 2, NIP 2, MIP 3, NIP 3, Reillex 402 and 425 tested with Sialic Acid (0.1M) with pH=8, pH=4.71 and pH=2.

Through the results obtained after the HPLC analysis, it is possible to conclude that there was not a preferable pH condition for all the material to achieve a higher retention. For MIP 1, Reillex 425 and 402, the most intense retention was reached in pH=4.71, and for MIP 2 and NIP 3 the same was equally achieved in pH=8. Although, a common condition that showed a lower retention for all the materials was an acid environment, with pH=2. Moreover, it was not obtained any negative retention for those materials.

However, for the 3 MIPs analysed, that were some conditions that lead to a lower retention than for the respective NIP. For instance, MIP 1 had a lower retention than NIP 1 in acid pH, and the same for MIP 2, which also revealed this behaviour in pH=4.71. Further, MIP 3 demonstrated lower retention than its respective NIP in pH=4.71 and pH=8. In this specific case, a not enough washing could not explain the higher retention for NIPs, once they were being tested in a batch analysis for the first time, so they had no contact with a sialic acid solution before.

4.1.2.3. 5th Batch

The fifth batch also had as main objective the evaluation of the materials in different pH conditions. In this batch, it was analysed through HPLC, MIP1 MIP 1, NIP1, MIP 6, NIP 6, MIP 7, NIP 7, MIP 1 4VBA and NIP 1 4VBA. In this test, a buffer solution was again used to obtain controlled pH values, and sialic acid solution with concentration 1.0 mmol/L.

The bar chart in Figure 12 shows the results obtained for Sialic Acid retention.

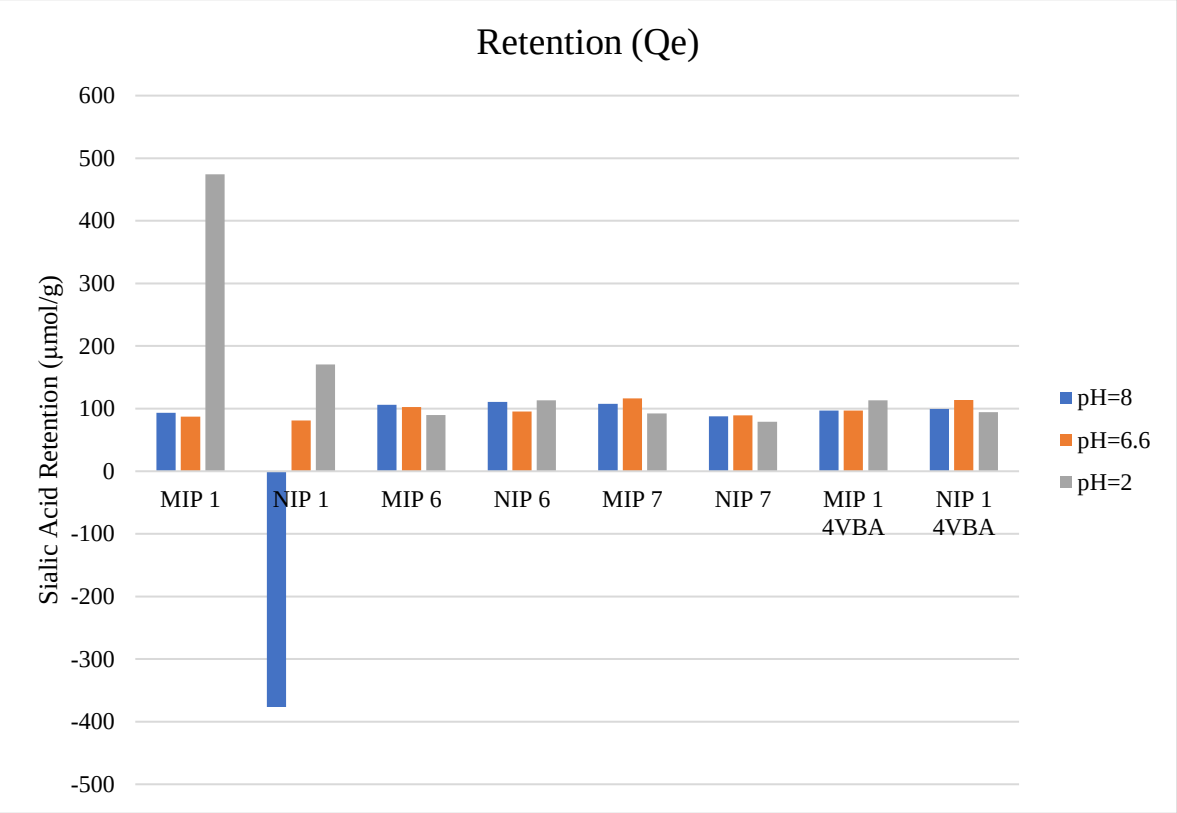


Figure 12 - Sialic Acid Retention MIP 1, NIP 1, MIP 6, NIP 6, MIP 7, NIP 7, MIP 1 4VBA and NIP 1 4VBA tested with Sialic Acid (1.0 mM) with pH=8, pH=6.6 and pH=2.

Considering the results obtained and shown in Figure 12, for MIP 1, with sialic acid concentration of 1.0 mM, the retention was higher for all pH conditions comparing to its respective NIP, and the most considerable retention was obtained in pH=2. Still, for NIP 1, a negative retention was seen in pH=8. A similar behaviour was observed for MIP 7, that now achieved a higher retention than NIP 7 in all the pH conditions, but with a higher retention in pH=6.6. For MIP 6 and MIP 1 4VBA, a lower retention than for their respective NIPs was demonstrated, both in pH=8, but also in pH=2 for MIP 6 and pH=6.6 for MIP 1 4VBA.

For MIP 1, comparing to the results in 4th Batch, the higher retention was now in acid pH, equally with this first analysis, that concluded that this behaviour was achieved in pH=4.71, that is also acid. Another material that shows a better retention in acid

environment is MIP 1 4VBA. MIP 6 demonstrates a higher retention in pH=8 and MIP 7, on the other hand, in pH=6.6.

CHAPTER V

5. CONCLUSION

This research had as main objectives the synthetisation and characterization of molecularly imprinted polymers (MIPs) for sialic acid adsorption. To evaluate and compare the efficiency of the synthesized MIPs, specifically their retention regarding sialic acid, two methods for sialic acid quantification were used, after batch adsorption, to also assess which would be the best approach for those materials and conditions.

Comparing the two methods used, the derivatization with thiobarbituric acid (TBA) with further analysis by visible UV, seemed to be the less reliable and error prone, once it has many steps and reagents inherent of the derivatization process, which could lead to errors passive to be subsequently loaded. For that reason, HPLC analysis to quantify sialic acid, once this analysis can be performed straight after the batch adsorption.

Batch adsorption test carried out with sialic acid solution with concentration 0.1 mol/L showed that MIP 1 was the one the highest sialic acid retention. Regarding the evaluation of this behaviour in different pHs, in an acid environment, MIP 1 showed highest retention, and MIP 2 and MIP 3 presented a largest retention in pH=8. Those three materials were synthesised using DMF as solvent, and sialic acid, used as template, had as concentrations 0.060 mol/L, 0.116 mol/L and 0.258 mol/L, respectively for MIP 1, MIP 2, and MIP 3.

Concerning the batch adsorption teste for sialic acid solution with concentration 1.0 mmol/L, MIP 1 again was the material with the largest retention, at pH=2 and, in addition, another material that revealed to retain more in acid pH was MIP 1 4VBA. Furthermore, MIP 7 exhibited a higher retention in pH=6.6 and MIP 6 in a basic environment. All analysed materials also were synthesised with DMF as a solvent, with the only difference for MIP, that had a solution with 58% DMF and 42% ACN. Additionally, the concentrations of sialic acid used as template were 0.060 mol/L, 0.019 mol/L, 0.186 mol/L and 0.160 mol/L, respectively for MIP 1, MIP 6, MIP 7 and MIP1 4VBA.

Furthermore, considering the monomers used to produce the different MIPs were 4-Vinylpiridine and 4-Vinylphenilboronic acid, the ones that had in their structure 4-Vinylpiridine showed a higher sialic acid adsorption in the analysed conditions.

Moreover, MIP 7 demonstrated a greater retention trend when tested with sialic acid solutions with higher concentrations. Besides, relating the concentration of sialic acid used as a template and the retention of this component after the synthetisation, the material with highest retention was MIP 1, which was synthesised with Sialic Acid 0.060 mol/L, and not the most concentrated solution used.

CHAPTER VI

6. FUTURE WORK

In order to improve and continue the exploitation of molecularly imprinted polymers with sialic acid as template molecule, and to better investigate other possibilities to produce polymers with sialic acid molecules in their structures, it is proposed the following research lines, taking into account the results obtained in this research:

- Extension of the investigation regarding the quantification of sialic acid adsorption in different conditions, namely pH, temperature and sialic acid concentration, to guarantee that the method of quantification is reliable and to obtain a deeper study and evaluation of the produced MIPs.
- Consideration of human body conditions for further evaluation, in order to evaluate the viability for application of the produced MIPs for virus targeting, such as Influenza, HIV and SARS CoV 2.
- Development of polymers with polyvalent interactions with sialic acid molecules, to inhibit the viral attachment and replication. In this research line, it is considered the mechanism used for virus replication. Influenza virus, for example, contain three primary proteins, which one is hemagglutinin, the most abundant, that binds to sialic acid present on the epithelial cell surface, allowing the viral replication. Polymers with sialic acid molecules in their extreme structures, can block the virus from attaching to cells, once hemagglutinin would bind to sialic acids from the polymers rather than the ones in the cells [119]–[121]. For those study, the obstacles regarding sialic acid quantification must be considered for the evaluation of those produced polymers, to facilitate the investigation.

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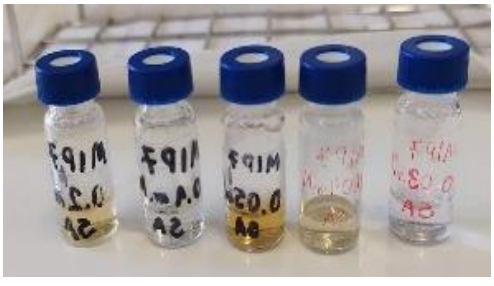
APENDIXES

APENDIX 1 – 1st Batch

1. Data regarding the analysis of 1st Batch using derivatisation with TBA (0.1 mM)

1st Batch					
Derivatization with TBA (0.1 mM) (1)					
MIP 7					
Initial Concentration (mmol/L)	0.03	0.04	0.05	0.1	0.2
Final Concentration (mmol/L)	0.015979	0.04187	0.048528	0.10253	0.140997
mass (mg)	20.5	20.3	19.8	20	19.6
Retention (µmol/g)	3.41983	-0.46062	0.371745	-0.63249	15.05174
NIP 7					
Initial Concentration (mmol/L)	0.03	0.04	0.05	0.1	0.2
Final Concentration (mmol/L)	0.022636	0.030774	0.039651	0.081817	0.206096
mass (mg)	19.9	19.9	20.6	19.7	20.2
Retention (µmol/g)	1.850129	2.318145	2.511933	4.615016	-1.50881
Derivatization with TBA (0.1 mM) (2)					
MIP 7					
Initial Concentration (mmol/L)	0.03	0.04	0.05	0.1	0.2
Final Concentration (mmol/L)	0.024856	0.044089	0.052227	0.117325	0.128421
mass (mg)	20.5	20.3	19.8	20	19.6
Retention (µmol/g)	1.254696	-1.00723	-0.56229	-4.33126	18.25986
NIP 7					
Initial Concentration (mmol/L)	0.03	0.04	0.05	0.1	0.2
Final Concentration (mmol/L)	0.026335	0.045569	0.059624	0.067022	0.197219
mass (mg)	19.9	19.9	20.6	19.7	20.2
Retention (µmol/g)	0.92079	-1.39921	-2.33597	8.370115	0.688484

2. Samples during derivatisation with TBA (0.1 mM)



3. Data regarding the analysis of 1st Batch using derivatisation with TBA (0.04 mM and 0.07 mM)

1st Batch					
Derivatization with TBA (0.04 mM)					
MIP 7					
Initial Concentration (mmol/L)	0.03	0.04	0.05	0.1	0.2
Reference Absorbance	0.0505	0.0445	0.0815	0.148	0.277
Exprerimental Absorbance	0.061	0.05	0.073	0.155	0.184
Equilibrium Concentration (mmol/L)	0.036238	0.044944	0.044785	0.10473	0.132852
mass (mg)	20.5	20.3	19.8	20	19.6
Retention (µmol/g)	-1.52137	-1.21769	1.316849	-1.18243	17.1296

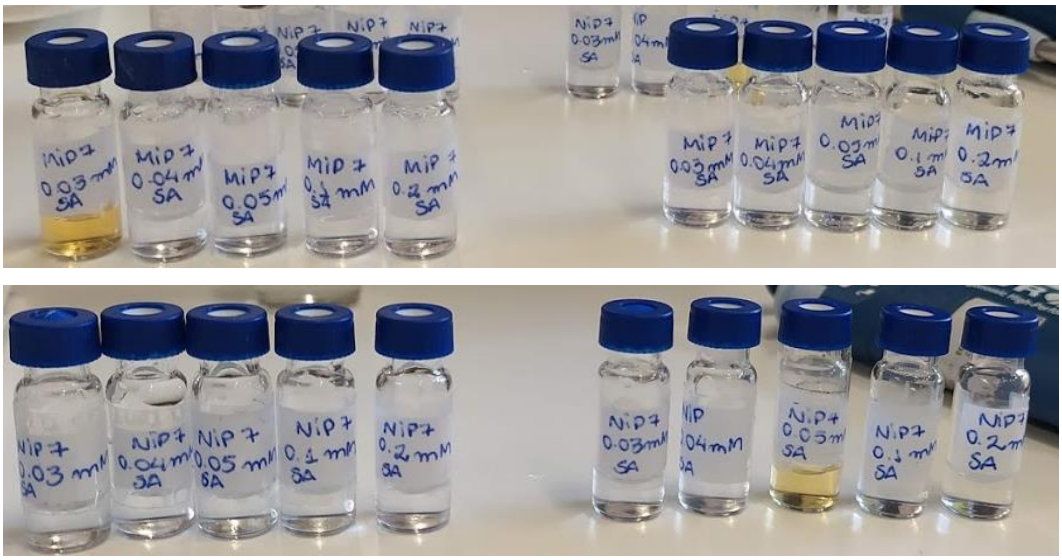
NIP 7					
Initial Concentration (mmol/L)	0.03	0.04	0.05	0.1	0.2
Reference Absorbance	0.0505	0.0445	0.0815	0.148	0.277
Experimental Absorbance	N/A	0.059	0.098	0.117	0.269
Equilibrium Concentration (mmol/L)	N/A	0.053034	0.060123	0.079054	0.194224
mass (mg)	19.9	19.9	20.6	19.7	20.2
Retention (µmol/g)	N/A	-3.2748	-2.45697	5.31623	1.429746

1st Batch					
Derivatization with TBA (0.07 mM)					
MIP 7					
Initial Concentration (mmol/L)	0.03	0.04	0.05	0.1	0.2
Reference Absorbance	0.0505	0.0445	0.0815	0.148	0.277
Exprerimental Absorbance	0.066	0.06	0.74	0.155	0.167
Equilibrium Concentration (mmol/L)	0.039208	0.053933	0.453988	0.10473	0.120578
mass (mg)	20.5	20.3	19.8	20	19.6
Retention (µmol/g)	-2.24583	-3.43167	-102.017	-1.18243	20.26081

NIP 7					
Initial Concentration (mmol/L)	0.03	0.04	0.05	0.1	0.2
Reference Absorbance	0.0505	0.0445	0.0815	0.148	0.277
Experimental Absorbance	0.046	0.063	0.09	0.107	0.276

Equilibrium Concentration (mmol/L)	0.027327	0.056629	0.055215	0.072297	0.199278
mass (mg)	19.9	19.9	20.6	19.7	20.2
Retention (µmol/g)	0.671675	-4.17819	-1.26571	7.031143	0.178718

4. Samples during derivatisation with TBA (0.07 mM and 0.04 mM)



APENDIX 2 – 2nd Batch

1. Data regarding the analysis of 2nd Batch using derivatisation with TBA (0.1 mM)

2nd Batch - Derivatisation				
MIP 7				
Concentration (mM)	1.5	1	0.5	0.2
UV Reference	0.684	1.052	0.686	0.363
UV abs	1.239	0.39	0.588	0.319
Retention	-0.8114	0.629278	0.142857	0.121212
Exp mass (mg)	19.9	19.5	20.9	21
Ce (mM)	2.717105	0.370722	0.428571	0.175758
Qe (µmol/g)	-305.805	161.3532	17.08817	5.772006
NIP 7				
Concentration (mM)	1.5	1	0.5	0.2
UV Reference	0.684	1.052	0.686	0.363
UV abs	1.355	0.637	0.545	0.315
Retention	-0.98099	0.394487	0.205539	0.132231
Exp mass (mg)	20.7	20.5	19.9	20.9
Ce (mM)	2.971491	0.605513	0.39723	0.173554
Qe (µmol/g)	-355.433	96.21627	25.82153	6.326861
Reillex 425				
Concentration (mM)	1.5	1	0.5	0.2

UV Reference	0.684	1.052	0.686	0.363
UV abs	0.453	0.843	0.466	0.296
Retention	0.337719	0.198669	0.3207	0.184573
Exp mass (mg)	19.4	20	20.4	20.1
Ce (mM)	0.993421	0.801331	0.33965	0.163085
Qe (µmol/g)	130.5616	49.6673	39.30143	9.182736
Reillex 402				
Concentration (mM)	1.5	1	0.5	0.2
UV Reference	0.684	1.052	0.686	0.363
UV abs	1.054	1.052	0.656	0.291
Retention	-0.54094	0	0.043732	0.198347
Exp mass (mg)	21.1	20.5	19.5	19.5
Ce (mM)	2.311404	1	0.478134	0.160331
Qe (µmol/g)	-192.276	0	5.606638	10.17165

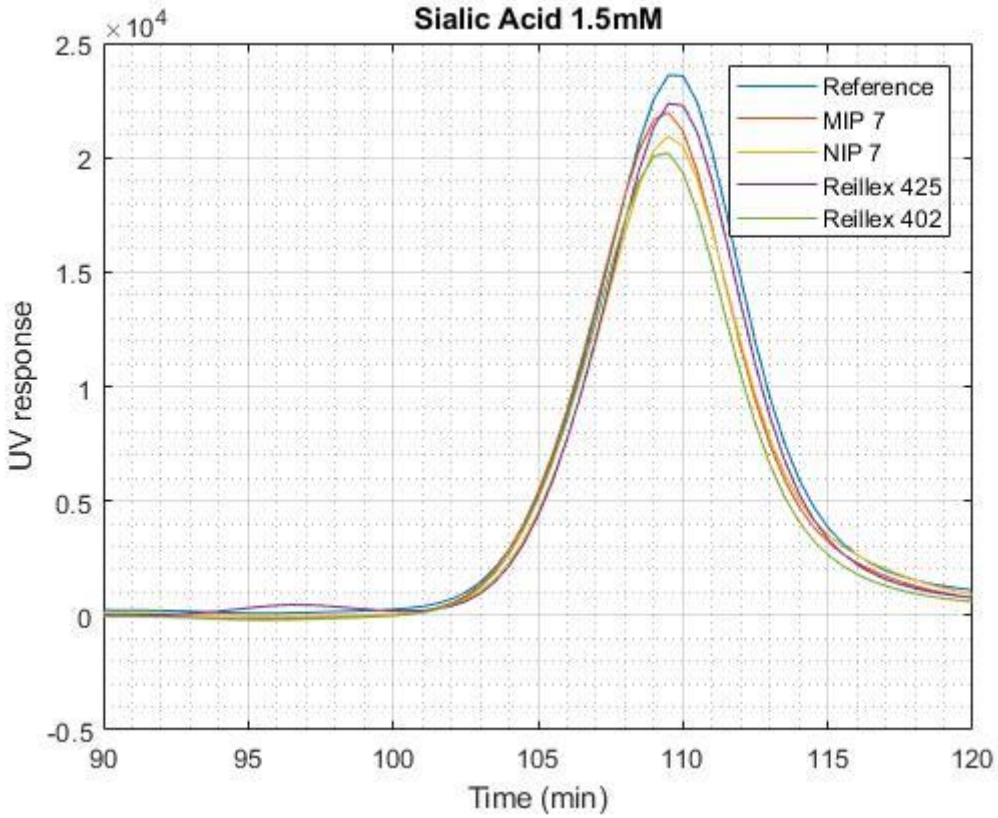
2. Data regarding the analysis of 2nd Batch using HPLC

		2nd Batch			
Sample	**Concentration (mM)**	**0.2**	**0.5**	**1**	**1.5**
Reference	Exp Mass (mg)	-	-	-	-
	Area	14282.72	47198.07	98092.75	147879.4
MIP 7	Exp Mass (mg)	19.9	19.5	20.9	21
	Area	12762.73	38815.85	95403.79	137624.3
NIP7	Exp Mass (mg)	20.7	20.5	19.9	20.9
	Area	11816.61	32800.61	85703.97	133691.1
Reillex 425	Exp Mass (mg)	19.4	20	20.4	20.1
	Area	13445.87	42313.08	80902.66	136514.2
Reillex 402	Exp Mass (mg)	21.1	20.5	19.5	19.5
	Area	17241.53	42958.53	84909.62	126722.8

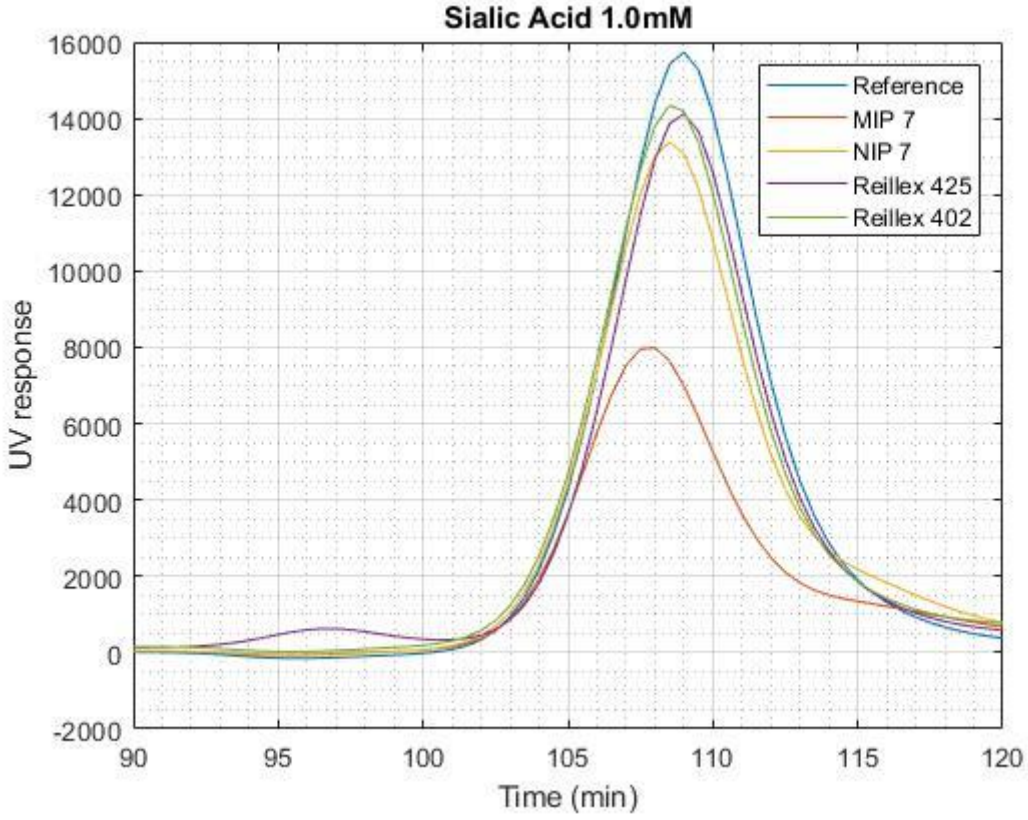
q (µmol/g)				
Concentration (mM)	**0.2**	**0.5**	**1**	**1.5**
MIP 7	5.347818479	22.76879	6.557998	24.76725
NIP 7	8.341239678	37.20042	31.7328	34.43018
Reillex 425	3.02018907	12.93746	42.95178	28.67721
Reillex 402	-9.818015802	10.95419	34.46014	55.02566
Ce (mM)				
Concentration (mM)	0.2	0.5	1	1.5
MIP 7	0.178715682	0.411202	0.972588	1.395978
NIP 7	0.165467268	0.347478	0.873703	1.356082
Reillex 425	0.188281666	0.44825	0.824757	1.384718
Reillex 402	0.241432027	0.455088	0.865605	1.2854

3. HPLC Chromatograms

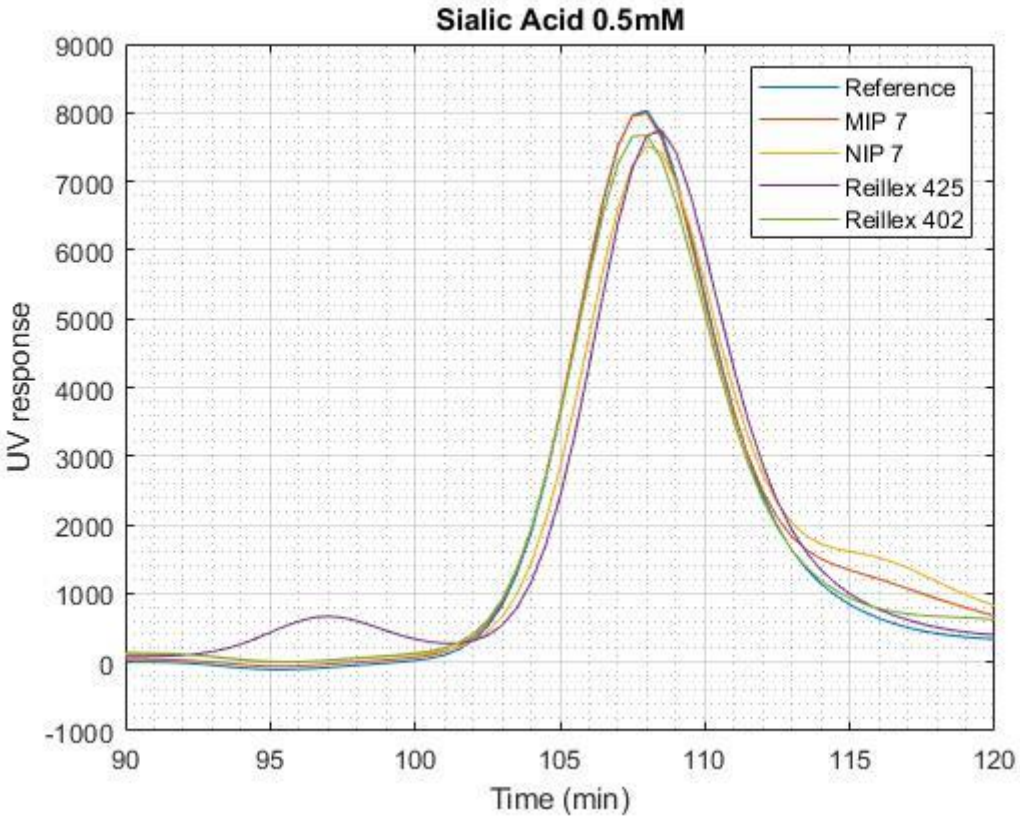
a. Sialic Acid 1.5 mM



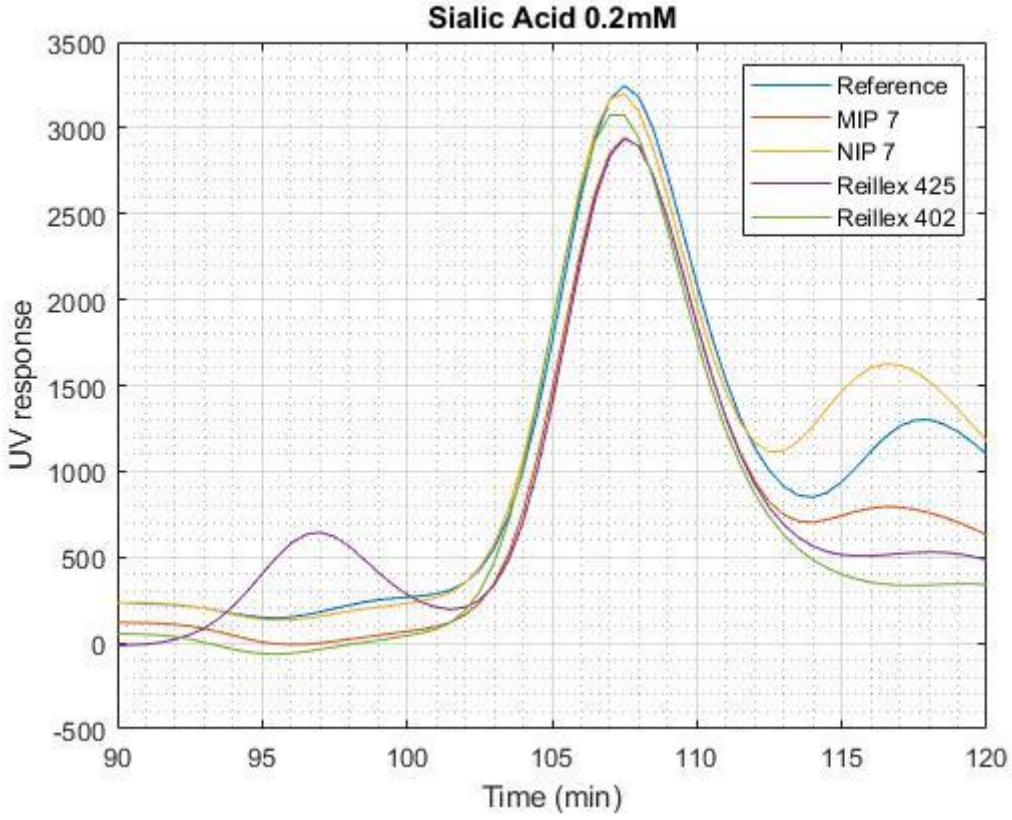
b. Sialic Acid 1.0 mM



c. Sialic Acid 0.5 mM



d. Sialic Acid 0.2 mM



APENDIX 3 – 3rd Batch

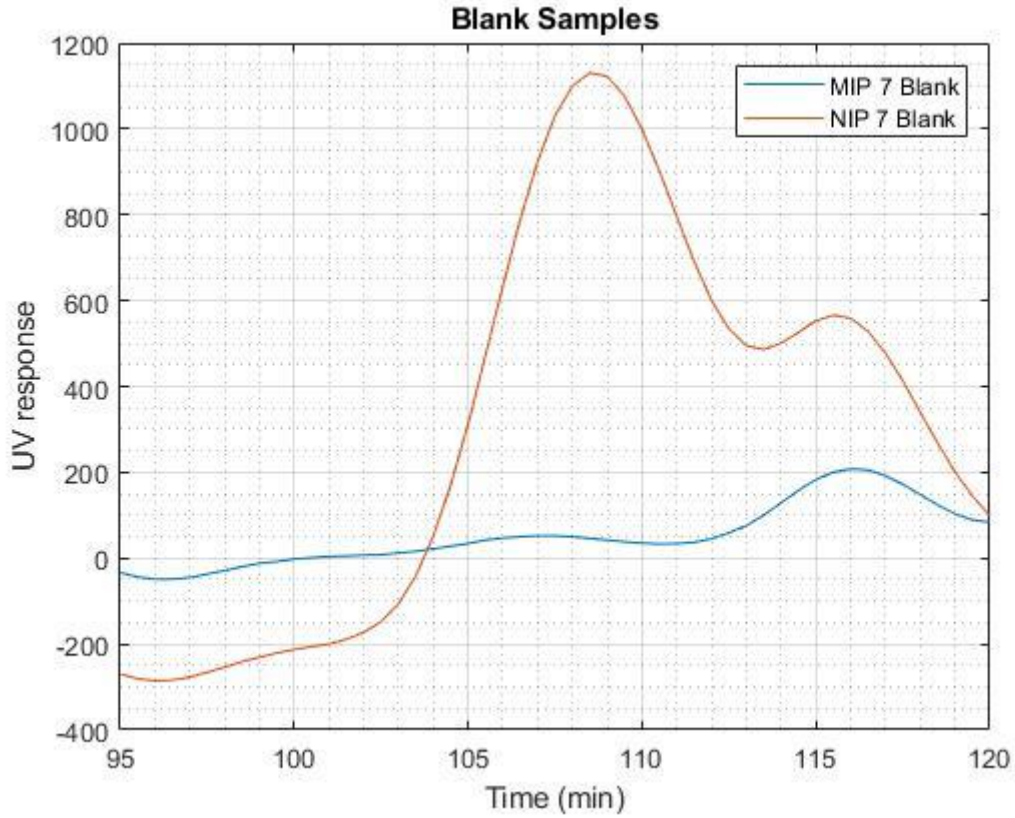
1. Data regarding the analysis of 3rd Batch using HPLC

		3rd Batch				
Sample	**Concentration (mM)**	**0.2**	**0.5**	**1**	**1.5**	**Blank**
Reference	Exp Mass (mg)	-	-	-	-	
	Area	13772.89	49545.52	97506.17	149653.2	
MIP 7	Exp Mass (mg)	20.5	19.2	20.4	21.4	19.6
	Area	15926.5	46986.43	92961.61	139845.6	
NIP7	Exp Mass (mg)	20	19.5	20.6	19.6	19.1
	Area	22381.28	48795.6	86862.99	130709.1	4850.722
Reillex 425	Exp Mass (mg)	21.1	19.3	19	20.2	
	Area	14823.16	41777.08	91037.28	131351.9	
Reillex 402	Exp Mass (mg)	19.1	20.4	20.1	20.9	
	Area	15265.6	44775.35	89109	135563	

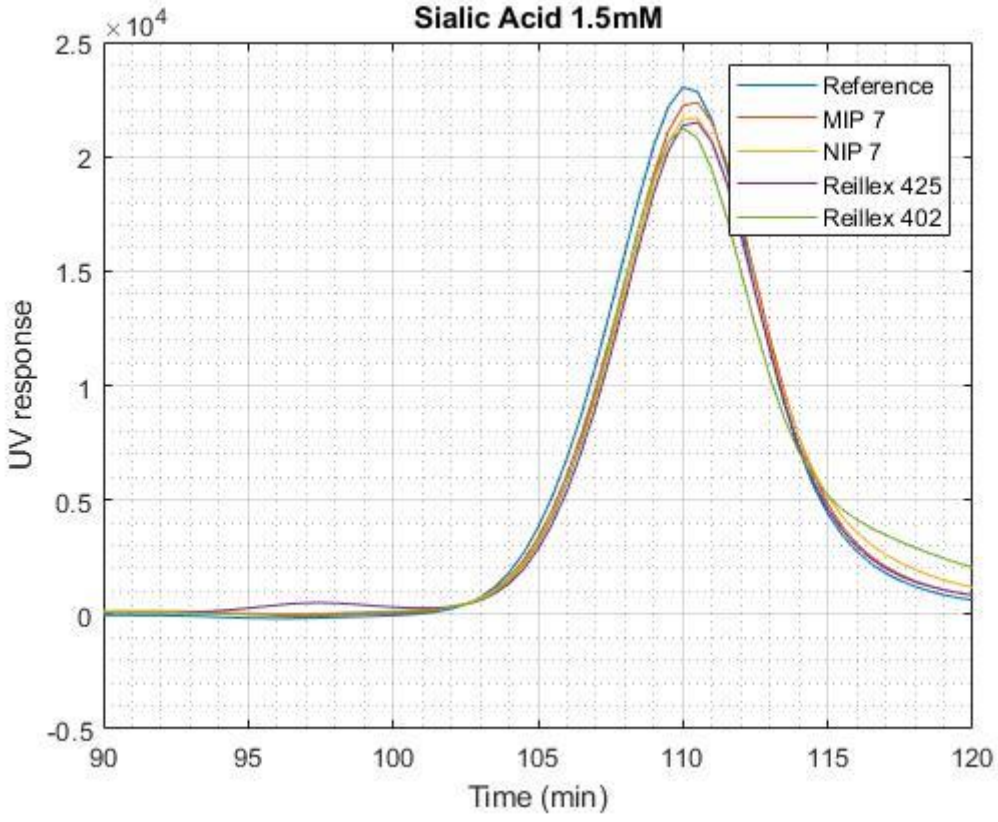
q (µmol/g)				
Concentration (mM)	0.2	0.5	1	1.5
MIP 7	-7.6E-06	6.725443	11.42351	22.96806
NIP 7	-31.2512	1.940522	26.49368	48.43885
Reillex 425	-3.61405	20.31012	17.4588	45.4054
Reillex 402	-5.67437	11.79885	21.42273	33.7868
Ce (mM)				
Concentration (mM)	0.2	0.5	1	1.5
MIP 7	0.231273	0.474174	0.953392	1.401697
NIP 7	0.325005	0.492432	0.890846	1.31012
Reillex 425	0.215251	0.421603	0.933657	1.316562
Reillex 402	0.221676	0.451861	0.913881	1.358771

2. HPLC Chromatograms

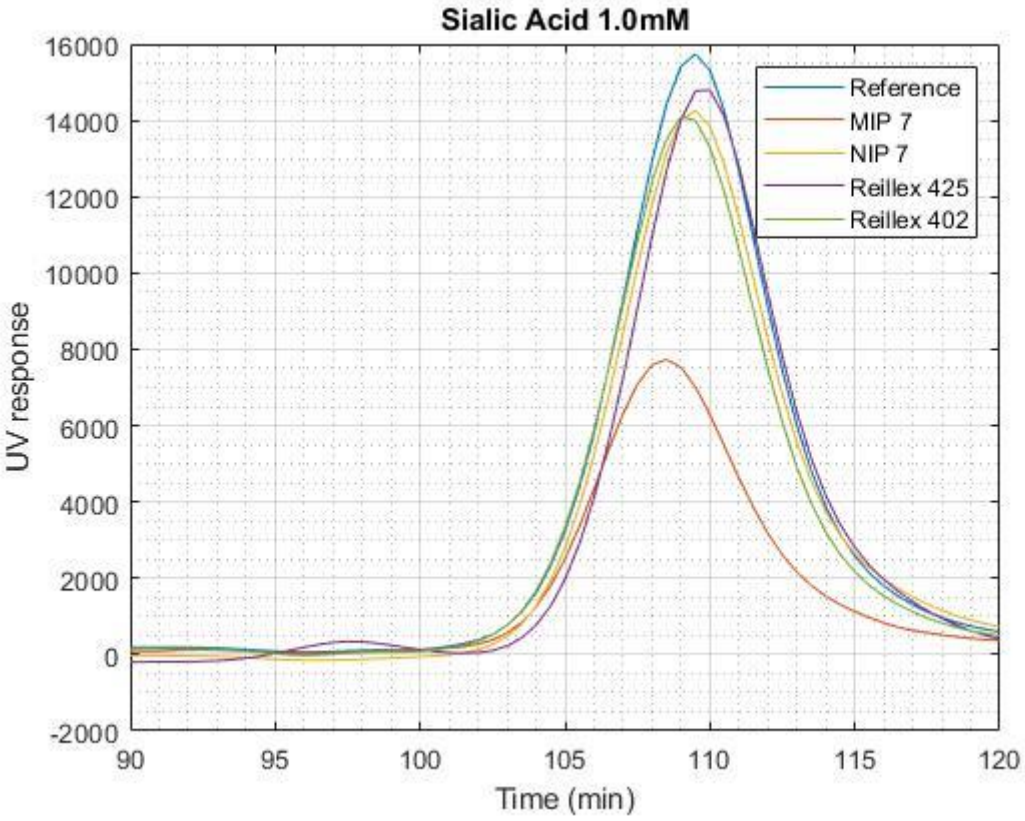
a. Blank



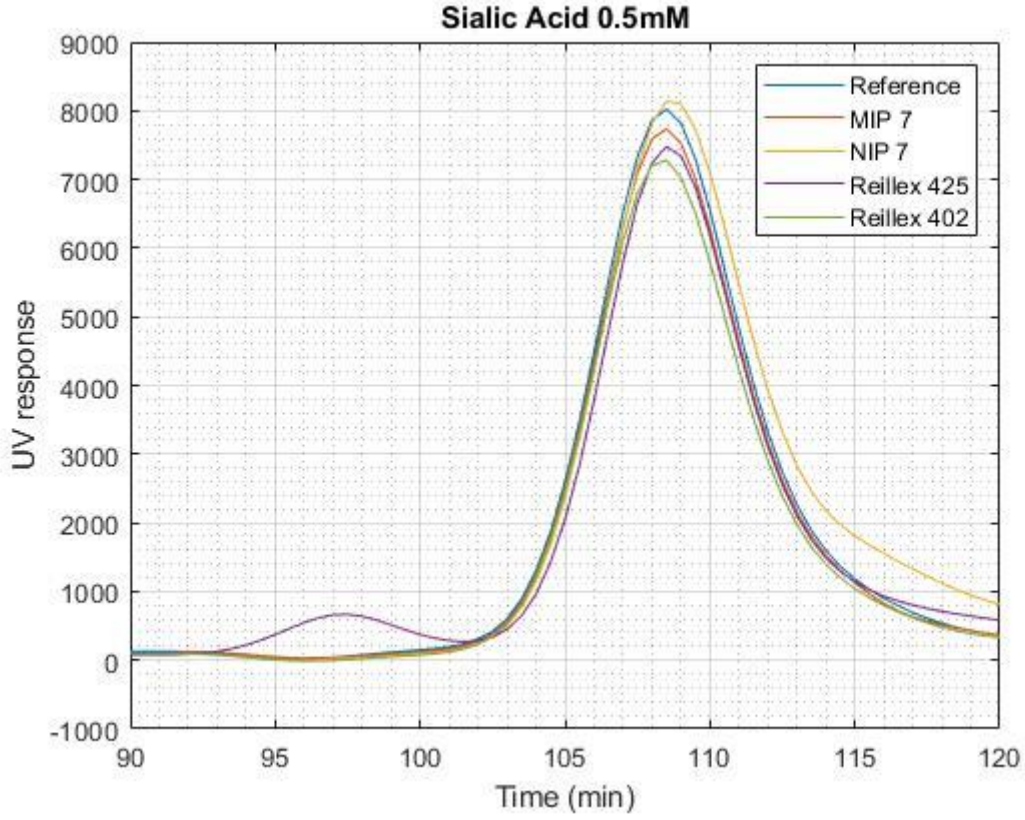
b. Sialic Acid 1.5 mM



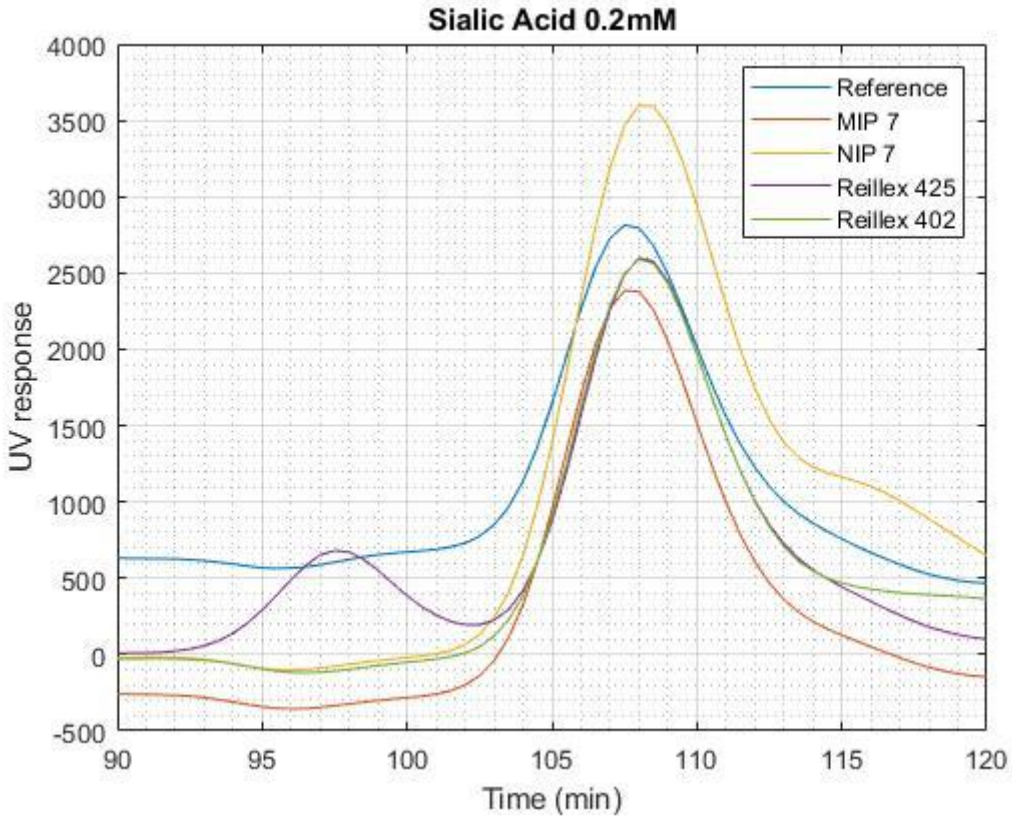
c. Sialic Acid 1.0 mM



d. Sialic Acid 0.5 mM



e. Sialic Acid 0.2 mM



APENDIX 4 – 4th Batch

1. Data regarding the analysis of 4th Batch using HPLC

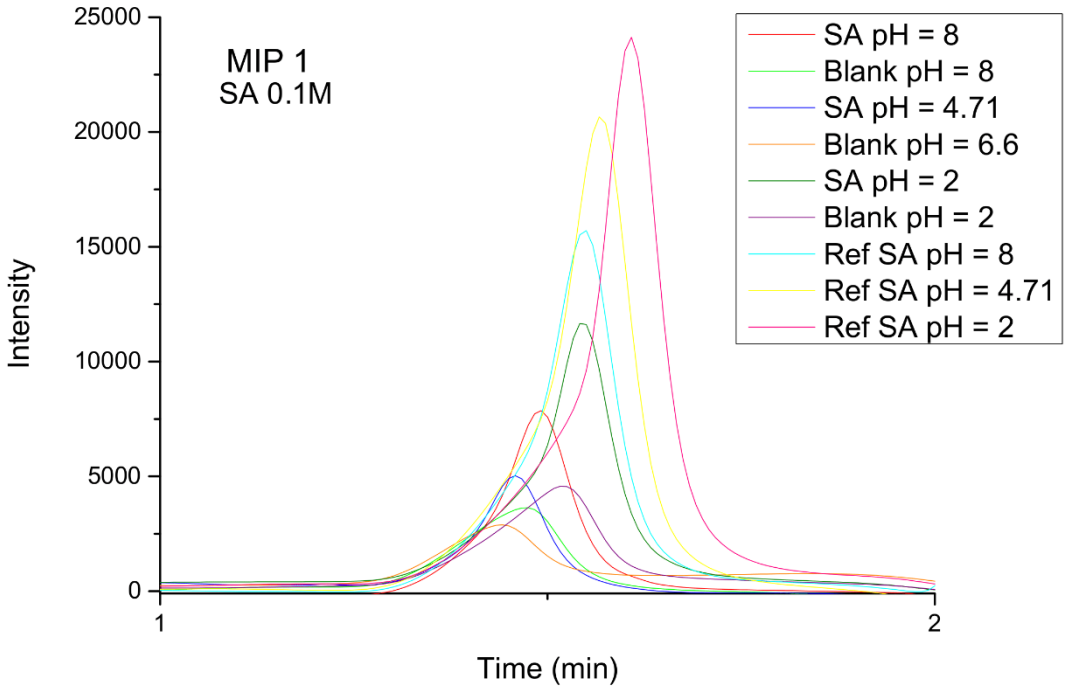
		Blank			SA 0.1M + Buffer		
		pH=8	**pH=6,6**	**pH=2**	**pH=8**	**pH=4,71**	**pH=2**
MIP 1	Mass (mg)	19.4	20.7	19.8	20.6	19.5	20.5
	Area	551.6305	346.7974	643.0913	984.2172	565.4686	1372.708
NIP 1	Mass (mg)	19.2	20.6	19.2	19.3	20.2	19.4
	Area	274.9181	414.4044	877.1191	1162.53	2212.401	1625.462
MIP 2	Mass (mg)	19.5	19.3	19.9	20.3	19.8	19.5
	Area	409.0868	487.7499	511.5178	984.8639	1365.661	1779.693
NIP 2	Mass (mg)	20.4	19.9	19.3	20	20.2	20.9
	Area	641.7403	386.2594	810.5187	2111.871	1179.462	1358.566
MIP 3	Mass (mg)	19.7	19.2	19.7	19.8	20.3	19.8
	Area	380.994	427.4108	812.7309	974.1724	1748.545	1749.949
NIP 3	Mass (mg)	20.5	19.8	19.7	19	19.4	20.8
	Area	460.7433	622.9657	559.6799	1072.914	1809.792	1517.786
Reillex 425	Mass (mg)	20.8	20.1	19.4	20.1	19.6	20.4
	Area	280.893	535.5022	711.4902	1801.353	1142.84	1425.375
Reillex 402	Mass (mg)	20.7	20.2	20.9	19.8	20.8	19.5
	Area	268.9212	607.8186	506.456	877.604	1347.598	1433.802

Reference	Area
pH=8	1952.373
pH=4.71	2657.186
pH=2	2804.939

		SA 0.1 M + Buffer		
		pH=8	pH=4.71	pH=2
MIP 1	New Area	432.5867	218.6712	729.6165
	Percentage	0.77843	0.887997	0.626292
	q (µmol/g)	7557.576	9107.664	6110.17
	Ce (mM)	22.15697	11.20028	37.37075
NIP 1	New Area	887.6117	1797.997	748.3433
	Percentage	0.545368	0.323346	0.733205
	q (µmol/g)	5651.479	3201.441	7558.816
	Ce (mM)	45.46323	92.09289	38.32994
MIP 2	New Area	575.7771	877.9108	1268.176
	Percentage	0.705089	0.669609	0.547878
	q (µmol/g)	6946.685	6763.725	5619.258
	Ce (mM)	29.49114	44.96634	64.9556
NIP 2	New Area	1470.13	793.2022	548.047
	Percentage	0.247003	0.701488	0.804614
	q (µmol/g)	2470.033	6945.424	7699.651
	Ce (mM)	75.29967	40.6276	28.07082
MIP 3	New Area	593.1783	1321.134	937.2177
	Percentage	0.696176	0.502807	0.665869
	q (µmol/g)	7032.078	4953.764	6725.947
	Ce (mM)	30.38243	67.66812	48.00403
NIP 3	New Area	612.1711	1186.826	958.1064
	Percentage	0.686448	0.553352	0.658422
	q (µmol/g)	7225.765	5704.661	6330.977
	Ce (mM)	31.35524	60.78892	49.07395
Reillex 425	New Area	1520.46	607.3378	713.8845
	Percentage	0.221225	0.771436	0.74549
	q (µmol/g)	2201.242	7871.793	7308.727
	Ce (mM)	77.87751	31.10767	36.56497
Reillex 402	New Area	608.6828	739.7794	927.3461
	Percentage	0.688234	0.721593	0.669388
	q (µmol/g)	6951.862	6938.393	6865.519
	Ce (mM)	31.17657	37.89129	47.49841

2. HPLC Curves

a. MIP 1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SA pH = 4.71
Blank pH = 6.6
SA pH = 2
Blank pH = 2
Ref SA pH = 8
Ref SA pH = 4.71
Ref SA pH = 2
Intensity
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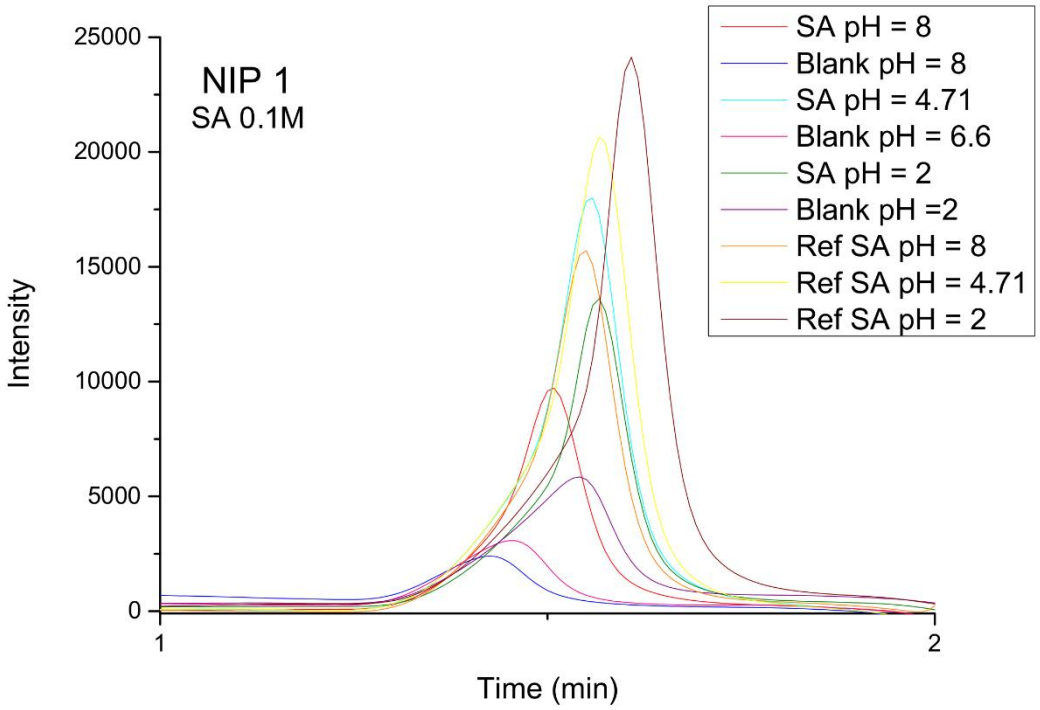
15000
10000
5000
0
1
2
Time (min)

b. NIP 1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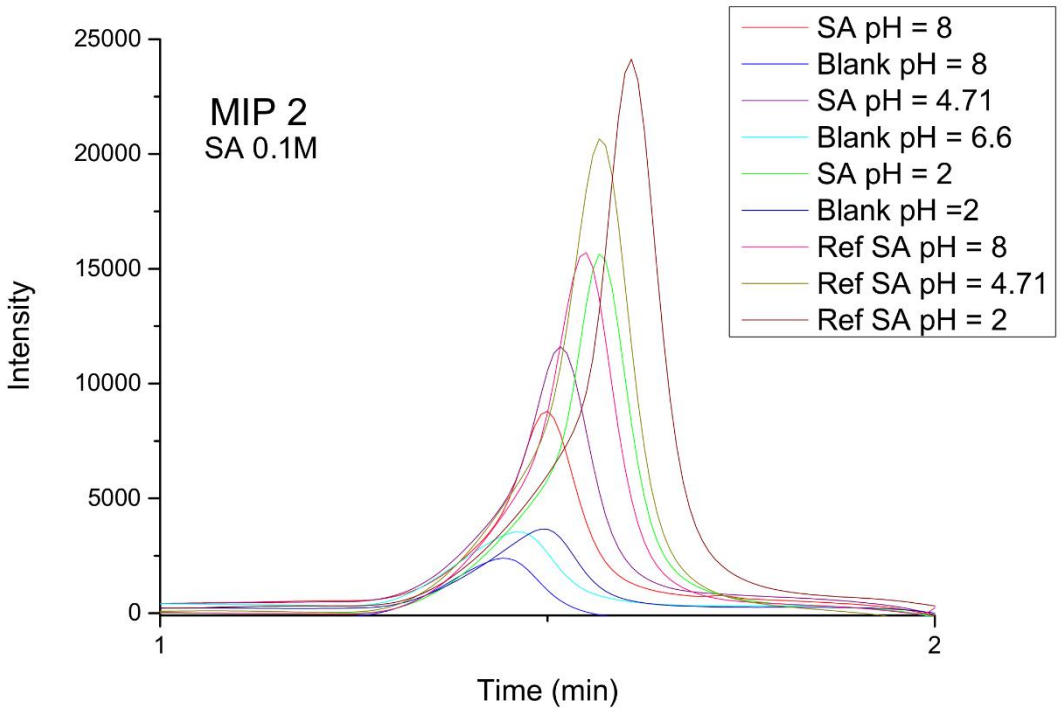
SA 0.1M
SA pH = 8
Blank pH = 8
SA pH = 4.71
Blank pH = 6.6
SA pH = 2
Blank pH =2
Ref SA pH = 8
Ref SA pH = 4.71
Ref SA pH = 2
Intensity
25000
20000
15000
10000
5000
0
1
2
Time (min)

c. MIP 2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SA 0.1M
SA pH = 8
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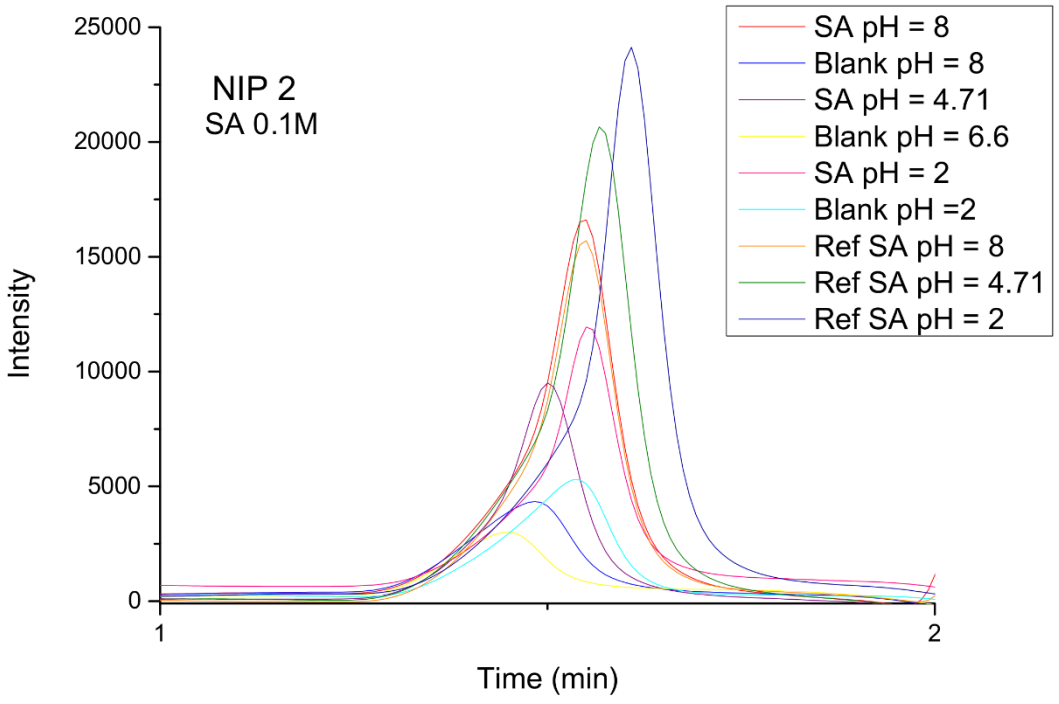
SA pH = 4.71
Blank pH = 6.6
SA pH = 2
Blank pH =2
Ref SA pH = 8
Ref SA pH = 4.71
Ref SA pH = 2
Intensity
25000
20000
15000
10000
5000
0
1
2
Time (min)

d. NIP 2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SA pH = 4.71
Blank pH = 6.6
SA pH = 2
Blank pH =2
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Ref SA pH = 4.71
Ref SA pH = 2
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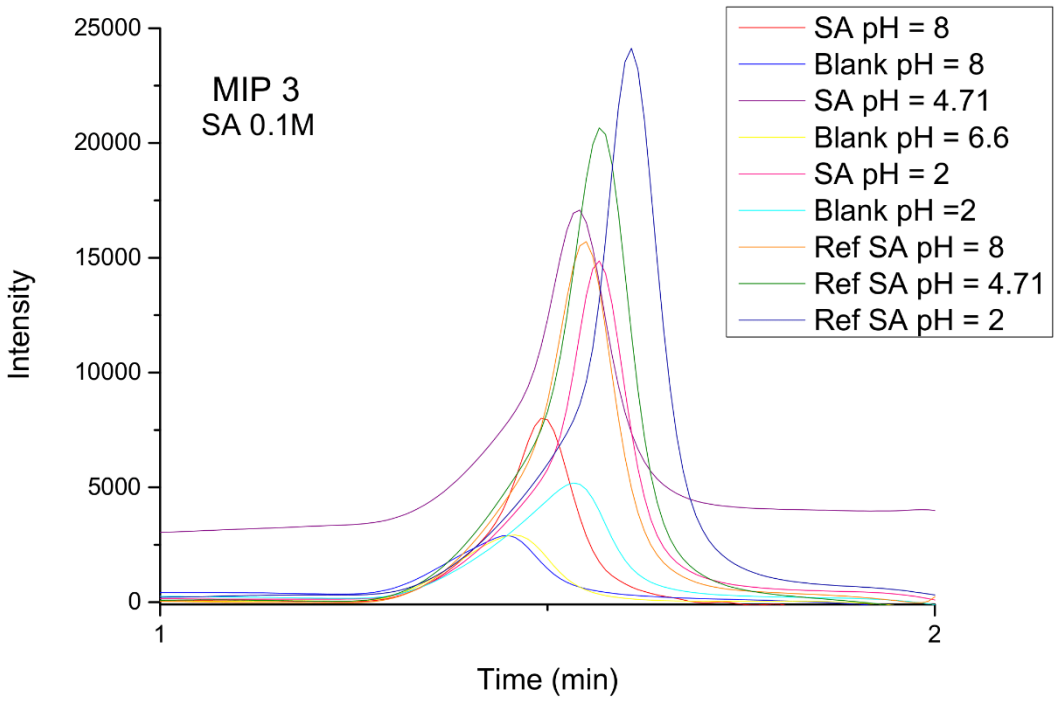
15000
10000
5000
0
1
2
Time (min)

e. MIP 3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Intensity
Time (min)
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SA pH = 4.71
Blank pH = 6.6
SA pH = 2
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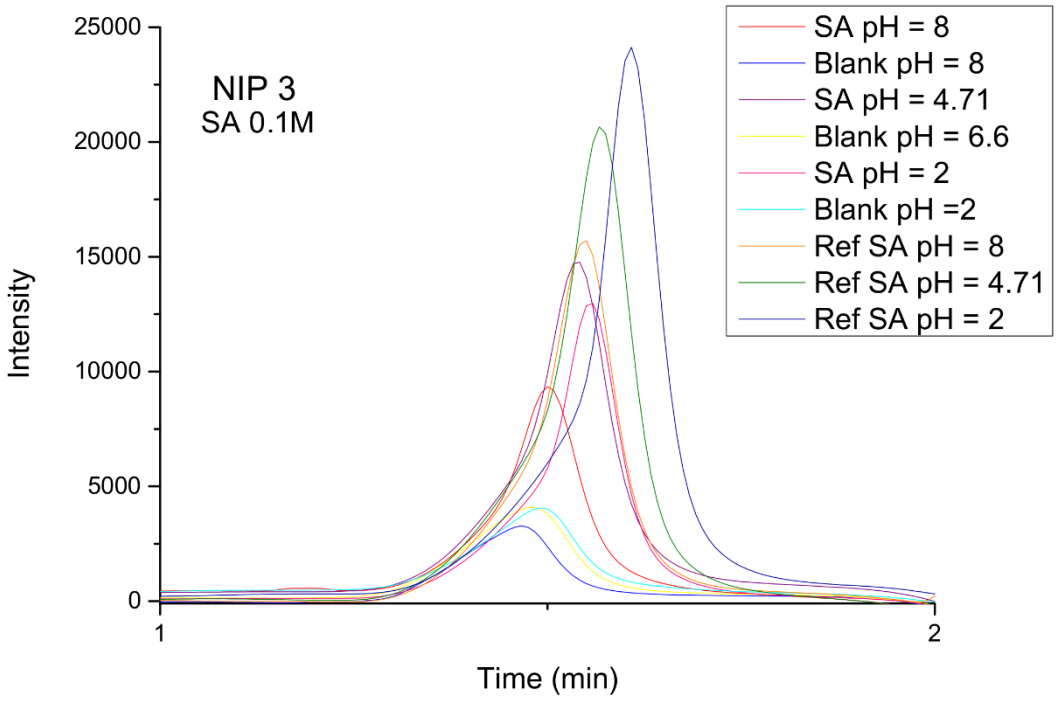
Ref SA pH = 4.71
Ref SA pH = 2

f. NIP 3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Intensity
Time (min)
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Blank pH = 6.6
SA pH = 2
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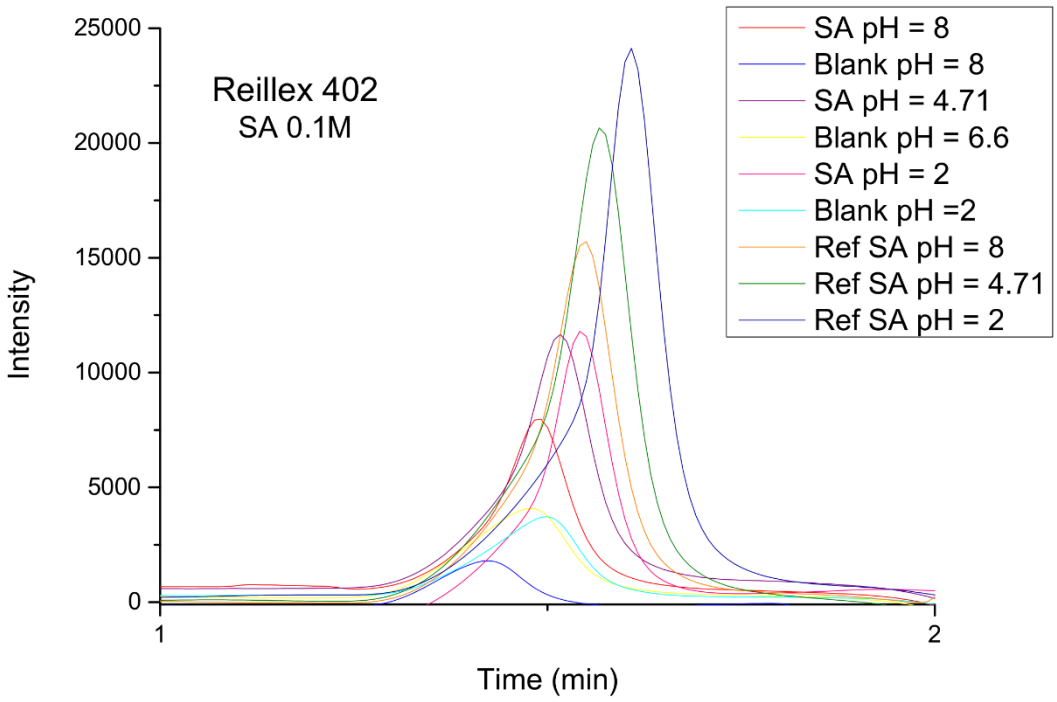
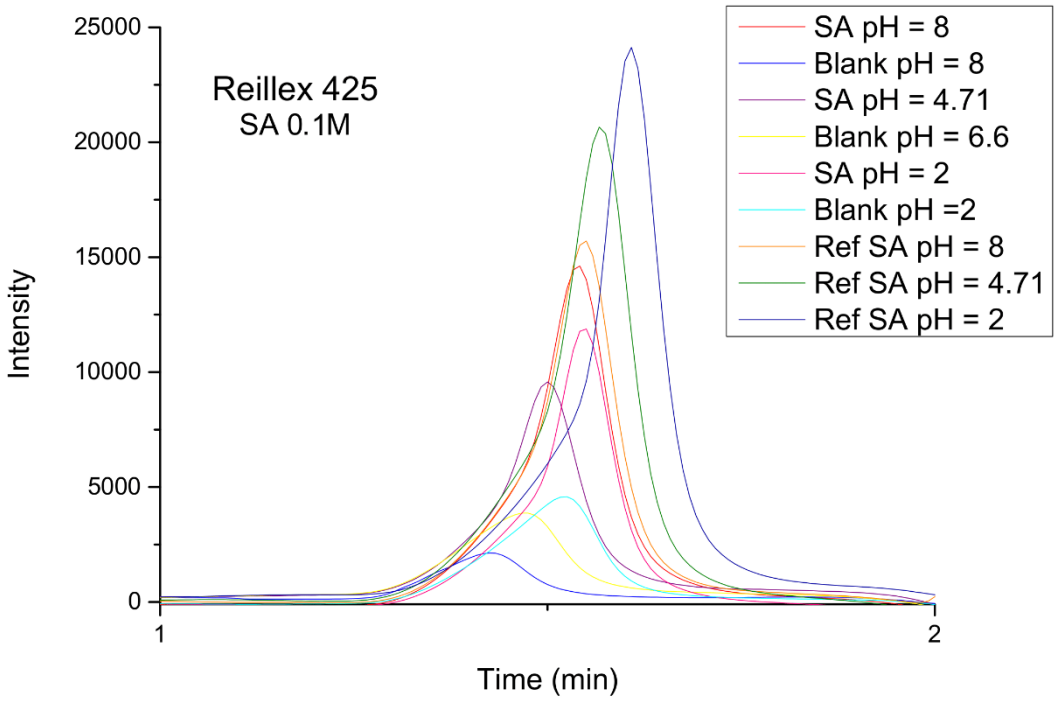
Ref SA pH = 4.71
Ref SA pH = 2

g. Reillex 402



h. Reillex 425



APENDIX 5 – 5th Batch

1. Data regarding the analysis of 5th Batch using HPLC

		Blank			SA 1 mM + Buffer		
		pH=8	**pH=6.6**	**pH=2**	**pH=8**	**pH=6.6**	**pH=2**
	Mass (mg)	20.6	20.1	20.2	20.1	20.2	20.2
MIP 1	Area	1683.436	2423.05	9111.54	1803.517	2653.167	1682.154
	Mass (mg)	19.6	20.7	19.4	19.2	19.4	19.2
NIP 1	Area	1814.691	2589.879	8559.869	10854.81	3154.136	1621.795
	Mass (mg)	20.6	20.1	19.3	20.3	19.9	20.7
MIP 6	Area	2000.347	2774.415	6793.944	1844.438	2724.266	7534.802
	Mass (mg)	20.3	20	19.1	20.9	20.1	19.8
NIP 6	Area	2012.768	2718.516	7556.76	1703.641	2827.463	6216.201
	Mass (mg)	19	20	20.3	20.2	19.6	20.3
MIP 7	Area	1858.358	3054.895	6889.53	1690.048	2679.543	7582.348
	Mass (mg)	19.9	19.2	20.9	20.6	19.9	20.3
NIP 7	Area	1801.319	2758.918	6584.871	1991.796	3055.554	8706.644
	Mass (mg)	20.6	19.4	19.1	20.6	20.4	19.1
MIP 1 4VBA	Area		2746.297	8996.902		2777.678	8098.863
	Mass (mg)	20.1	20.6	20.1	19.1	19.2	19.7
NIP 1 4VBA	Area	1720.256	3024.665	8486.765	1813.376	2772.738	9248.845

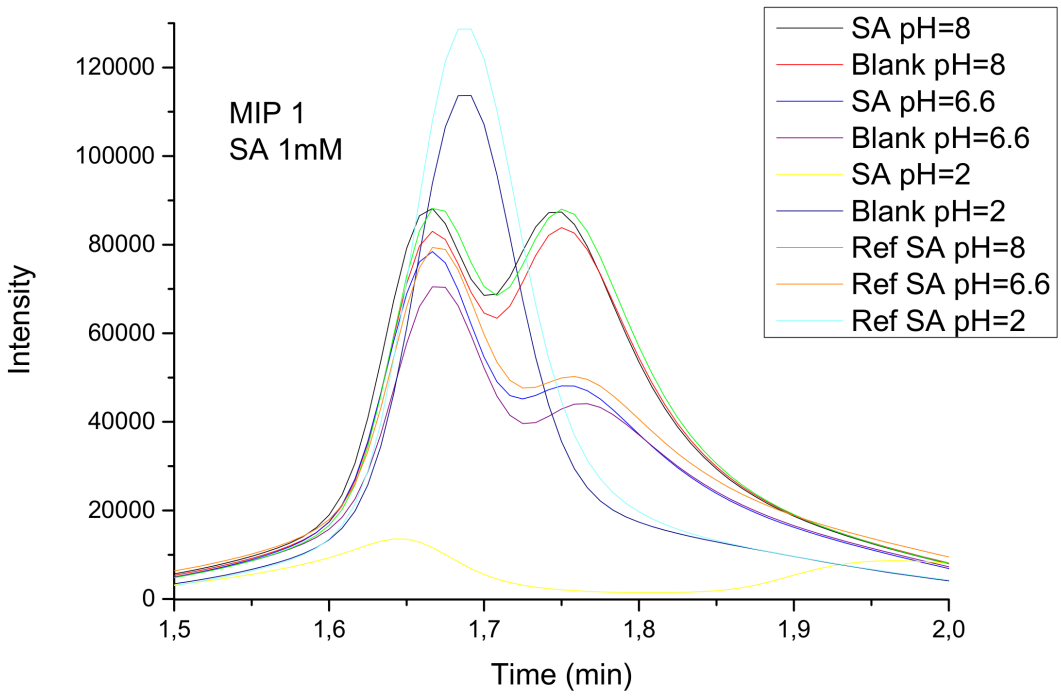
Reference	**Area**
pH=8	1959.85
pH=6.6	2653.425
pH=2	10854.81

		SA 1 mM + Buffer		
		pH=8	**pH=6.6**	**pH=2**
MIP 1	New Area	120.0813	230.1165	-7429.39
	Percentage	0.938729	0.882585	4.790793
	q (μmol/g)	93.4059	87.38462	474.336
	Ce (mM)	0.061271	0.117415	-3.79079
NIP 1	New Area	9040.115	564.2566	-6938.07
	Percentage	-3.61266	0.787348	1.639171
	q (μmol/g)	-376.318	81.16988	170.7469
	Ce (mM)	4.612657	0.287908	-3.5401
MIP 6	New Area	-155.909	-50.1494	740.8584
	Percentage	1.079551	1.0189	0.931748
	q (μmol/g)	106.3597	102.402	90.024
	Ce (mM)	-0.07955	-0.02559	0.378018
NIP 6	New Area	-309.126	108.9474	-1340.56
	Percentage	1.15773	0.958941	1.123499
	q (μmol/g)	110.7875	95.417	113.4848
	Ce (mM)	-0.15773	0.05559	-0.68401

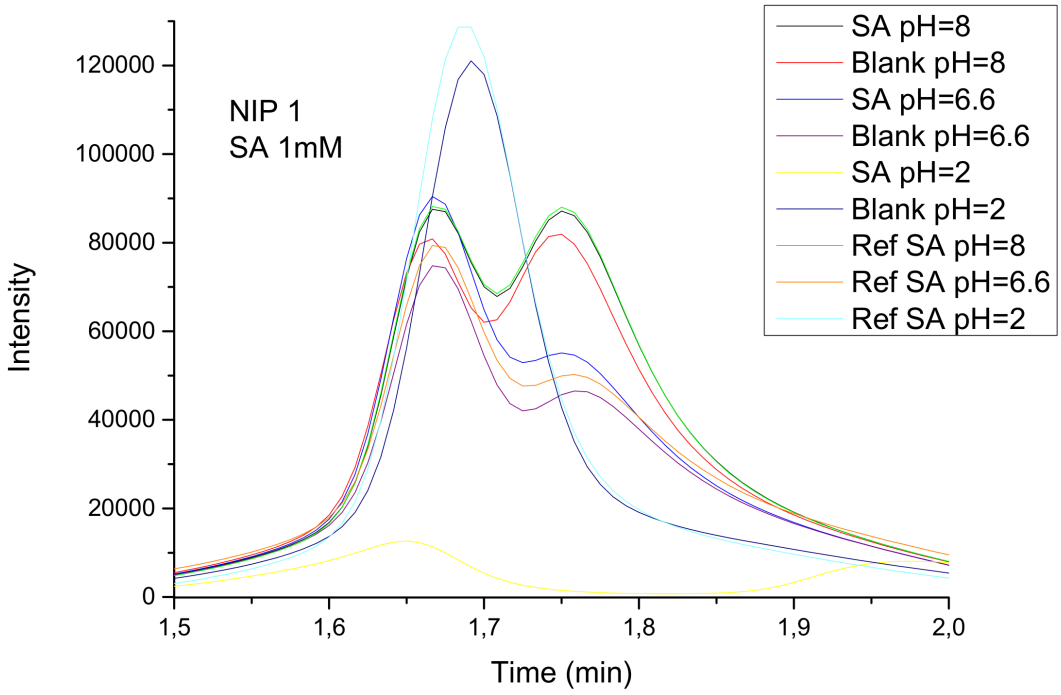
	New Area	-168.311	-375.352	692.818
	Percentage	1.085879	1.141459	0.936174
	q (µmol/g)	107.5128	116.4754	92.2339
MIP 7	Ce (mM)	-0.08588	-0.19152	0.353506
	New Area	190.4774	296.6357	2121.773
	Percentage	0.90281	0.888206	0.804532
	q (µmol/g)	87.65148	89.26698	79.26419
NIP 7	Ce (mM)	0.09719	0.151356	1.08262
	New Area	0	31.3813	-898.039
	Percentage	1	0.988173	1.082732
MIP 1	q (µmol/g)	97.08738	96.87973	113.3751
4VBA	Ce (mM)	0	0.016012	-0.45822
	New Area	93.11945	-251.927	762.0807
	Percentage	0.952486	1.094944	0.929793
NIP 1	q (µmol/g)	99.7368	114.0567	94.39525
4VBA	Ce (mM)	0.047514	-0.12854	0.388847

2. HPLC Curves

a. MIP 1



b. NIP 1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SA pH=6.6
Blank pH=6.6
SA pH=2
Blank pH=2
Ref SA pH=8
Ref SA pH=6.6
Ref SA pH=2
Intensity
Time (min)
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60000
40000
20000
0
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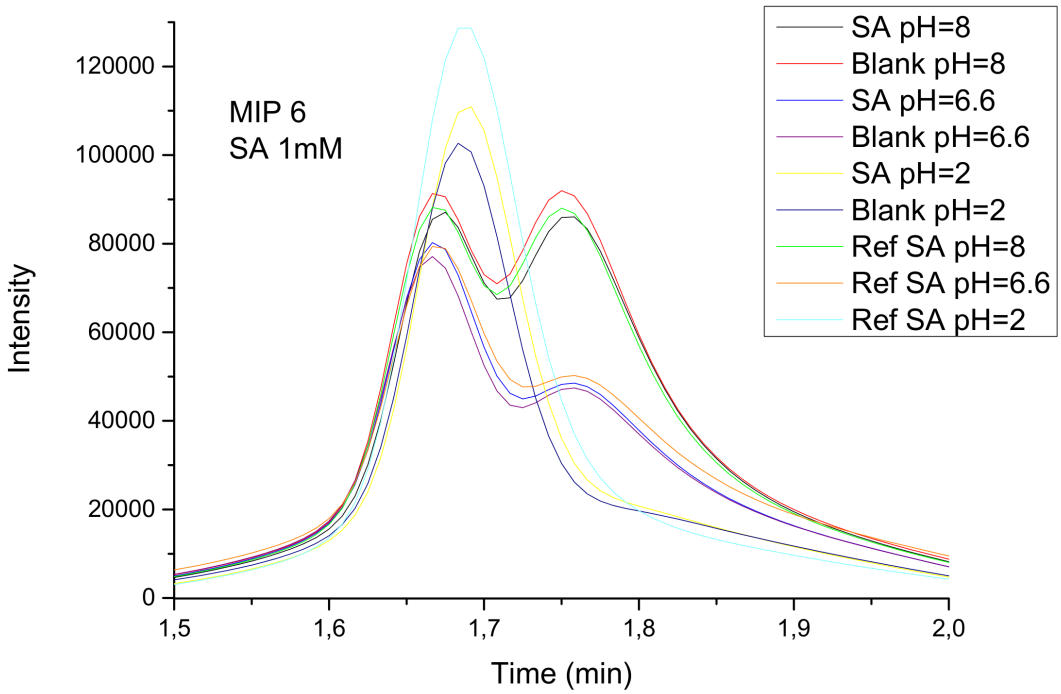
1,6
1,7
1,8
1,9
2,0

c. MIP 6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SA 1mM
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SA pH=6.6
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SA pH=2
Blank pH=2
Ref SA pH=8
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Time (min)
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100000
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40000
20000
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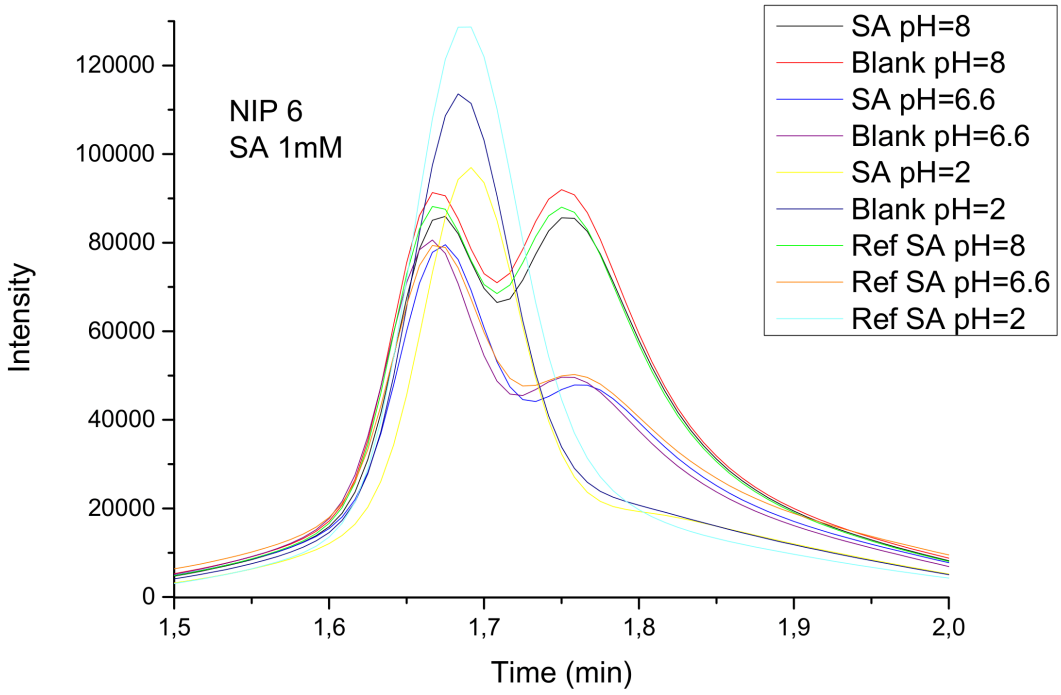
1,6
1,7
1,8
1,9
2,0

d. NIP 6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SA 1mM
SA pH=8
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SA pH=6.6
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Ref SA pH=8
Ref SA pH=6.6
Ref SA pH=2
Intensity
Time (min)
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20000
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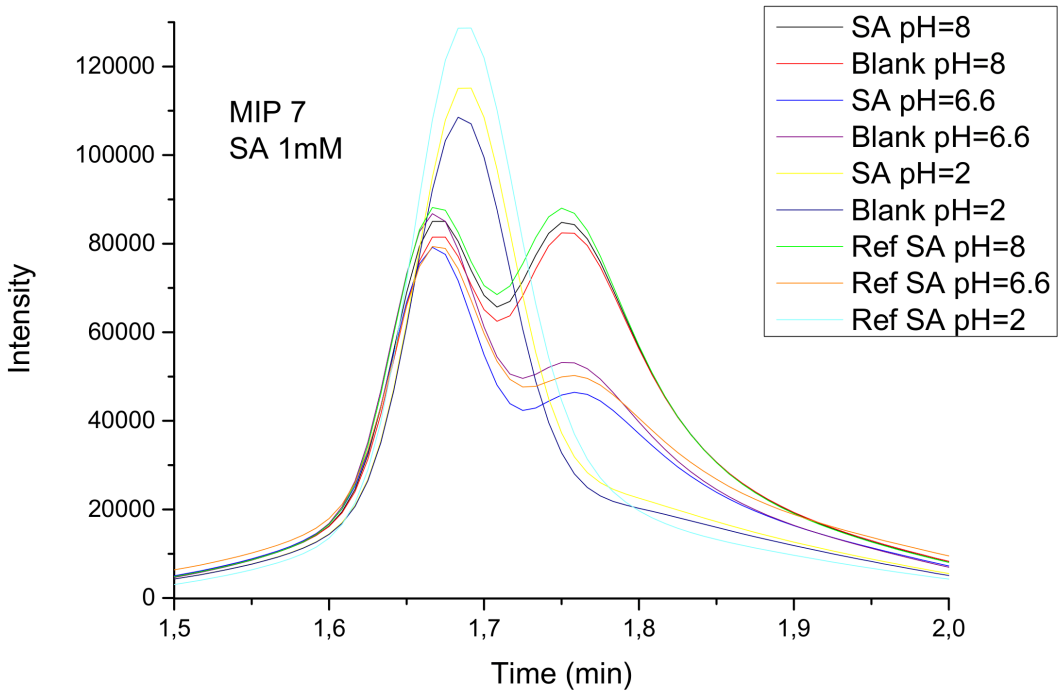
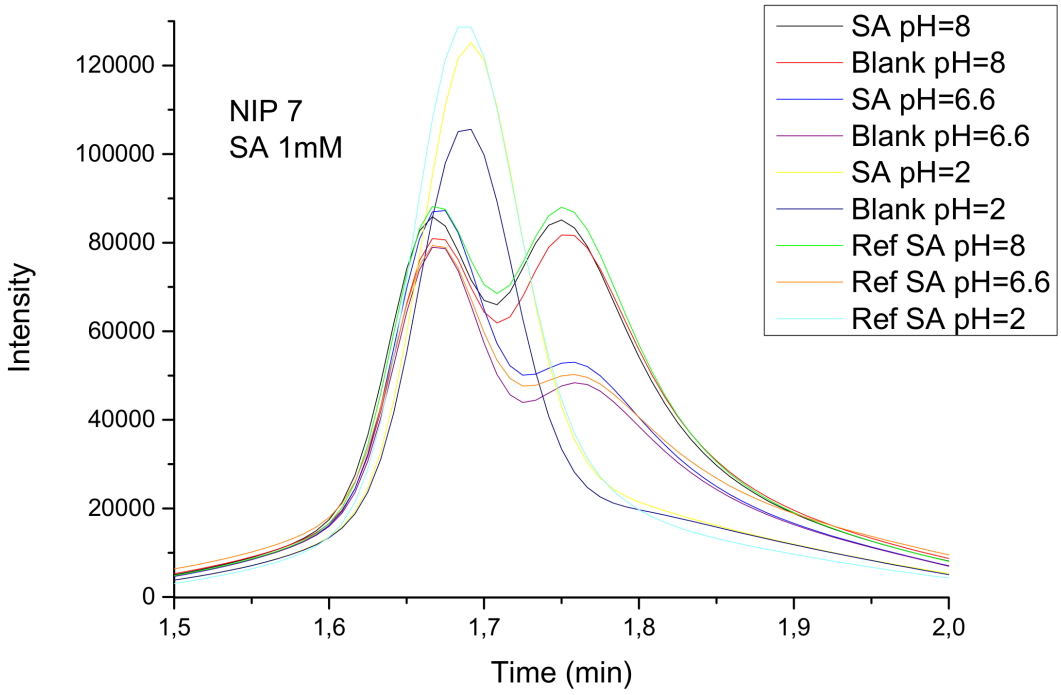
2,0

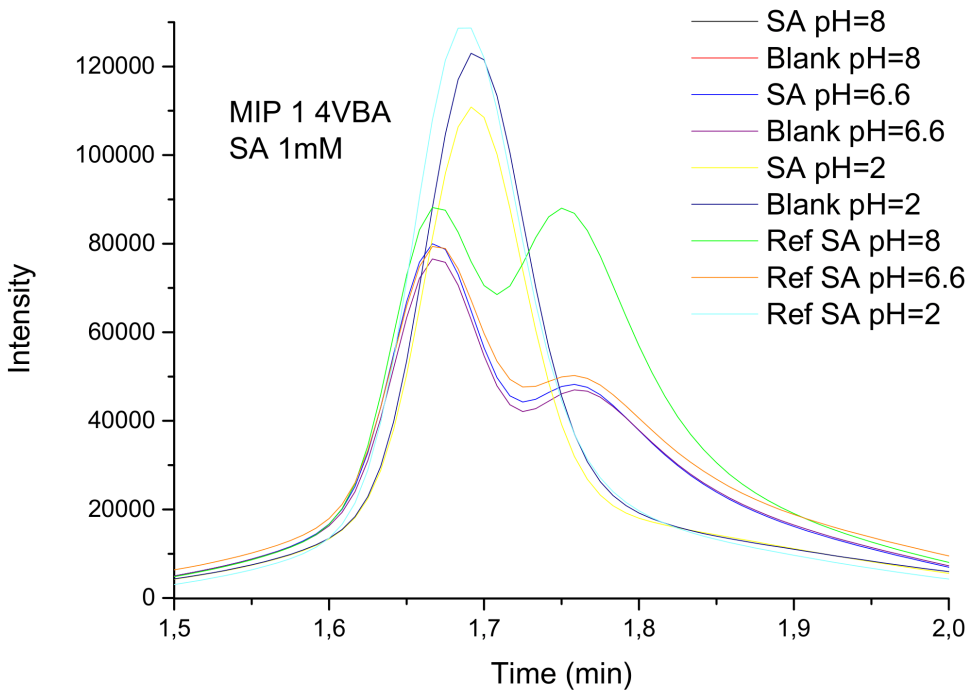
e. MIP 7


MIP 7
SA 1mM
SA pH=8
Blank pH=8
SA pH=6.6
Blank pH=6.6
SA pH=2
Blank pH=2
Ref SA pH=8
Ref SA pH=6.6
Ref SA pH=2
Intensity
Time (min)
120000
100000
80000
60000
40000
20000
0
1,5
1,6
1,7
1,8
1,9
2,0

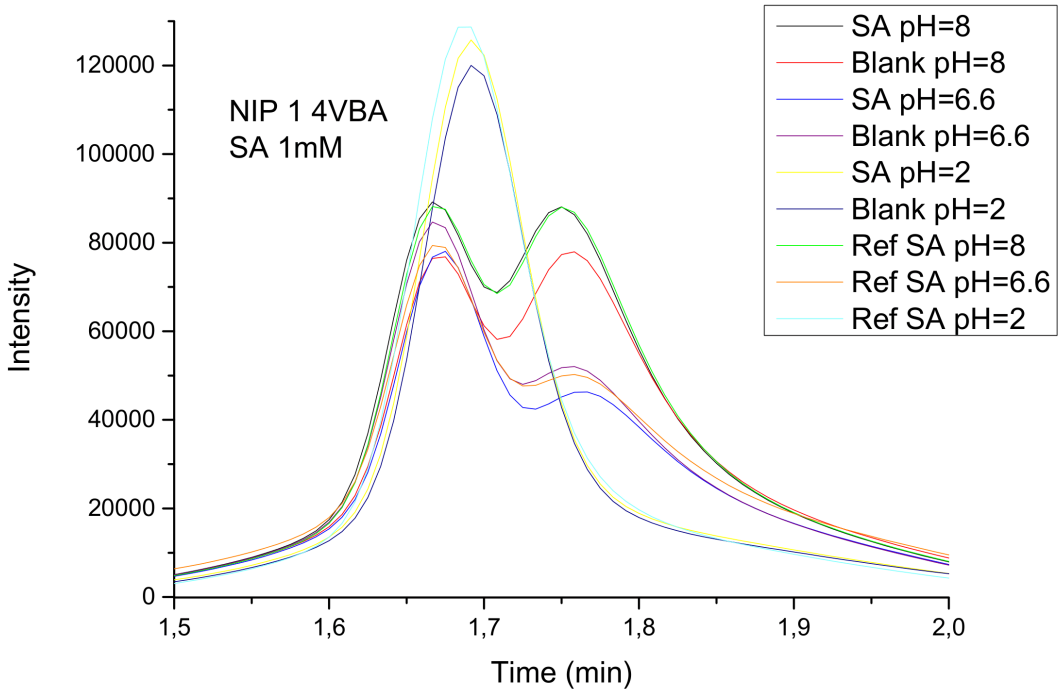
f. NIP 7



g. MIP 1 4VBA



h. NIP 1 4VBA


NIP 1 4VBA
SA 1mM
Intensity
Time (min)
120000
100000
80000
60000
40000
20000
0
1,5
1,6
1,7
1,8
1,9
2,0
SA pH=8
Blank pH=8
SA pH=6.6
Blank pH=6.6
SA pH=2
Blank pH=2
Ref SA pH=8
Ref SA pH=6.6
Ref SA pH=2