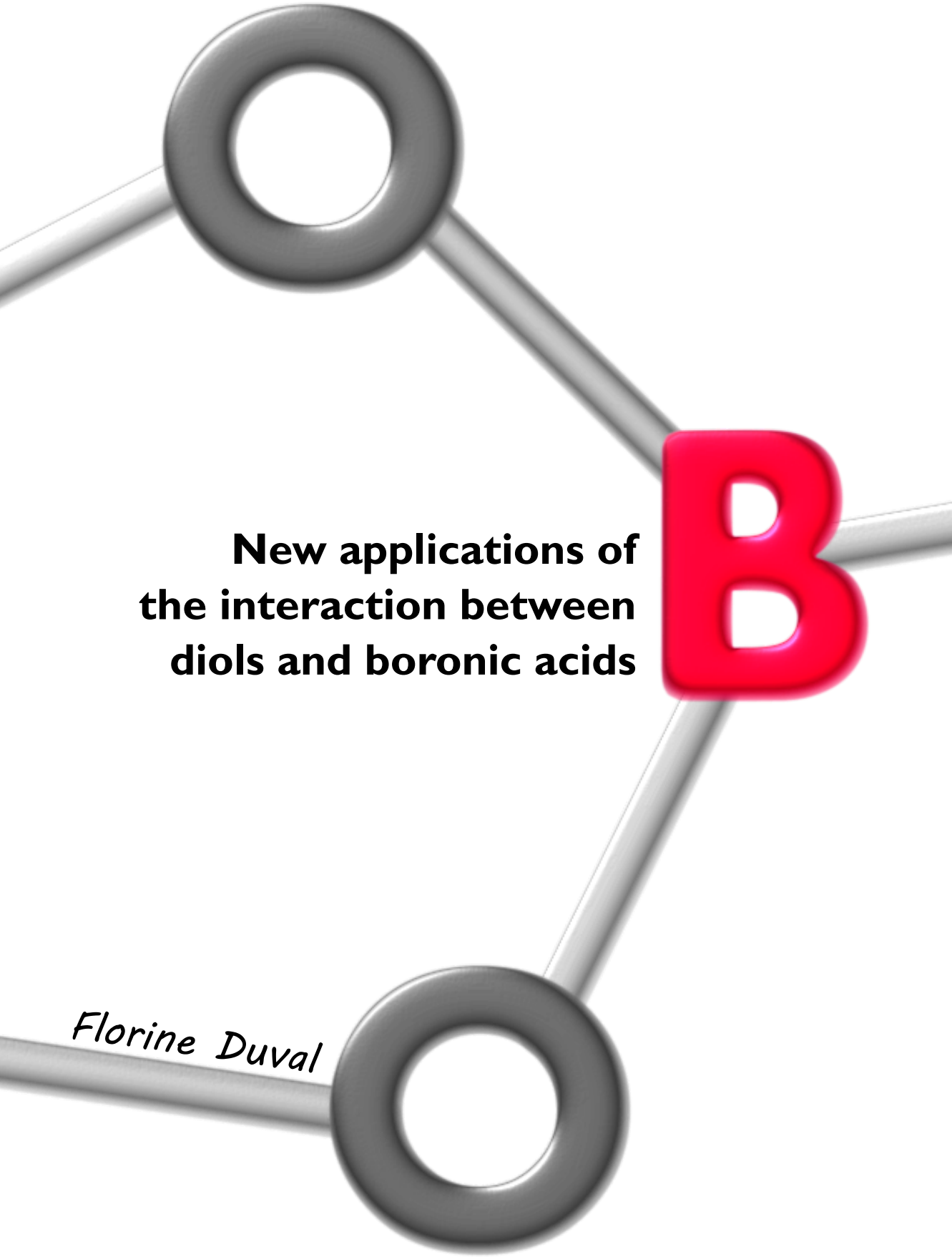

New applications of the interaction between diols and boronic acids

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

New applications of the interaction between diols and boronic acids

Florine Duval

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 9 October 2015
at 4 p.m. in the Aula.

Florine Duval

New applications of the interaction between diols and boronic acids,

140 pages.

PhD thesis, Wageningen University, Wageningen, NL (2015)

With references, with summary in English

ISBN 978-94-6257-471-7

To the memory of my father,

builder with fascination for science

Table of contents

List of abbreviations

2D-TLC	two-dimensional thin-layer chromatography
AACPBA	(4-allylaminocarbonyl)-phenylboronic acid
Ab	antibody
Abs	antibodies
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACN	acetonitrile
APBA	3-aminophenylboronic acid
ARS	alizarin Red S
Boc	*tert*-butyloxycarbonyl
Boc_2O	di-*tert*-butyl dicarbonate
Boc-Cys	N-a-t-butyloxycarbonyl-L-cysteine
CHES	2-(cyclohexylamino)ethanesulfonic acid
CPBA	4-carboxyphenylboronic acid
DCM	dichloromethane
DEAM-PS	diethanolamine-functionalized polystyrene resin
DIPEA	N,N-diisopropylethylamine
DME	1,2-dimethoxyethane
DMF	dimethylformamide
DMPA	2,2-dimethoxy-2-phenylacetophenone
DMSO	dimethyl sulfoxide
DPBA	diphenylborinic acid 2-aminoethyl ester
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDC.HCl	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide HCl
ESI-HRMS	electrospray ionization high-resolution mass spectrometry
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
Fmoc	fluorenylmethyloxycarbonyl
g	gram(s)
h	hour(s)
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HOBt	hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HSA	human serum albumin
IgG	immunoglobulin G
LOD	limit of detection
mAU	milli arbitrary units
MeOH	methanol
mg	milligram(s)
MIDA	N-methyliminodiacetic acid
min	minute(s)
mL	milliliter(s)
mmol	millimole(s)
MPBA	4-methoxyphenylboronic acid
NCBI	National Center for Biotechnology Information
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
PBA	phenylboronic acid
PBS	phosphate-buffered saline
PEEK	polyetheretherketone
PS	polystyrene
PTFE	polytetrafluoroethylene
RM	reaction mixture
RP	reversed-phase
RT	room temperature
S/N ratio	signal to noise ratio
tBu	*tert*-butyl
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin-layer chromatography
UV	ultraviolet

Chapter 1
Introduction

1 When boronic acids and diols meet

1.1 Theory

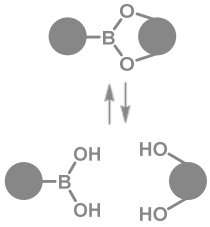
Imagine a dancefloor full of men and women, moving alone in all directions. When a man and a woman bump into each other, if the chemistry is right, then chances are that they start holding hands, making them tightly bound to each other. Chances that a man and a woman form a dancing pair depend on many factors, which are among others:

Ambient conditions

Chances that a man and a woman bump into each other are higher when the dancefloor is busy and people are moving a lot, than when the dancefloor is quiet and people are standing still.

Affinity

If the man's arms are too far from each other, or pointing in different directions, then the woman might not be able to hold both his hands.



Imagine a solution of diols and boronic acids, moving alone in all directions. When a diol and a boronic acid bump into each other, if the chemistry is right, then chances are that they react together, making them tightly bound to each other. Chances that a diol and a boronic acid form a boronic ester depend on many factors, which are among others:

Ambient conditions

Chances that a diol and a boronic acid bump into each other are higher when the solution is concentrated and molecules are moving a lot, than when the solution is diluted and molecules are standing still.

Affinity

If the diol's OH groups are too far from each other, or pointing in different directions, then the boronic acid might not be able to react with it.

To hold a woman's hands, the man needs to point both his arms towards her.
The woman needs to be attractive enough, so that the man wants to hold her hands.

Competition

If there are many women around the dancing pair, or more attractive ones, chances are high that the man goes with another woman.
If there are many men around the dancing pair, or men with more inviting arms, chances are high that the woman goes with another man.

To react with a boronic acid, the diol needs to point both its OH groups towards it.
The boronic acid needs a low pK_a, so that the diol wants to react with it.

Competition

If there are many boronic acids around the boronic ester, or more acidic ones, chances are high that the diol goes with another boronic acid.
If there are many diols around the boronic ester, or diols with a better conformation, chances are high that the boronic acid goes with another diol.

Interactions between men and women are far more complex than interactions between boronic acids and diols, and outside the scope of this thesis. From now on, therefore, only boronic acids and diols will be discussed. A boronic acid B^0 has a vacant valence orbital, which makes it a Lewis acid. By accepting a hydroxyl group, the conjugate base B^- is formed (Figure 1). In presence of a 1,2-diol L, studies have shown that mainly, B^0 first reacts with L to form the corresponding boronate ester B^0L, which is then converted into the more stable B^-L (in aqueous solutions).[1] These reactions are reversible and, as differently explained above, the equilibria depend on many factors. In the next page, more of these factors, specific to boronic acids and diols, are explained.

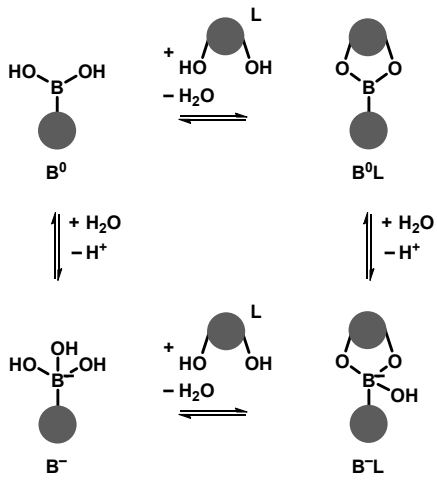
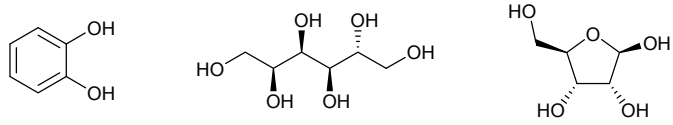


Figure 1. Possible equilibria in aqueous solutions of boronic acids and diols.[1]

pH, diol pK_a and boronic acid pK_a. The optimal pH for boronate ester formation, in general, is about half-way between the pK_a of the boronic acid (e.g., 8.70 for phenylboronic acid) and the pK_a of the diol (e.g., 12.38 for glucose).[1] To enable binding at neutral pH, therefore, efforts have been made in lowering the pK_a of boronic acids.[1]

Boronic acid structure. The structure of the boronic acid influences its pK_a significantly, and hence also its ability to bind with diols in given conditions.[1] For example, if phenylboronic acid is considered, its pK_a is made higher by electron-donating groups (e.g., methoxy-) and lower by electron-withdrawing groups (e.g., trifluoromethyl-) on the phenyl ring, affecting the stability of the resulting boronate ester. Moreover, bulky groups close to the boronic acid may hinder the binding with diols.

Diol structure. A more favorable geometry of the 1,2-diol normally results in a higher affinity for the boronic acid.[1] For example, catechol has a perfectly planar cis-1,2-diol, whereas sorbitol, although it contains several 1,2-diols, needs to rotate away from its ideal conformation in order to create a planar 1,2-cis-diol. Sorbitol, in turn, is *able* to create a planar 1,2-diol, whereas ribose cannot freely rotate the relevant C-C bond and can thus only display a *near*-planar cis-diol. These differences directly result in differences of association constants with phenylboronic acid (Figure 2).[2]



	Catechol	D-sorbitol	D-ribose
K_{eq} (M^{-1})	830	370	24

Figure 2. Association constants (K_{eq}) with phenylboronic acid at pH 7.4, 0.10 M phosphate buffer[2]

Competition. The proportions of boronate esters that are formed strongly depend on the composition of the solution. For example, in a mixture containing phenylboronic acid and equal amounts of catechol and D-sorbitol, more phenylboronate esters of catechol than of D-sorbitol will be found. However, if a large excess of D-sorbitol is added, more phenylboronate esters of D-sorbitol than of catechol will be found.

1.2 Well-explored applications

The boronic acid-diol interaction has found many applications. A few of them are highlighted here, some of which are illustrated in Figure 3 (next double page).

Many boronic acid-based sensors for sugars have been developed, with efforts in making these sensors selective for specific sugars. For example, particular attention has been given to the selective and sensitive detection of glucose for the monitoring of glucose levels in blood of diabetic patients, and the development of glucose-responsive materials that automatically release insulin when glucose levels are too low.[3-5]

Glycans, formed by several monosaccharides attached to each other, are found in many proteins (thus glycoproteins). Various materials that contain boronic acid moieties have been used for the reversible capture (≈ fishing) of glycoproteins from complex mixtures, facilitating their detection, detailed analysis or purification. Glycoproteins have also been immobilized on boronic acid-functionalized surfaces, enabling further applications that rely on the properties of these glycoproteins (e.g., detection of antigens through the use of surface-bound antibodies, see section 2 of this chapter). Furthermore, as glycans are also found on cell surfaces, boronic acid-containing materials are being developed for the detection or capture of cancer cells, which overexpress certain glycans at their surface.[6-9]

The other way around, the boronic acid-diol interaction has been exploited for the detection of boronic acids at the surface of newly developed materials (e.g., for the applications above).[10-12] Alizarin red S (ARS) is widely used for this, as it shows low fluorescence in its free form, and high fluorescence when it is bound to a boronic acid via its catechol moiety (see chapters 4 and 5).

In organic synthesis, diols are used for the protection of boronic acids,[13] and boronic acids are used for the protection and activation of diols, and for the catalysis of reactions involving the formation of diols.[14] Furthermore, diol-functionalized materials have been developed to function as protecting groups of boronic acids and facilitate their modification and isolation (see chapter 3), and vice versa.[14]

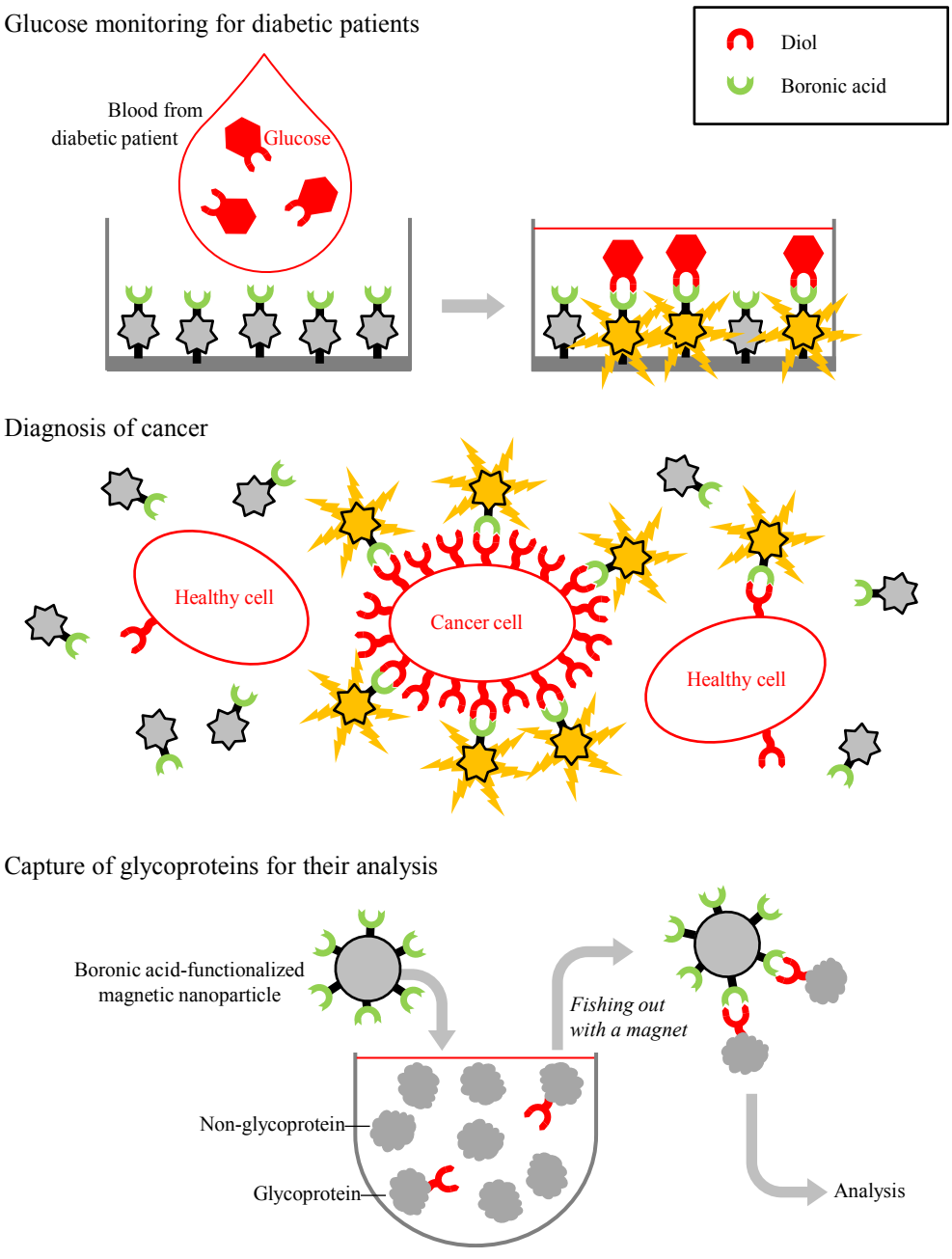


Figure 3. Some known applications of the boronic acid-diol interaction.

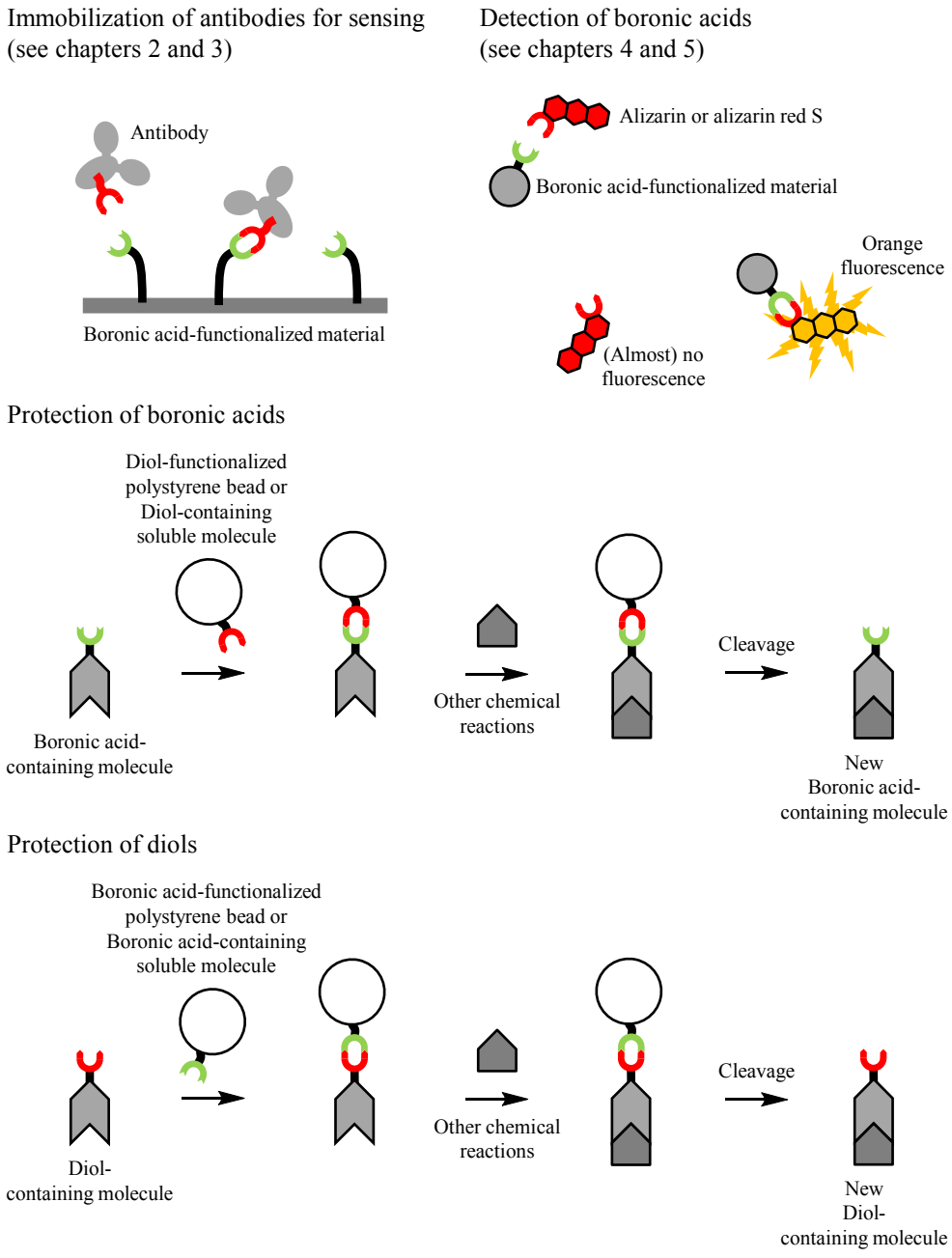


Figure 3. *(Continued).*

2 From diagnosis of depression to boronic acids

If you feel depressed, you may go to your doctor, who will ask you many questions. Your answers will indicate whether you actually suffer from depression, and which type of depression you may have. This way to diagnose depression is subjective and may lead to inappropriate treatments. This lengthens the disease, or might even fully hamper a cure. Knowing this fact, Brainlabs B.V. started a project for the development of a device that would enable the diagnosis of depression through urine analysis. Instead of asking questions, the doctor would put a urine drop on a chip, wait for a few minutes and read the results. This multidisciplinary project involved several companies and institutions, who worked on different aspects of the project: finding which biological markers (= biomarkers) in urine would give the best evidence of the different types of depression, performing clinical studies with depressed and non-depressed people, making suitable chips… Functionalizing these chips to enable sensitive detection of the biomarkers was the mission of Wageningen University, in particular my PhD project within the Laboratory of Organic Chemistry.

How to functionalize a chip in such a way that it is able to detect a biomarker? One way of doing this would be by immobilizing specific antibodies in a specific way on the surface of such a chip. Antibodies are, roughly seen, Y-shaped proteins that bear two binding sites (Figure 4, top left), regions that recognize and bind to substances such as bacteria, viruses, toxins, hormones or other molecules (= antigens) in a specific way: each antibody is specialized in one antigen. Thus, one popular way of detecting a particular biomarker (= antigen) for diagnostic purposes is by performing an immunoassay. The use of such an assay can, for certain classes of assays, be described as follows (Figure 4, top right): 1) immobilizing the corresponding antibody on a chip, 2) incubating this chip with the sample (e.g., urine), which results in binding of the biomarker (= antigen) to the antibody, 3) incubating the chip with a solution that contains a fluorescently labelled antibody, which binds to the biomarker, 4) measuring the fluorescence on the surface of the chip, which is related to the concentration of biomarker in the sample.

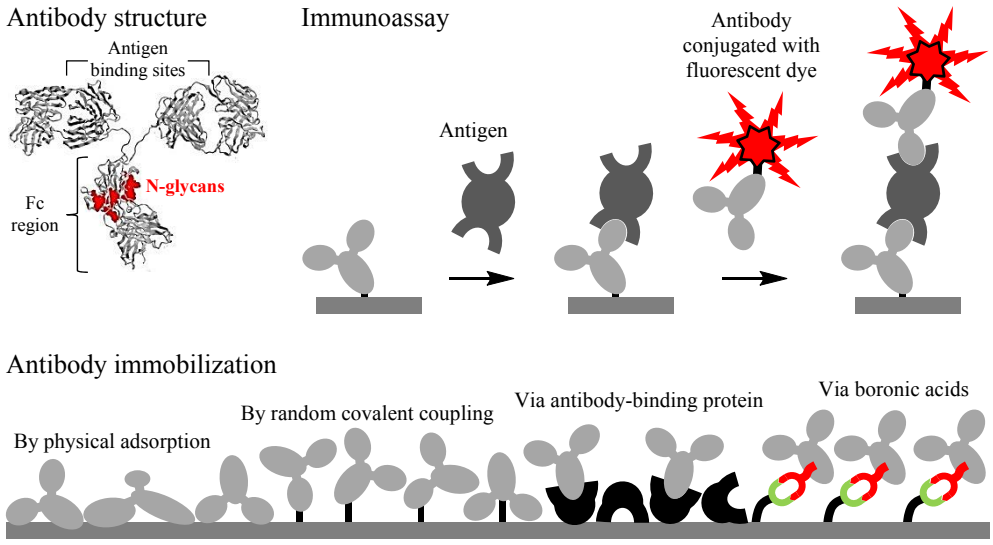


Figure 4. Antibody structure, immobilization (not exhaustive), orientation and immunoassay principle.

To diagnose depression, biomarkers would need to be detected in a similar manner at very low concentrations (~ 1 pM) in urine. One key factor of the method sensitivity is the way in which antibodies are immobilized on the surface of the chip: statistically, if very few antigen molecules are present in solution, many antibody binding sites need to be available for capturing at least some antigen molecules. The number of available antibody binding sites depends on the number of antibodies immobilized on the surface (related to the efficiency of the immobilization procedure), their integrity (which may be affected by harsh reaction conditions), and their orientation.

Various strategies have been developed for protein (thus antibody) immobilization.[15,16] Physical adsorption is the most simple and widely used way (Figure 4, bottom left): when a hydrophobic support, such as a polystyrene plate, is incubated with an antibody solution, the antibodies spontaneously stick to the surface via several of their own hydrophobic sites. However, the antibodies may have difficulties keeping their original shape, and they may be released from the surface again. These problems, resulting in loss of available binding sites, can be solved by covalent immobilization strategies (Figure 4, bottom). The most

common one relies on the reaction between an activated ester on the surface (e.g., N-hydroxysuccinimide ester) and an amine on the outside of the antibody (more precisely one of the side chains of lysine residues), to form a stable amide bond. The antibody is attached to the surface in a stable way, and if the conditions are right, it also conserves its shape. However, both described immobilization strategies have a serious limitation, namely they are "random": there is no control over the orientation of the antibody, which may stand upright, stand upside down, lie flat or lie on its side. Thus, statistically, many binding sites will not be available for antigen binding, hence the sensitivity for antigen detection will not be optimal. To solve this problem, strategies for "oriented" (or "site-specific") immobilization of antibodies have been developed. For example, an antibody-binding protein (protein A, G, A/G or L) is immobilized onto the surface. When the surface is incubated with the antibody solution, the antibody-binding protein captures the antibody via its Fc chain only. As a result, both binding sites are available for antigen binding (Figure 4, bottom). Although antibody orientation is optimal with this strategy, the expensive antibody-binding proteins are immobilized on the surface in a random way, resulting in relatively few sites that are available for capturing the antibodies. Other oriented antibody immobilization strategies, however, do not depend on additional proteins, and take advantage of the sugars (N-glycans) that are present in the antibody Fc chains. It is possible to oxidize the diols in the N-glycans with periodate, and let the obtained aldehydes react with amines on the surface, resulting in oriented and covalent antibody immobilization. This method, however, requires prior treatment of the antibody with periodate, which might result in degradation of the antibody. Alternatively, the N-glycans can be modified via a series of enzymatic and chemical reactions, to provide an antibody derivative that can be clicked onto a surface in a well-defined manner.[17] This method yields highly defined antibody orientations, but does require extensive antibody modifications. Fortunately, there is a simpler (although less strongly binding) alternative to immobilize antibodies via their N-glycans (thus via their Fc chains), namely to let these N-glycans react with surface-bound boronic acids (Figure 4, bottom right).

Antibody immobilization through the reaction between boronic acids on the surface and diols in the antibody N-glycans would present several advantages for the detection of biomarkers for the diagnosis of depression: 1) it is cheaper to modify surfaces with boronic acids than with antibody-binding proteins, which is important for the manufacturing of disposable chips, 2) no prior modification of the antibodies is required, making it easy to immobilize many different antibodies on the same surface, 3) and most importantly, the antibodies are immobilized via their Fc chain, leaving their binding sites available for antigen binding and resulting in a high sensitivity for the detection of biomarkers at low concentrations.

3 From one chapter to another: outline of this thesis

While biomarkers for depression were being investigated by the project partners, our work focused on the development of a general strategy to immobilize antibodies on surfaces via boronic acids. From preliminary experiments and gradual "discoveries" from the literature, we became aware of the many challenges that this immobilization strategy faces, giving rise to **chapter 2**, which discusses several important points that need to be taken into account when one plans to immobilize antibodies via boronic acids.

One big issue being the reversibility of the reaction between boronic acids and diols, hence the possible release of the antibody from the surface, we designed and synthesized boronic acid-containing linkers that would enable the oriented *and* irreversible immobilization of antibodies. This work is described in **chapter 3**.

While synthesizing boronic acid-containing linkers, analysis of the reaction mixtures was inconclusive, as it was difficult to see which spots on thin-layer chromatography (TLC) plates corresponded to boronic acids. To solve this problem, we developed a new TLC staining method based on the reaction between boronic acids and alizarin, resulting in fluorescent spots where boronic acids are present. **Chapter 4** presents this work in detail.

Although TLC is very useful to synthetic chemists, analysis of reaction mixtures by high-performance liquid chromatography (HPLC) is sometimes necessary for more accurate information or for optimization of preparative HPLC conditions. **Chapter 5** presents the development and applicability of a method for the on-line HPLC detection of boronic acids using alizarin.

Looking back, some useful findings have been made, some things may have been done differently, and some results may be the starting point of further developments. **Chapter 6** provides a critical discussion on the work described in this thesis.

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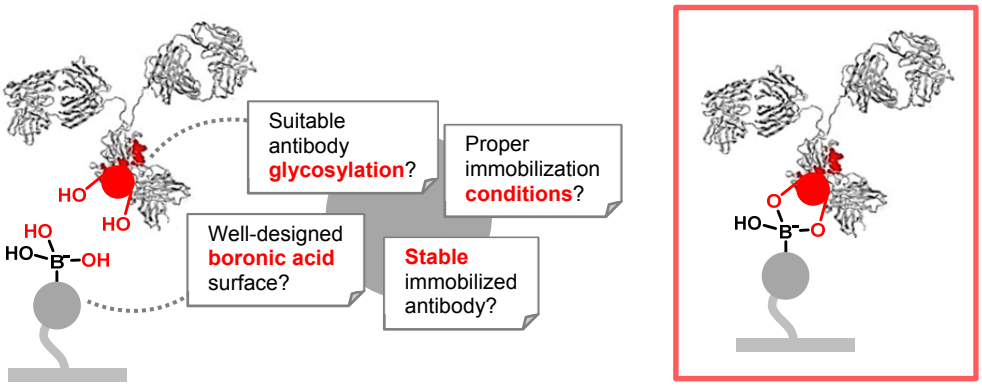
Chapter 2
Key steps towards the oriented immobilization of antibodies using boronic acids



Oriented immobilization of antibodies using boronic acids presents a strong potential for improving immunoassay performance but is not widely used yet, possibly because of difficulties encountered in its implementation. How to choose the boronic acid structure and how should it be attached to the surface? How to choose an antibody that will bind to the boronic acid? In which conditions should the binding take place for an effective oriented antibody immobilization? How to make sure that the antibody stays on the surface? This chapter provides answers to these questions through analysis of the literature and personal suggestions, and thereby intends to facilitate the development of this promising antibody immobilization strategy.

This chapter is a slightly modified version of the following publication: F. Duval, T.A. van Beek, H. Zuilhof, *Key steps towards the oriented immobilization of antibodies using boronic acids,* Analyst, 2015, DOI: 10.1039/C5AN00589B.

1 Introduction

Antibody (Ab) immobilization is an important step in the preparation of devices for the detection of various analytes (= antigens) such as proteins, bacteria, viruses, drugs and toxins. Antibodies (Abs) are proteins and protein immobilization strategies, which have been reviewed in detail,[1,2] can be divided into two categories: random and oriented (or site-specific). Oriented immobilization is especially important for Abs in immunosensing applications, as their binding sites need to be kept available for antigen binding. Using oriented immobilization, signal enhancement factors of over 200 have been obtained,[3] displaying the significance of Ab orientation. Strategies for oriented Ab immobilization, which have recently been reviewed,[4,5] aim at attaching the Ab to the surface via its Fc region (Figure 1, right edge), far away from the binding sites. Abs are N-glycosylated (i.e., carbohydrate chains are attached to the amide nitrogen of Asn residues of Abs and thus defined as N-glycans) in their Fc regions, thus a proper orientation of the antibody can be achieved by immobilization via its N-glycans. This has mainly been done in two ways, both taking advantage of available vicinal diols in the N-glycans: 1) by oxidation of diols into aldehydes, which then react with nucleophiles attached to the surface, or 2) by reaction of diols with boronic acids attached to the surface. The present chapter focuses on the second approach.

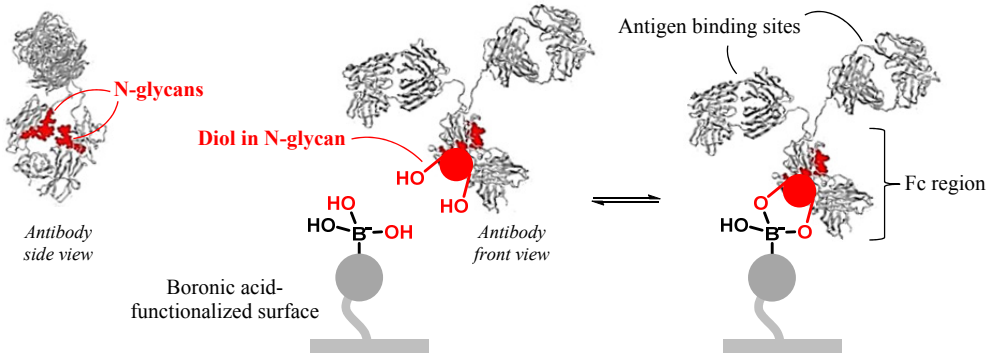


Figure 1. Principle of oriented antibody immobilization using boronic acids.

Boronic acids react with 1,2-diols to form boronate esters (see chapter 1). This reaction has been used for many applications, including the separation and immobilization of glycoproteins (including antibodies).[6] Oriented Ab immobilization using boronic acids (through the same reaction) has been published for the first time in 2009 by Lin et al.,[7] and appeared in another ten papers since then.[8-17] An overview of these articles is given in Table 1.

Paper code	**Main feature**	**Ref.**
Ab-BA 1	First Ab immobilization via boronic acid	7
Ab-BA 2	Novel immunosensing application	8
Ab-BA 3	Novel immunosensing application	9
Ab-BA 4	Novel immunosensing application	10
Ab-BA 5	Novel immunosensing application	11
Ab-BA 6	Boronic acid on zwitterionic polymer brushes	12
Ab-BA 7	Probing of the available binding sites	13
Ab-BA 8	Direct assessment of Ab orientation	14
Ab-BA 9	Microarray of 71 Abs	15
Ab-BA 10	Novel immunosensing application	16
Ab-BA 11	Novel immunosensing application	17

Table 1. Overview of the publications that report antibody immobilization via boronic acids. The "paper codes" are used in this chapter to quickly refer to these papers.

This immobilization method appears very attractive, as it presents several advantages over more common ones: it leaves the binding sites available for antigen binding (oriented vs random immobilization), it can be done in one step without former antibody modification (vs oxidation with periodate, use of antibody fragments or enzymatic modification of the N-glycans), and it is relatively economical (vs Fc-chain binding proteins such as protein A/G). Moreover, several comparative studies (in Ab-BA 1, 2, 7 and 10) suggested that the highest antigen detection sensitivity was achieved when Abs were immobilized using boronic acids.

The number of papers reporting this immobilization strategy, however, remains limited (11 papers, four of them having been reviewed by Wang et al.[6]). So the question arises: where

does its lack of popularity come from? We believe that many researchers may have tested Ab immobilization via boronic acids, that likely problems were encountered in most cases, and that therefore few successful studies were published. Although promising, this immobilization strategy is not as simple as it seems. In this chapter, meant as a tutorial review, the reader is guided through several points that need to be considered to allow development of the full potential of this still very appealing approach.

2 Step-wise guide

2.1 Preparation of boronic acid-functionalized surface

The first step towards a successful Ab immobilization via boronic acids is a surface modification with the right molecules.

Boronic acid structure. The interaction between a boronic acid and a diol strongly depends on the structure of both partners.[18] This interaction, hence the Ab immobilization efficiency, may be enhanced by a boronic acid with a low pK_a value, which can be obtained by electron-withdrawing groups or intramolecular interactions (e.g., in Wulff-type boronic acids and benzoboroxoles).[18,19] To achieve a correct Ab orientation, secondary interactions between the boronic acid and Ab should be minimized: a hydrophilic boronic acid would help to minimize hydrophobic interactions, and ionic interactions may be prevented by using a boronic acid that is not negatively charged at high pH (e.g., Wulff-type boronic acid or benzoboroxole).[19] 3-Aminophenylboronic acid (APBA) was used in nine out of the eleven Ab-BA papers, but this choice was never justified and, in view of the above, may provide room for improvement.

Spacer length and structure. The N-glycans are nested between the Ab Fc chains, and relatively far from the C-terminus/"bottom" (Figure 1, left). If the boronic acid is directly attached to the surface without spacer or with a too short one, then the Ab may not be immobilized or it may be forced to "lie" on the surface, which is not optimal for antigen binding. In contrast, if a long and flexible spacer separates the boronic acid from the

surface, then the Ab has more freedom and it can "stand". Thus we expect that the antigen binding capacity was relatively high when Abs were immobilized on zwitterionic polymer brushes (Ab-BA 6, Figure 2) that had been functionalized with boronic acids.

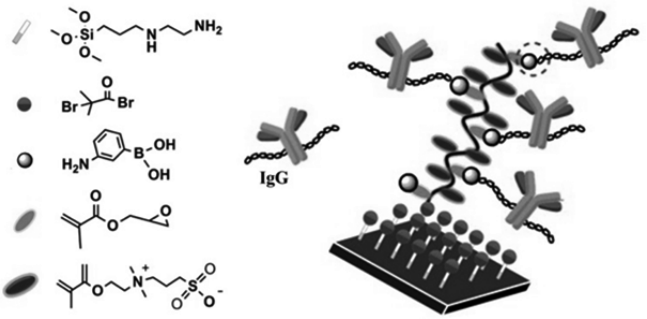


Figure 2. Antibody immobilization via boronic acids on zwitterionic polymer brushes.[12] Adapted with permission from ref. 12. Copyright 2015 American Chemical Society.

Antifouling surface. In Ab-BA 1, 6, 8 and 10, which report the reaction of an amine-containing boronic acid molecule with an active ester (e.g., N-hydroxysuccinimide, NHS) or epoxide on the surface, the active esters or epoxides remaining after the reaction were deactivated with ethanolamine. This is required to prevent Ab nucleophilic groups (such as the $-NH_2$ of lysine residues) from reacting with surface-bound esters or epoxides, hence yielding a random orientation again.

Furthermore, an antifouling coating helps to minimize other types of unwanted (e.g., electrostatic or hydrophobic) interactions between Ab and surface. Poly(ethylene) glycol is widely used for its antifouling properties, and zwitterionic polymer brushes prevent the nonspecific adsorption of biomolecules even more effectively.[20,21] Thus, it may be a good idea to prepare boronic acid-containing zwitterionic polymer brushes, as was done in Ab-BA 6 (Figure 2). Alternatively, commercially available substrates with antifouling coatings decorated with reactive groups such as NHS or epoxide may be modified with a boronic acid that contains an appropriate reactive group (such as APBA). One has to keep in mind, however, that some antifouling polymers bind with boronic acid groups, possibly resulting in a decreased availability of the boronic acid groups for subsequent Ab binding. This

problem may be encountered with dextran, which has been used as boronic acid blocking agent in Ab-BA 1 and 6, and with polymers of unknown structure, based on our own experience with proprietary coatings of some commercial antifouling slides.

Characterization of the boronic acid-functionalized surface. In order to pinpoint causes of possibly unsuccessful Ab immobilization, it is useful to know whether the surface actually is modified with boronic acids. In Ab-BA 3, 4, 8 and 11, changes in surface properties before and after modification with APBA suggested that a chemical change indeed occurred, but did not prove the boronic acid integrity. In Ab-BA 2 and 6, however, X-ray photoelectron spectroscopy (XPS) revealed the presence of boron, and in Ab-BA 6, narrow-scan XPS data more accurately indicated the presence of boronic acids on the surface. XPS, though, is not available to every researcher and we would like to suggest a much simpler and more widely applicable method. Alizarin and its more water-soluble derivative alizarin red S (ARS) strongly bind with boronic acids to form fluorescent complexes (see chapters 4 and 5). Outside the field of Ab immobilization, boronic acid-functionalized surfaces have been incubated with ARS, and characterization by fluorescence imaging then allowed for an even quantitative indicator of the presence of surface-bound boronic acid moieties.[22-26]

2.2 Choice of antibody with suitable glycosylation

Many different Abs were used in the Ab-BA papers. Few of them were fully described and the rationale behind their selection was never explained. Considering the intrinsic properties of an Ab, a key factor for its successful immobilization is the composition of the N-glycans at its Fc chain. These N-glycans must present diols that have a suitable conformation for binding with boronic acids. Many monosaccharides bind with boronic acids, but when they are attached to each other, relatively weak interactions between boronic acids and the formed glycans are enabled. Sialic acid, however, has a side chain with a free and flexible glycol function that may bind stronger to boronic acids.[18] Based on this, the presence of sialic acids in the Ab N-glycans is highly favorable, and possibly even required for a successful immobilization. The Ab N-glycans vary with cell line, animal

species, culture conditions and even from batch to batch, resulting in the presence or absence of sialic acids at the glycan chain ends.[27]

Analysis of Ab candidates for immobilization is therefore advisable. Ab glycosylation may be analyzed using mass spectrometric and chromatographic techniques,[28] or more simply, Abs may be screened for sialic acids using the periodic acid-Schiff method[29] (we used a commercial glycoprotein carbohydrate estimation kit). The affinity of Abs for boronic acids may also directly be assessed by boronate affinity chromatography using a commercially available boronate column,[30,31] although the results will also depend on the properties of the column and the binding conditions (see 2.3).

2.3 Antibody immobilization on the boronic acid-functionalized surface

Binding conditions. Depending on the binding conditions, (glyco)proteins can bind to boronic acids not only via the boronic acid-diol complexation, but also via secondary interactions, such as hydrophobic, ionic[19] and charge-transfer interactions.[31] As these interactions would surely result in a partly random Ab immobilization, optimization of the binding conditions is essential. For example, the ionic strength should be low enough to minimize hydrophobic interactions and high enough to minimize ionic interactions.[19] The pH should be low enough to preserve the integrity of the biomolecules and high enough to minimize charge transfer interactions between boronic acids on the surface and amines in the antibody.[31] These effects are illustrated in the article of Azevedo et al. who studied the binding of pure human IgG (antibody, glycosylated protein), human recombinant insulin (non-glycosylated protein) and human serum albumin (HSA, poorly glycosylated protein) by boronate affinity chromatography in various conditions (Figure 3).[31] When PBS with pH 7 was used, almost all the IgG was bound, as well as a significant amount of HSA and insulin. PBS with pH ≈ 7 was used in Ab-BA 3, 4, 5, 9, 10 and 11, in which no proof of the Ab orientation is given. Thus, it might be that some of the Abs were actually physically adsorbed in a random way on the boronic acid-functionalized surfaces. In contrast, Figure 3 shows a very good selectivity for IgG when HEPES at pH 8.5 was used. HEPES was used in Ab-BA 8, although the pH was unfortunately not mentioned. This paper happens to be the only one in which the actual Ab orientation was studied (see top of page 23).

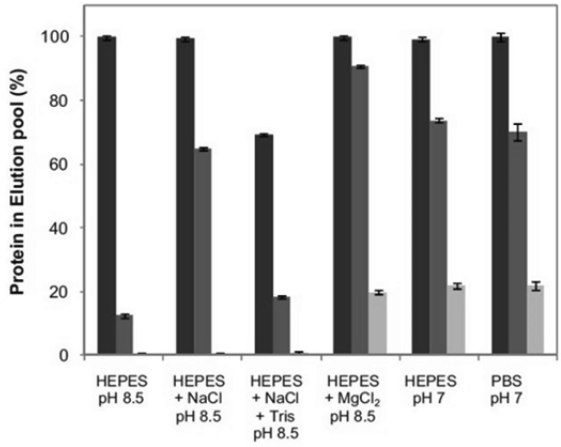


Figure 3. Binding of proteins to boronic acids as displayed by percentage of bound and then eluted IgG (dark), albumin (middle), and insulin (light) in different adsorption buffers: (i) 20 mM HEPES at pH 8.5; (ii) 20 mM HEPES, 150 mM NaCl at pH 8.5; (iii) 20 mM HEPES, 150 mM NaCl, 15 mM Tris at pH 8.5; (iv) 20 mM HEPES, 150 mM $MgCl_2$ at pH 8.5; (v) 20 mM HEPES at pH 7; and (vi) 10 mM phosphate, 150 mM NaCl at pH 7. Reproduced with permission.[31]

Reaction time. Although the incubation in Ab-BA 1, 2, 4, 6, 7, 10 and 11 was done for 12 h or more at 4 °C, it was shown in Ab-BA 8 that the Ab immobilization was complete within 20 min.

Blocking. After Ab immobilization, the remaining surface binding sites (any sites that may attract unwanted proteins in subsequent experiments, e.g., by boronic acid-carbohydrate, hydrophobic or electrostatic interactions) were blocked with dextran (Ab-BA 1, 6 and 10), bovine serum albumin (Ab-BA 3, 4, 5, 6, 8, 10 and 11), casein (Ab-BA 7), milk (Ab-BA 9) or left unblocked (Ab-BA 2). In Ab-BA 1, five different compounds (glucose, glycerol, dextran, ethylene glycol and ethanolamine) were tested for blocking. Dextran was selected as it resulted in the most effective suppression of non-specific binding, although we expect that it may expel the Ab from the surface upon binding with the boronic acid.

Characterization of the antibody-functionalized surface. In order to pinpoint causes of possibly unsuccessful antigen binding, it is useful to figure out whether the Ab is bound to the surface in the expected way. Although Ab-BA 1, 4, 5, 6, 7 and 11 reported experiments

that indicated that the Abs were successfully immobilized on the surfaces, the orientation of the Abs was directly assessed in Ab-BA 8 only: dual polarization interferometry showed upon binding of the Ab a thickness increase of 8.44 ± 0.86 nm and a mass increment of 3.33 ± 0.40 ng/mm^2. These values indicated that the Abs were attached to the surface via their Fc chain ("end-on" conformation), based on Ab dimensions of 14.2 × 8.5 × 4.0 nm and a previously reported mass increment of 3.70 ng/mm^2 for an end-on oriented Ab. More information about the techniques to study Ab orientation can be found in a previous review by our group.[4]

2.4 Stability

The reaction between a boronic acid and a diol to form a boronate ester is reversible, which may strongly disturb Ab immobilization. Diol content and pH of the solution both have a strong influence on the equilibrium.

Effect of competing diols on the stability of the immobilized antibody. In Ab-BA 5 and 6, it was shown that sorbitol solutions were able to release the Ab from the surface. In Ab-BA 1, a western blot (Figure S4 in Ab-BA 1) suggests a poor stability of the Ab-functionalized material in serum. In order to prevent the competition of soluble diols with the immobilized Ab, APBA was added to serum before incubation of the Ab-functionalized material with this serum. Subsequent antigen binding was apparently improved, but we are doubtful about the validity of this treatment: APBA may compete with the surface-bound boronic acid and expel the Ab from the surface, resulting in a loss of antigen binding capacity and consequently a wrong measurement of antigen concentration in the sample.

If the antigen is a protein, then chances are high that it is glycosylated.[32] Therefore, it may expel the Ab from the surface, resulting in erroneous measurements. Blocking antigen diols with APBA may not only cause the problem described in the previous paragraph, but also cause blocking of the antigen epitopes or nonspecific interactions between antigen and surface. However, if the antigen is big enough and the Ab layer is dense enough, then steric hindrance may prevent the antigen from accessing the boronic acid.[33] If steric hindrance is

not sufficient, then we recommend a synthetically involved but potentially effective solution: make the boronic acid-mediated immobilization irreversible (see chapter 3).

Effect of pH on the stability of the immobilized antibody. Treatment with acidic solutions, as reported in Ab-BA 6 (pH 3) and 8 (glycine pH 2.5), resulted in dissociation of the Ab from the surface. In Ab-BA 1, a western blot (Figure S2 in Ab-BA 1) suggests an increasing dissociation of the Ab from the surface when the pH was increased from 7 to 12. If the immunoassay conditions stay in a "safe" pH range and the sensor surface is not intended for reuse, then this should not cause a problem. If regeneration of the sensor surface with an acidic or basic solution is required, then one must keep in mind that not only the antigen, but also part or all of the Ab will be washed away, depending on the regeneration conditions.

3 Conclusion and outlook

Ab immobilization using boronic acids requires substantial preliminary work: preparing a boronic acid-containing antifouling surface (most likely via several chemical steps), finding an Ab with suitable N-glycans, finding binding conditions that result in effective and oriented Ab immobilization, and making sure that the immobilized Ab is not expelled from the surface by competing diols or harsh regeneration conditions.

However, once the basic requirements are met and initial optimizations are done, Ab immobilization via boronic acids may result in a much higher sensitivity for antigen detection than what can be achieved by random immobilization, and an improvement of cost and simplicity compared to other strategies for oriented Ab immobilization.

Future research in this field may give precious information about trends in the glycosylation of Abs and their affinity for boronic acids, and explore the effect of boronic acid structure, surface architecture and binding conditions on Ab immobilization and orientation. This could lead to the development of Ab immobilization via boronic acids towards a well-established method within the fabrication of high-performance biosensors. Moreover,

further knowledge on the interactions between boronic acids and antibodies may lead to improvements in related fields, such as the purification or site-specific labelling of antibodies and, more generally, glycoproteins.

Reversibility of the binding between boronic acid and diol, which remains an obstacle of Ab immobilization that relies only on this binding, has led to a strategy for oriented *and* irreversible Ab immobilization. This strategy is described in chapter 3.

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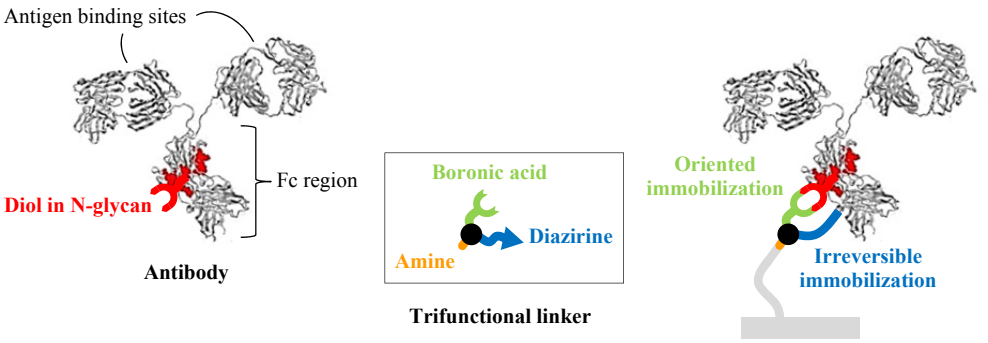
Chapter 3
Design and synthesis of linkers for the oriented and irreversible immobilization of antibodies



To enable the oriented and irreversible immobilization of antibodies, two linkers were designed with an amine for surface attachment, a boronic acid for capturing antibodies via the N-glycans in their Fc chain, and a diazirine for irreversible immobilization upon UV irradiation while maintaining antibody orientation. From a diazirine building-block that was obtained in three steps, the first linker was synthesized in four steps and the second linker was synthesized in three steps. Diol-functionalized silica was used for the chromatography of two boronic acid-containing intermediates, this method being novel (to the best of our knowledge) and likely based on boronic acid-diol interactions. High-resolution mass spectrometry, through matching exact masses, matching isotope patterns and observation of species corresponding to the esterification of boronic acids with MeOH, confirmed that both linkers were synthesized successfully.

Florine Duval, Han Zuilhof and Teris A. van Beek are authors of this chapter.

1 Introduction

The oriented immobilization of antibodies using boronic acids, as reviewed and discussed in chapter 2, seems very promising. However, because the boronic acid-diol complexation is reversible, it is difficult to apply this immobilization strategy to the fabrication of sensors for the analysis of sugar-containing samples, or sensors that need to be regenerated using acidic or basic solutions. When the surface is incubated with a sample to analyze or a regeneration solution, the antibody may be washed away from the surface. Therefore, we asked ourselves the following question: how to orient antibodies on surfaces using boronic acids, and immobilize them in an irreversible way while maintaining their orientation?

2 Idea and design

2.1 Development of the idea

The idea of oriented and irreversible antibody immobilization was triggered by the article of Abad et al. from 2002, which describes the immobilization of a peroxidase glycoprotein on a mixed epoxy-boronic acid monolayer (Figure 1).[1] As the diols in the glycoprotein glycans react fast with the surface-bound boronic acid, the glycoprotein concentrates on the surface. The surface-bound epoxide groups and the glycoprotein nucleophilic groups (such as amines) come in close proximity, and are therefore able to react with each other in spite of the slow kinetics of the reaction between an epoxide and a nucleophile. The formed bond is very stable and the peroxidase glycoprotein is tightly bound to the surface.

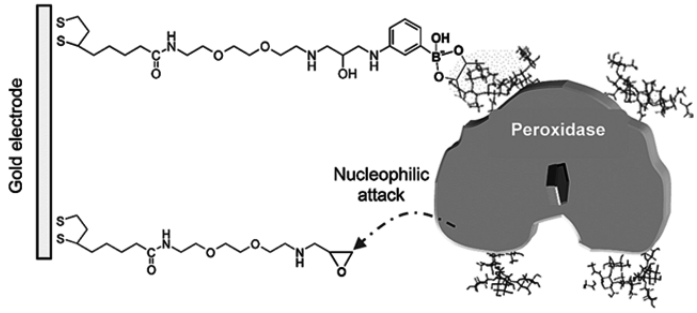


Figure 1. Immobilization of peroxidase on mixed epoxy-boronic acid monolayer. [1] Reprinted with permission from 1. Copyright 2015 American Chemical Society.

Can the same mixed epoxy-boronic acid monolayer directly be used for the oriented immobilization of antibodies? No: first, the antibody N-glycans (see chapter 2) would bind to the boronic acid on the surface. Second, the antibody would move around the obtained anchor point and almost every part of the antibody would be able to access the epoxide groups on the surface. This would result in a random immobilization of the antibody, or maybe worse: the antibody sites that are away from the N-glycans, including the antigen binding sites, may be even more susceptible to react with the epoxide groups.

Therefore, we thought of designing a surface bearing a long spacer, at the end of which a boronic acid and an epoxide, close to each other, would be present. First, the boronic acid would react with a diol in the antibody N-glycans. Second, the epoxide would react with a nucleophile on the antibody in the vicinity of the N-glycans. At the end, regardless of the reversibility of the binding between diol and boronic acid, the antibody would be bound to the surface via its Fc chain in an irreversible way.

However, the use of an epoxide would present the following drawbacks: 1) The epoxide should be formed just before antibody immobilization, on the sensing surface, and the surface may not resist the harsh conditions (such as peracid or concentrated base) that are necessary for the oxidation of an alkene (common stable precursor of epoxides) into an epoxide. 2) Whereas most epoxides in a reaction mixture will react with a nucleophile *after* the boronic acid reacts with a diol, some epoxide moieties will react *before* their

neighboring boronic acid, resulting in improper orientation of the antibody. 3) The reaction of epoxides with the nucleophilic groups present on proteins is slow. On the one hand, this is what makes the approach possible. On the other hand, the reaction may therefore also be inefficient as some epoxide groups may hydrolyze before they react with the antibody. 4) Enough nucleophiles should be present in the close proximity of the N-glycans for the antibody to be immobilized in an irreversible way.

To circumvent all these problems together, we decided to replace the epoxide by a photoreactive group. Photoreagents such as aryl azides, benzophenones and diazirines attached to surfaces have been used for protein immobilization upon irradiation with UV light.[2] Diazirines, in particular, require a shorter irradiation time than benzophenones and a higher irradiation wavelength than aryl azides, thereby minimizing protein degradation. Upon irradiation with 365 nm light, diazirine groups generate carbenes, which rapidly insert into C-C, C-H and X-H (X = heteroatom) bonds, thereby forming covalent bonds with neighboring (bio)molecules (Figure 2, example with C-H bond).[3] Trifluoromethyl-aryldiazirines are among the most chemically and thermally stable of all types of diazirines and the formed carbenes do not participate in intramolecular rearrangements. For these reasons, trifluoromethylaryldiazirines are most commonly used.[4]

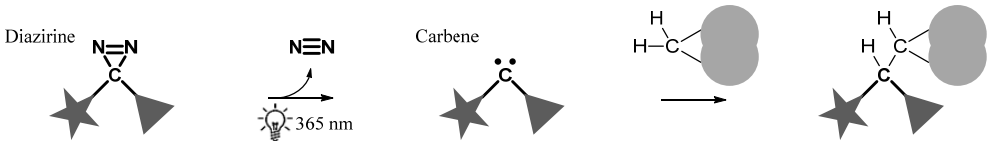
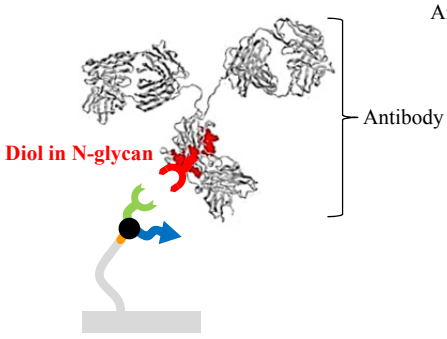


Figure 2. Chemistry of the photoimmobilization of (bio)molecules with diazirines.

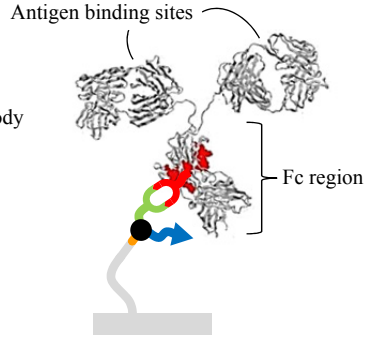
In order to prepare a surface bearing a long spacer, at the end of which a boronic acid and a diazirine would be present, we envisaged the synthesis of a trifunctional linker that would contain 1) a primary amine for surface attachment, 2) a boronic acid and 3) a diazirine. The obtained linker would then be attached to a surface bearing a long spacer (e.g., polyethylene glycol) that is terminated with a reactive ester (e.g., N-hydroxysuccinimide ester). Such surfaces are commercially available. The strategy for oriented and irreversible antibody immobilization via boronic acid and diazirine is summarized in Figure 3.

1) **Activated ester**-coated sensor surface is incubated with **trifunctional linker**.

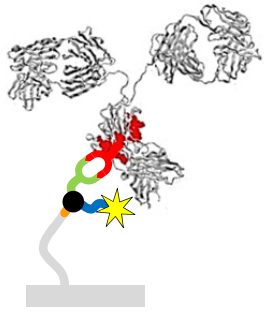
2) **Amine** reacts with **activated ester** and trifunctional linker is attached to surface.



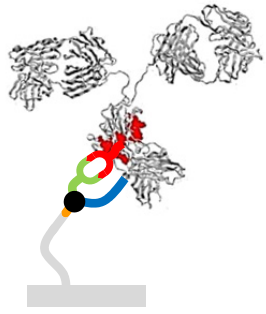
3) Trifunctional linker-coated surface is incubated with antibody.



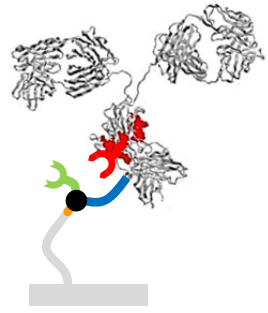
4) **Boronic acid** binds with **diol**, antigen binding sites are kept away from the surface.



5) Upon irradiation with UV light, **diazirine** is converted into a reactive **carbene**.



6) Carbene reacts immediately with a C-C, C-H or X-H bond (X = heteroatom) in antibody backbone.



7) If boronic acid breaks up with diol, antibody is still bound to the surface, binding sites are still away from the surface.

Figure 3. Strategy for oriented and irreversible antibody immobilization via boronic acid and diazirine.

2.2 Linker design and synthetic approach

The described molecules are depicted in Figure 4.

Diazirine building-block. In order to introduce a trifluoromethylaryldiazirine in the linkers, compound **1** was chosen as it is commercially available and contains a carboxylic acid group that would allow straightforward attachment to an amine-containing molecule. When the boronic acid is bound to the diol in the antibody N-glycan and the diazirine is converted into a reactive carbene, the carbene should have enough freedom to reach the antibody. To allow this, we chose to introduce a spacer within the trifunctional linker. Diamine **2** would be connected to carboxylic acid **1** and the obtained diazirine building-block **3** would present a diazirine on one side and a primary amine on the other side for attachment to the rest of the linker.

Linker A. 3-Aminophenylboronic acid **4** (APBA) was chosen as the starting material for the introduction of a boronic acid group in the linker. As discussed in chapter 2, other boronic acids may result in a stronger interaction with diols, but APBA seemed a good starting point for proof-of-principle experiments: it is by far the most commonly used boronic acid for antibody immobilization (see chapter 2), and using the same structure would allow an easier comparison of our strategy with known Ab immobilization via boronic acids. For the linker backbone, glutamic acid **5** was chosen as it conveniently contains an amine for attachment of the finished linker to the surface, a carboxylic acid for reaction with the amine group of APBA and another carboxylic acid for reaction with the amine group of the diazirine building-block **3**.

Linker B. Having difficulties with the synthesis and purification of **Linker A** intermediates, **Linker B** was designed from the need for a more straightforward synthetic route towards a linker with the desired functional groups (amine, boronic acid and diazirine). Addition reactions between thiols and terminal alkenes, namely thiol-ene "click" reactions, are known to be very easy, versatile and efficient reactions, often directly yielding pure products.[5] Therefore, this type of reaction seemed attractive for the synthesis of boronic acid-containing compounds, which are typically difficult to purify. The terminal

alkene of commercially available boronic acid **6** would "click" with the thiol of cysteine **7**, which also contains an amine for attachment of the finished linker to the surface and a carboxylic acid for reaction with the amine group of the diazirine building-block **3**.

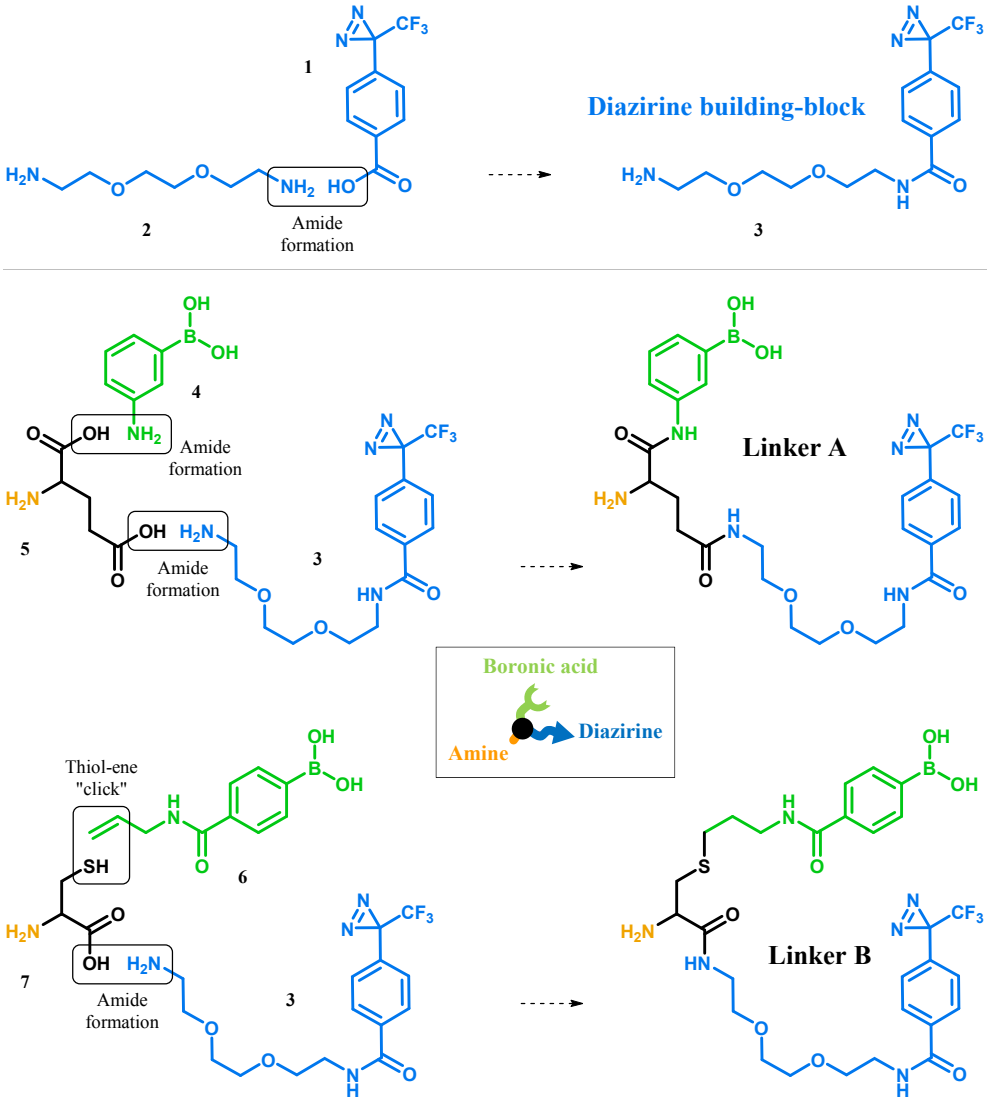


Figure 4. Design and synthetic approach of diazirine building-block, **Linker A** and **Linker B**.

3 Results and discussion

3.1 Synthesis of the diazirine building-block

It may be possible to directly couple diamine **2** with carboxylic acid **1** and obtain diazirine building-block **3** in one step (Figure 5B). One molecule of diamine **2** can potentially react with two molecules of carboxylic acid **1**, but if a large excess of **2** is used, it may promote the formation of **3** as a major compound. However, a mixture of diamine **2**, desired product **3** and by-product **3a** would be unavoidable. The delicate purification process would result in a partial loss of compound **3**, and thus a waste of expensive compound **1**. To avoid this, we chose to first protect diamine **2** on one side with a Boc group, then couple the obtained product **8** with compound **1**, and finally remove the Boc group (Figure 5A).

Boc protection encountered the same issue as described above, as a mixture of diamine **2**, desired product **8** and by-product **8a** was obtained (Figure 5C). However, the reagent for Boc protection di-*tert*-butyl dicarbonate (Boc_2O) is cheap and widely available, unlike compound **1**. The reaction was performed at a relatively large scale, and a sufficient amount of amine **8** was isolated (3.02 g, 59% yield). After coupling amine **8** with carboxylic acid **1**, purification of the obtained amide **9** by silica flash chromatography, and subsequent removal of the Boc group in typical conditions, the HCl salt of diazirine building-block **3** was isolated in a quantitative yield over the last two steps. The effort of working in the dark to prevent degradation of the diazirine moiety proved fruitful (although the synthesis has not been carried out in full light for yield comparison, NMR analysis of compound **1** before and after exposure to laboratory light for 2 h showed about 10% degradation of the material, which had shown excellent stability in the dark for several days).

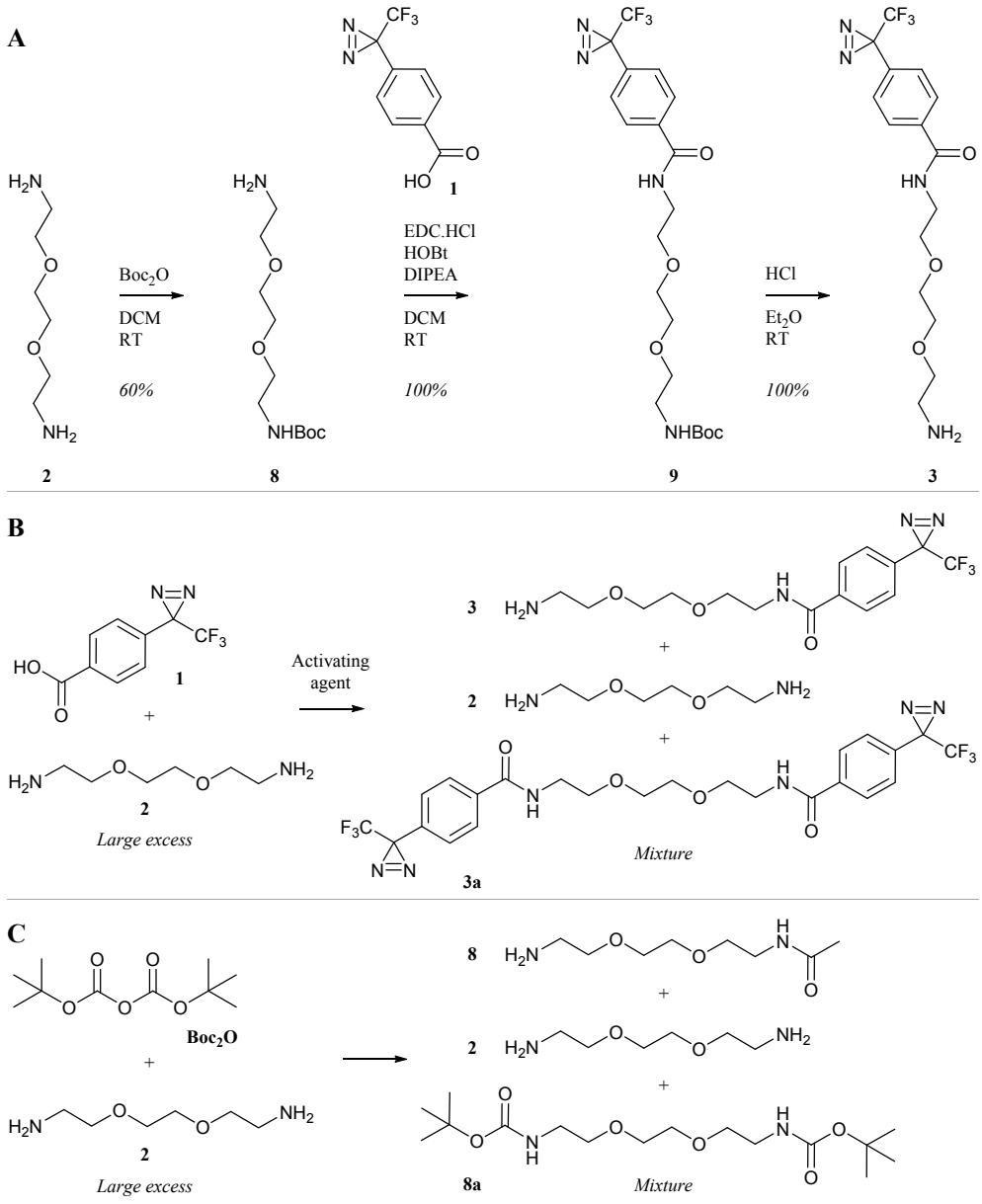


Figure 5. Synthesis of the diazirine building-block: **A**. Synthetic strategy as performed; **B**. Synthetic strategy in one step, which was not performed as it results in an undesired mixture of compounds and waste of the diazirine starting material; **C**. First step of the synthetic strategy as performed, showing the obtained by-products.

3.2 Synthesis of linker A

The synthesis of **Linker A**, as depicted in Figure 6, was done in four steps.

1) Amide formation. The first step towards **Linker A** consisted of the coupling between the amine of commercially available APBA **4** and the carboxylic acid of commercially available protected glutamic acid **10**, which was performed in DCM and in the presence of EDC.HCl as activation reagent. Liquid-liquid extraction was not deemed suitable to isolate product **11**, as many boronic acid-containing compounds have an amphiphilic character. This makes the extraction of such compounds from aqueous solutions difficult.[6] Therefore, the reaction mixture was directly purified by silica flash chromatography, and the desired product **11** was obtained in 30% yield.

2) Carboxylic acid deprotection. Removal of the *tert*-butyl group from the remaining carboxylic acid was achieved in TFA/DCM 1:1, typical conditions for this type of reaction. It did not require any purification and afforded compound **12** in a 98% yield.

3) Amide formation. The coupling of amine-containing diazirine building-block **3** and carboxylic acid **12** was performed in DCM, with a slight excess of **3**, in presence of EDC.HCl and DIPEA. TLC showed complete conversion of acid **12**. After a failed attempt to isolate compound **13** by silica flash chromatography, the reaction was repeated and the desired product was successfully isolated by means of diol-silica flash chromatography (see 3.4). Compound **13** contained major impurities by ^{1}H NMR, but it was used as such for the next step in the hope that the final product would be easier to purify.

4) Amine deprotection. Removal of the Fmoc protecting group was performed in 20% piperidine in DMF, typical conditions for this type of reaction. This is normally a relatively clean reaction, as only two products are formed (plus release of CO_2): the free amine and the Fmoc by-product. In our case, however, ^{1}H NMR showed that a complex mixture was obtained, and too little material was available for any purification attempt. Electrospray ionization high-resolution mass spectrometry (ESI-HRMS) (Figure 13) did indicate the presence of the desired final compound, **Linker A**.

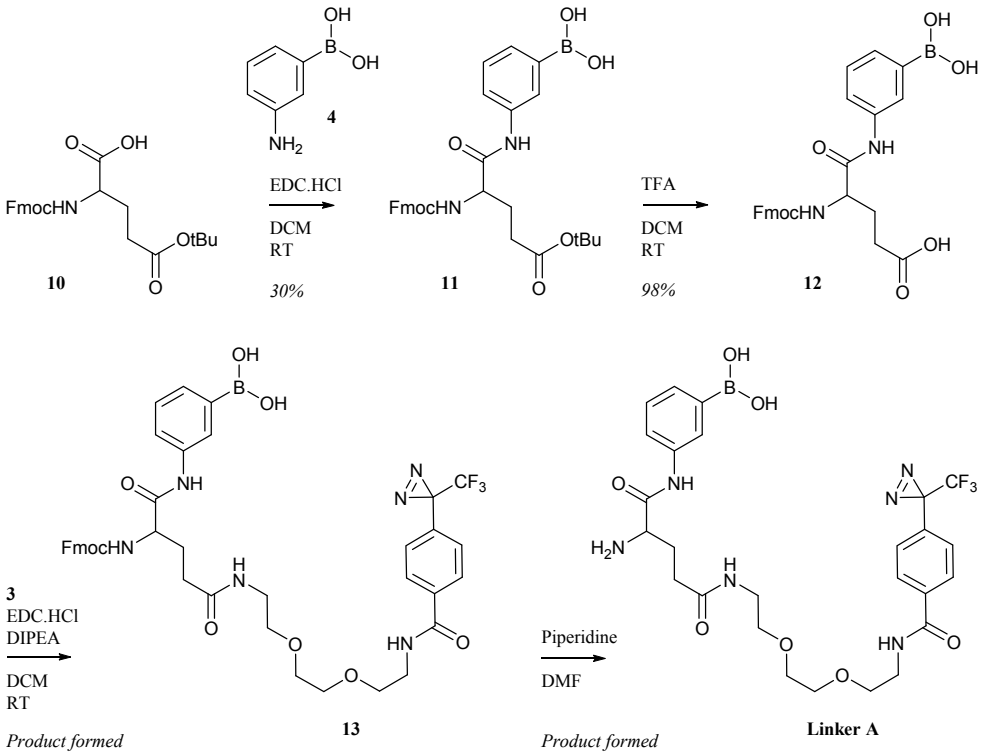


Figure 6. Synthesis of **Linker A**.

3.3 Synthesis of linker B

The synthesis of **Linker B**, as depicted in Figure 7, was done in three steps.

1) Thiol-ene "click". The first step consisted of a radical thiol-ene reaction between the thiol of commercially available Boc-protected cysteine **14**, and the alkene of commercially available boronic acid **6**. A large excess of **14** was used in order to push the desired reaction to completion. DMPA was the radical initiator, and the mixture was irradiated with UV (365 nm). This reaction was done four times:

1. At test scale (15 mg alkene), the reaction was monitored by TLC, which showed near-complete conversion of the alkene after 40 min.

2. To ensure complete conversion of the alkene, the reaction was repeated (100 mg alkene) and run for 2 h. Diol-silica flash chromatography (see 3.4) effectively removed DMPA and excess thiol **14**. The desired product **15** was isolated, but some impurities were present. Subsequent purification by preparative reversed-phase HPLC resulted in a much higher purity as shown by ^{1}H NMR analysis.

3. The reaction was repeated at a larger scale (500 mg alkene). After two failed attempts to purify the product by reversed-phase flash column chromatography (no separation occurred), the recovered material was purified by diol-silica flash chromatography (see 3.4) as in the previous reaction. When further purification was attempted by preparative reversed-phase HPLC, analysis of the obtained fractions by ESI-HRMS did not show the desired product. Instead, the data suggested that the boronic acid had oxidized to a phenol, and the thio-ether had oxidized to a sulfoxide (see chapter 6 for further discussion).

4. The reaction was repeated under the same conditions (500 mg alkene), and compound **15** was directly isolated by diol-silica flash chromatography (see 3.4). ^{1}H NMR suggested the presence of some impurities, but no further purification was attempted as it might result in degradation of the product.

2) Amide formation. The coupling of carboxylic acid **15** with amine-containing diazirine building-block **3** was performed in DCM, with a slight excess of **3**, in presence of EDC.HCl

and DIPEA (same conditions as for the synthesis of **Linker A**). TLC (using the method described in chapter 4) showed the presence of various boronic acid-containing compounds, thus diol-silica flash chromatography did not seem to be a good option for isolation of the desired product. Preparative TLC was tested, without success. Purification by preparative reversed-phase HPLC, however, afforded a trace (unknown) amount of compound **16**, as suggested by ^{1}H NMR and confirmed by ESI-HRMS. The material was not pure, but the available amount did not allow any further purification attempts.

3) Amine deprotection. Removal of the Boc group from compound **16** was carried out using HCl in Et_2O and ESI-HRMS proved the presence of the desired final compound, **Linker B**.

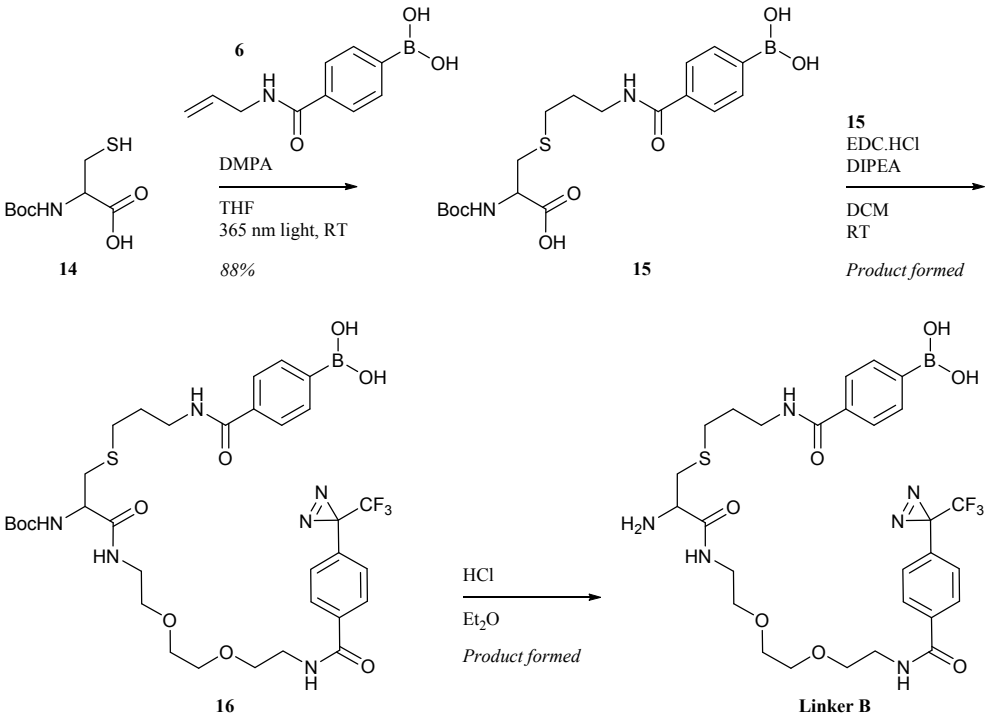


Figure 7. Synthesis of **Linker B**.

3.4 Diol-silica column chromatography of boronic acids

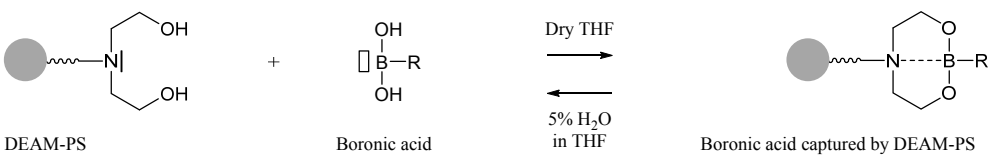
Boronic acids are difficult to purify by silica flash chromatography. In our own experience, many compounds could not be recovered from the column, or were obtained in low yields (such as compound **11**). This results from the tendency of boronic acids to stick to silica (probably by esterification with the silanols), even when highly polar eluents are used.[6] To circumvent this problem, we looked for an alternative way to purify the synthesized boronic acid-containing compounds.

Several polystyrene (PS)-based resins have been developed for the capture and release of boronic acids (see Table 1 for a brief overview), which is based on the pH/solvent-dependent affinity between boronic acid and diol (or triol).

Ref.	Authors	Ligand	Capture	Release
7	Carboni et al.	1,3-diol	Refluxing THF	Refluxing MeOH, THF and DCM
8, 9	Hall et al.	Diethanolamine	THF	THF/H_2O/HOAc (90/5/5) or THF/H_2O (9/1)
10	Yang et al.	Catechol	DCM, THF or toluene	THF/H_2O/HOAc (90/5/5)
11	Liu et al.	Trometamine	THF	THF/H_2O/HOAc (90/5/5)

Table 1. Polystyrene-based resins for the capture and release of boronic acids.

In particular, Hall et al. have developed a diethanolamine-functionalized PS resin (DEAM-PS), which has become commercially available and enables efficient capture, derivatization and release of boronic acids in mild conditions (Figure 8).[8,9] The binding is not only based on boronic acid-diol complexation, but also on an additional interaction between the boronic acid (= Lewis acid) and the tertiary amine (= Lewis base) of PS-bound diethanolamine.



DEAM-PS Boronic acid Boronic acid captured by DEAM-PS

Figure 8. Capture and release of boronic acids by diethanolamine-functionalized polystyrene resin (DEAM-PS)

We prepared a small amount of DEAM-PS using Hall's procedure. Briefly, chloromethyl PS resin (Merrifield resin) was treated with an excess of diethanolamine, resulting in nucleophilic substitution of the chloride with the amine. The obtained material was not characterized, but it was tested for boronic acid capture and release. In our hands, the capture of APBA in dry THF and its release in 5% water in THF resulted in about 50% APBA recovery.

In parallel, we got acquainted with commercially available diol-functionalized silica (diol-silica, Figure 9). Silica powder seemed easier to work with than PS resin: it does not (have to) swell, so it is compatible with many solvents, it does not break under mechanical stress so mixtures can be vigorously stirred, and it does not stick to glassware. According to the manufacturer, diol-silica is meant for the scavenging of boronic acids (e.g., excess boronic acid in a Suzuki reaction), and subsequent release of the boronic acids from the material would require harsh acidic or basic conditions. However, test capture and release of APBA with diol-silica under the same conditions as with DEAM-PS gave similar results as DEAM-PS, although the binding mode was different (no amine). Furthermore, diol-silica was added to the water-free mixture resulting from the reaction of boronic acid-containing intermediate **12** with an excess of diazirine building-block **3**, reasoning that the obtained boronic acid **13** should be captured selectively (if it was the only boronic acid present in solution). After separating the diol-silica from the solution and washing it with dry THF, the bound molecules were released using 5% water in THF. TLC of the obtained material suggested the presence of boronic acid **13**, although it was impure.

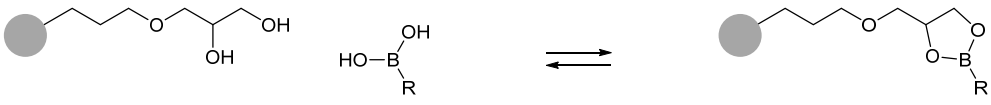


Figure 9. Capture and release of boronic acids by diol-functionalized silica.

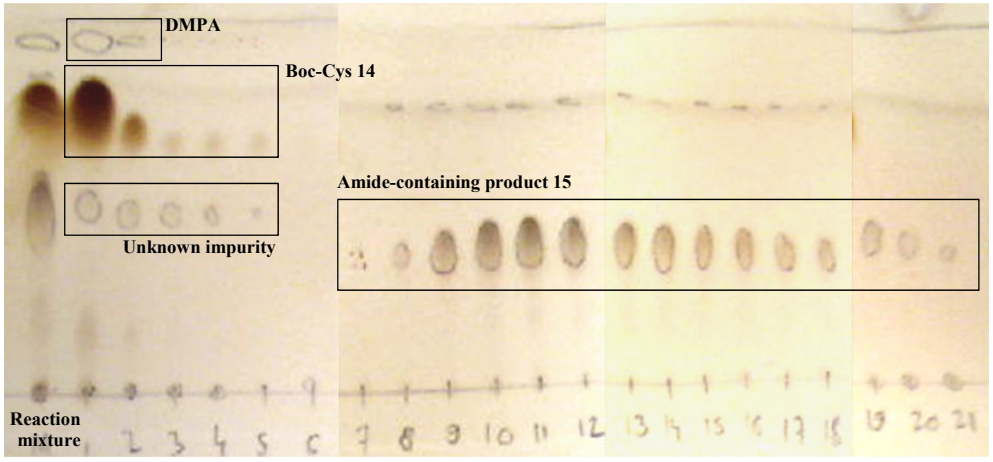
Diol-silica is well known as a versatile stationary phase in chromatography, as it is neutral, moderately polar and able to form hydrogen bonds with solvents and analytes. It has been used in normal-phase, size-exclusion,[12] hydrophilic interaction and reversed-phase chromatography[13] for the separation of diverse compounds. In particular, diol-silica has

sometimes been used in “manual” column chromatography for the purification of organic compounds.[14,15]

The retention of boronic acids on a diol-silica column has once been reported in 1992.[16] The effect of pH of the mobile phase (consisting of 0.1 M phosphate solution of which pH was varied from 4 to 9) and of the boronic acid structure on the retention time was studied. Although the results did not lead to general conclusions, some of them suggested that boronic acids with lower pK_a values had higher retention times, and that ortho-substituted phenylboronic acids could not be retained, most probably because of steric hindrance. This paper has never been cited, and no further work in this direction could be found in the literature.

As the capture and release of our boronic acid-containing intermediate **13** by diol-silica looked promising, we tested diol-silica as stationary phase for the flash chromatography of this compound. Although product and impurities ran very close to each other on silica TLC, the diol-silica column enabled the rapid elution of these impurities in a gradient of MeOH in EtOAc, and then the slow elution of boronic acid **13** when 1% acetic acid was added to the eluent. The obtained material still contained major impurities, but the fact that some material was recovered from the column was already an improvement compared to previously attempted silica flash chromatography. Diol-silica flash chromatography was also tested for the purification of boronic acid **15**, which proved particularly effective (Figure 10). According to silica TLC, boronic acid **15** and an apparently boronic acid-free impurity had similar polarities, and Boc-cysteine **14**, which was still present in large excess in the reaction mixture, was difficult to remove by silica flash chromatography. However, **14**, DMPA and the unknown impurity were quickly eluted from the diol-silica column, whereas boronic acid **15** was eluted at a later stage and was obtained in a satisfying purity according to ^{1}H NMR. Diol-silica, therefore, seems a promising alternative to bare silica for the column chromatography of boronic acid-containing compounds.

Ninhydrin staining: amino acids



Alizarin staining: boronic acids (method described in chapter 4)

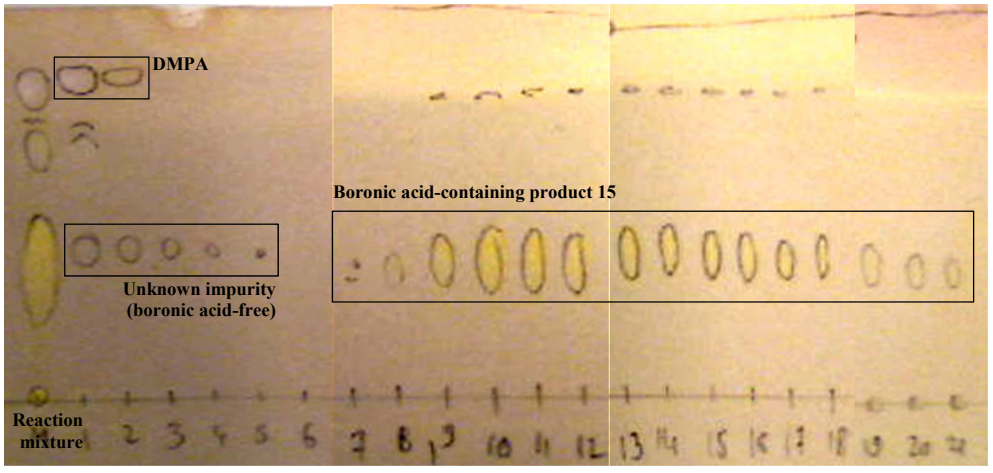


Figure 10. Purification of boronic acid-containing intermediate **15** by diol-silica column chromatography. Silica TLC of the column fractions (10% MeOH and 1% acetic acid in EtOAc). Spots circled by pencil: visible under 254 nm light.

3.5 Mass spectrometry of boronic acids

All synthesized boronic acid-containing compounds were dissolved in MeOH and analyzed by ESI-HRMS, and the obtained mass spectra presented a particular feature: species corresponding to the reaction of the compounds with one or two molecules of MeOH were identified (Figure 11). This is supported by the work of Wang et al. who studied the chemistry of boronic acids under electrospray conditions and observed the same phenomenon.[17] We found this very useful because it proved, in addition to the matching exact masses, the presence of boronic acid moieties with great confidence. All observed masses are given in paragraph 4.5 and mass spectra of **Linker A** and **Linker B** are given in Figure 13 and Figure 14, respectively. Moreover, as shown in Figure 12, the main species observed in the analysis of both linkers in positive mode and negative mode revealed isotope patterns that were in excellent agreement with the simulated ones.

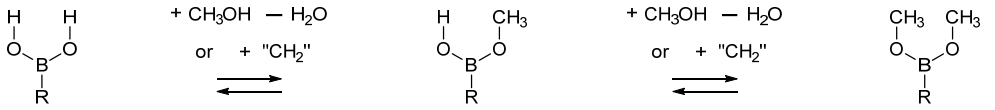
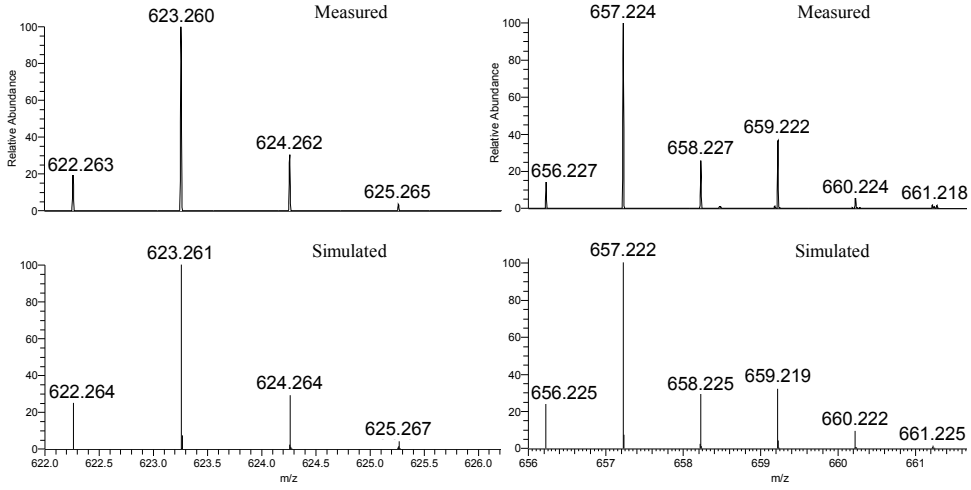


Figure 11. Reaction of boronic acids with MeOH as observed in mass spectra.

Linker A

Positive mode: $C_{27}H_{35}O_7N_6BF_3$ ($M + CH_2 + H^+$)

Negative mode: $C_{27}H_{34}O_7N_6BClF_3$ ($M + CH_2 + Cl^-$)



Linker B

Positive mode: $C_{29}H_{38}O_7N_6BF_3NaS$ ($M + CH_2 + Na^+$)

Negative mode: $C_{29}H_{38}O_7N_6BClF_3S$ ($M + CH_2 + Cl^-$)

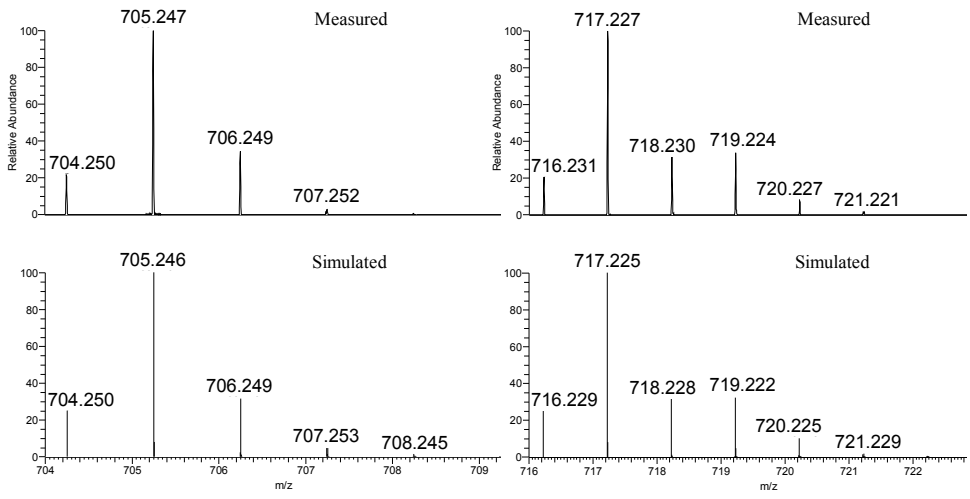


Figure 12. Measured and simulated isotope patterns of the largest quasi-molecular ions observed in the HRMS analysis of linkers A and B.

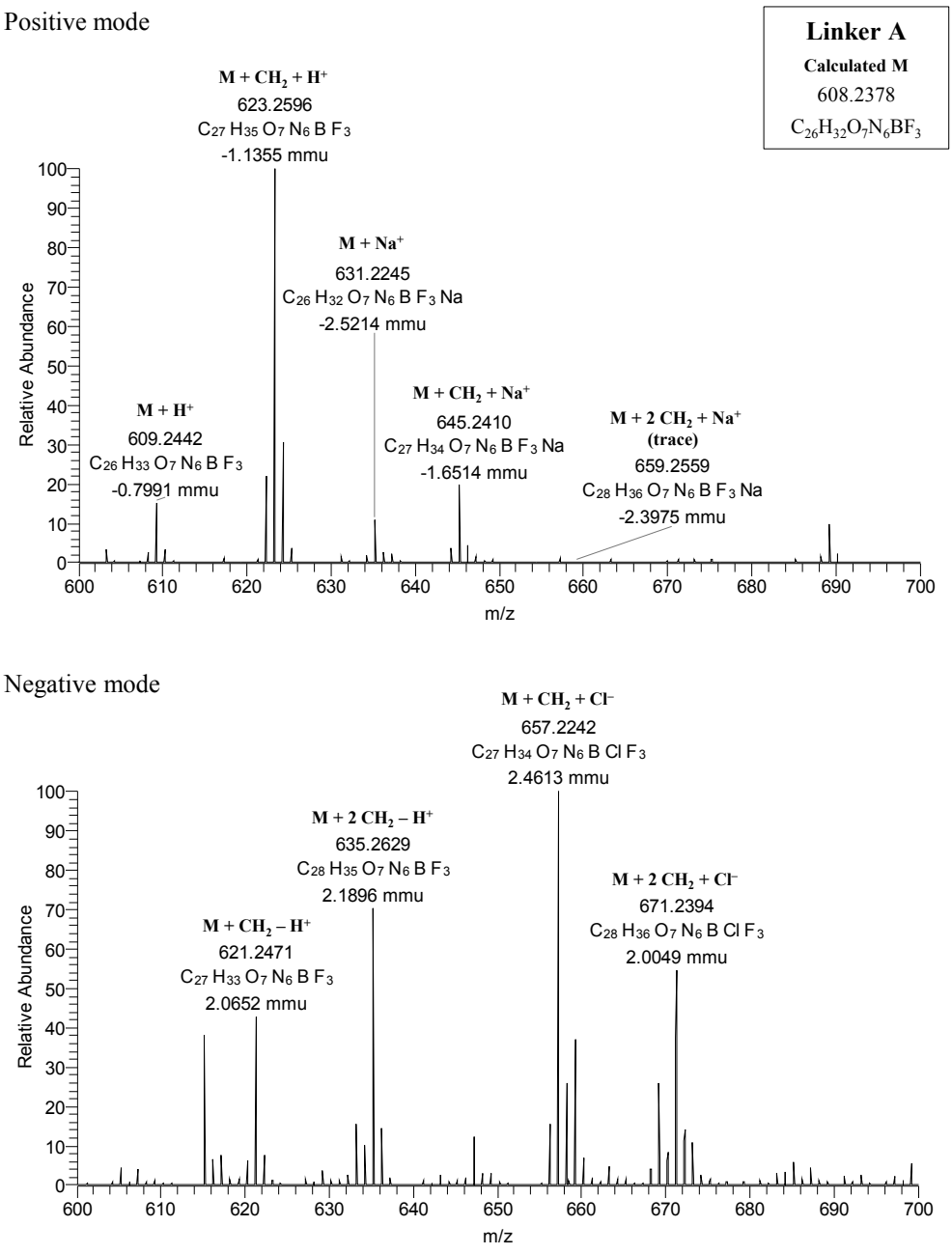


Figure 13. High-resolution mass spectra of **Linker A**.

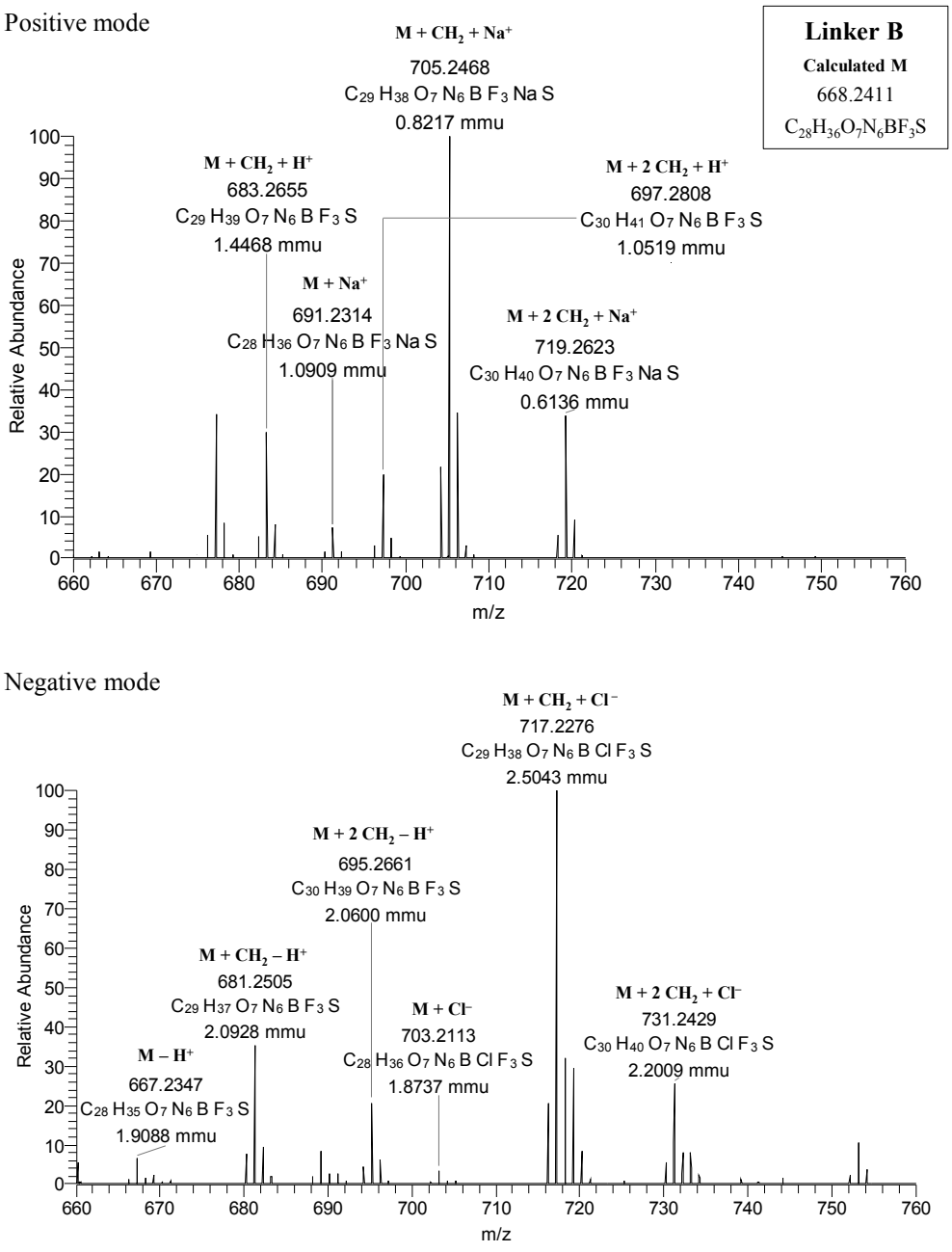


Figure 14. High-resolution mass spectra of **Linker B**.

4 Experimental section

4.1 General information

All solvents and reagents were obtained commercially and used as received unless stated otherwise. Moisture-sensitive reactions were performed under Ar atmosphere and with oven-dried flasks. All experiments involving compounds that contain a diazirine group were carried out at RT or lower temperature and in the dark or with minimal light. Diol-functionalized silica was obtained from Screening Devices.

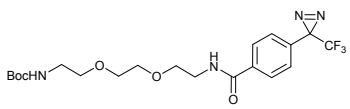
Reactants and reagents. 2,2′-(ethylenedioxy)diethylamine, HOBt, DIPEA, 3-aminophenylboronic acid (APBA), Fmoc-Glu(OtBu)-OH, piperidine and DMPA were from Sigma-Aldrich. Di-*tert*-butyl dicarbonate (Boc_2O) and TFA were from Acros Organics. 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzoic acid was from TCI Europe. EDC.HCl was from Fischer. 4-Allylaminocarbonyl)phenylboronic acid and N-alpha-*tert*-butyloxycarbonyl-L-cysteine were from ABCR. HCl 1 M in Et_2O was from Alfa-Aesar.

Solvents. For reactions, DCM and THF, both from Sigma-Aldrich, were purified using a Pure Solv 400 solvent purification system (Innovative Technology, Amesbury, USA). DMF was from VWR. DCM and EtOAc from Acros Organics, acetic acid and heptane from VWR, MeOH and methyl *tert*-butyl ether from Biosolve and ammonia (25% in water) from Merck were used for purifications. $CDCl_3$ from Acros Organics and CD_3OD from Roth were used for NMR analysis.

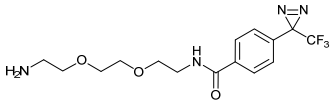
Apparatus. ^1H NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer. Chemical shifts are reported in parts per million (ppm), with tetramethylsilane as internal standard. High-resolution mass spectrometry analyses were recorded on a Thermo Fisher Scientific Exactive High-Resolution mass spectrometer, using electrospray ionization. The preparative HPLC setup consisted of two LC-8A pumps, SIL-10AP auto-injector, FRC-10A fraction collector and SPD-M10Avp diode array detector from Shimadzu, and an Alltima C18 column (250 × 22 mm, 5 µm) from Alltech.

4.2 Diazirine building-block

8. A solution of 2,2′-(ethylenedioxy)diethylamine **2** (15.5 mL, 106.2 mmol) in dry DCM (200 mL) was cooled to 0 °C. A solution of di-*tert*-butyl dicarbonate (Boc_2O, 4.52 g, 20.7 mmol) in dry DCM (50 mL) was added over 40 min. The reaction mixture was allowed to warm up to RT and stirred overnight at RT. The reaction mixture was purified by flash chromatography (silica, 1% to 20% MeOH/ammonia 9:1 in DCM), to give 3.02 g of compound **8** (59%) and 1.55 g of a mixture of compounds **8** and **8a**. Compound **8**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 9H), 2.84 (m, 2H), 3.27 (m, 2H), 3.50 (m, 4H), 3.58 (apparent s, 4H).



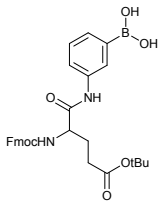
9. A solution of 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzoic acid **1** (500 mg, 2.17 mmol, 1 eq), **8** (701 mg, 2.82 mmol, 1.3 eq), HOBt (440 mg, 3.26 mmol on anhydrous basis, 1.5 eq) and DIPEA (1.14 mL, 6.52 mmol, 3 eq) in dry DCM was cooled to 0 °C. EDC.HCl (625 mg, 3.26 mmol, 1.5 eq) was added, the reaction mixture was allowed to warm up to RT and stirred overnight. TLC showed complete conversion of **8**. The reaction mixture was concentrated under vacuum, and the material was purified by flash chromatography (30 g silica, 30% to 70% EtOAc in heptane) to give 1.04 g yellow oil ("104%", probably near-quantitative yield and unknown impurities). ^{1}H NMR (400 MHz, CD_3OD) δ 1.37 (s, 9H), 3.15 (m, 2H), 3.45 (t, 2H), 3.50-3.64 (m, 8H), 7.30 (d, 2H), 7.87 (d, 2H).



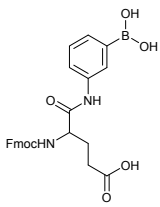
3. Compound **9** (1.04 g, 2.17 mmol + impurities) was dissolved in HCl 1 M in Et_2O (30 mL), and stirred at RT for 48 h. TLC (EtOAc) showed complete conversion of **9** (incomplete after 24 h). The reaction mixture consisted of a white suspension and a wax on the bottom of the flask. NMR analysis showed that the desired product was only in the wax. The liquid was removed, the wax was dissolved in 2 mL MeOH, 10 mL HCl 1 M in Et_2O was added, which caused precipitation, and this mixture was stirred for 1 h (to make sure that the product was only in HCl salt form). The mixture was concentrated under vacuum to

give 890 mg waxy solid (quantitative yield). ^{1}H NMR (400 MHz, CD_3OD) δ 3.04 (m, 2H), 3.54 (m, 2H), 3.64 (m, 8H), 7.30 (d, 2H), 7.87 (d, 2H).

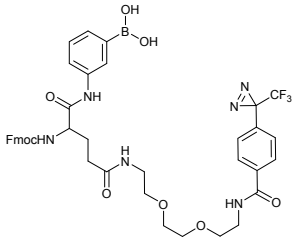
4.3 Linker A



11. A solution of APBA **4** (259 mg, 1.89 mmol, 1 eq) and Fmoc-Glu(OtBu)-OH **10** (965 mg, 2.27 mmol, 1.2 eq) in dry DCM was cooled to 0 °C. EDC.HCl (905 mg, 4.72 mmol, 2.5 eq) was slowly added, and the reaction mixture was stirred for five days (a shorter time may have been sufficient). The reaction mixture was concentrated under vacuum and purified by flash chromatography (silica, 50% to 100% EtOAc in heptane, and 5% MeOH in EtOAc), to give 309 mg solid (30%). ^{1}H NMR (400 MHz, CD_3OD) δ 1.43 (s, 9H), 1.9 (m, 1H), 2.1 (m, 1H), 2.36 (t, 2H), 4.20 (t, 1H), 4.27 (m, 1H), 4.39 (m, 2H), 7.00-8.00 (several signals, 12H).

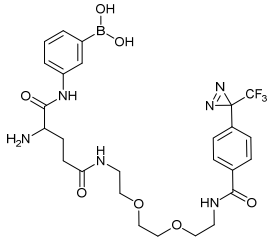


12. Compound **11** (112 mg, 0.206 mmol) was dissolved in DCM (3 mL). TFA (3 mL) was added and the reaction mixture was stirred at RT overnight. TLC (5% MeOH and 1% acetic acid in EtOAc) showed complete conversion of **11** and a single new spot by UV and by alizarin staining (method described in chapter 4). The reaction mixture was concentrated under vacuum to give 98 mg light-brown solid (98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.02 (m, 1H), 2.17 (m, 1H), 2.46 (t, 2H), 4.15-4.35 (m, 2H), 4.41 (m, 2H), 7.10-7.90 (multiple signals, 12H).



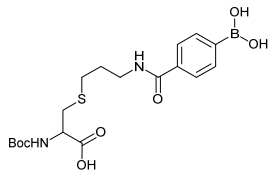
13. A solution of **12** (38 mg, 0.078 mmol), **3** (41 mg, 0.10 mmol, 1.3 eq) and DIPEA (34 µL, 0.2 mmol, 2 eq) in dry DCM (5 mL) was cooled to 0 °C. EDC.HCl (30 mg, 0.16 mmol, 2.0 eq) was slowly added, the reaction mixture was allowed to warm up to RT and stirred overnight at RT. TLC (10% MeOH and 1% acetic acid in EtOAc) showed complete conversion of **12**. The RM was concentrated from 5 mL to 2-3 mL and purified by flash chromatography (8.4 g diol-silica, gradient of 0 to 5% MeOH in EtOAc and then 10%

MeOH and 1% acetic acid in EtOAc). The fractions that looked purest by TLC were concentrated under vacuum at RT to give about 40 mg oil. ^{1}H NMR suggested but did not confirm that the desired product was present. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.94 (acetic acid + 1H, based on chemical shift in Fmoc-Glu(OH)-APBA), 2.10 (m, 1H), 2.28 (t, 2H), 3.10-3.80 (several signals, 12H + MeOH + impurities), 4.10-4.25 (several signals, 1H + 1H), 4.33 (m, 1H), 6.40-8.00 (several signals, 16H), major unknown impurities.



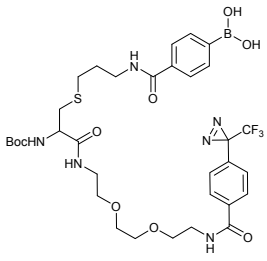
Linker A. A solution of **13** in DMF (0.8 mL) and piperidine (0.2 mL) was stirred at RT. When TLC (10% MeOH and 1% acetic acid in EtOAc) showed complete conversion of **13**, DMF and piperidine were evaporated using a high-vacuum pump (pressure between 0 and 1 mbar) with a liquid nitrogen trap. The mixture (oil and solid) was partitioned between methyl tert-butyl ether (6 mL) and water (6 mL). Not all material was dissolved, so some ammonia was added: the water layer reached a pH of about 10-11. Both layers separated very slowly. According to TLC analysis, both layers contained boronic acid-containing compounds, and only the organic layer contained Fmoc by-product. The water layer was concentrated under vacuum. NMR analysis of the obtained material was inconclusive, but ESI-HRMS confirmed the presence of the desired final compound.

4.4 Linker B

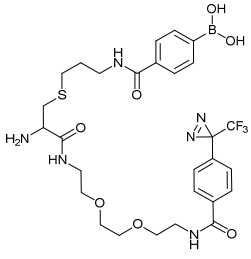


15. (4-Allylaminocarbonyl)phenylboronic acid **6** (500 mg, 2.44 mmol, 1 eq), N-alpha-*tert*-butyloxycarbonyl-L-cysteine **14** (2.70 g, 12.2 mmol, 5 eq) and DMPA (625 mg, 2.44 mmol, 1 eq) were dissolved in dry and stabilizer-free THF (10 mL). The resulting solution was stirred and irradiated at 365 nm for 2 h. TLC (10% MeOH and 1% acetic acid in EtOAc) showed complete conversion of **6**. The reaction mixture was concentrated to a volume of about 5 mL, and purified by flash chromatography (28 g diol-silica, 0 to 20% MeOH in EtOAc) to give 920 mg solid/oil (88%). The obtained material was not very pure, but it was used as such in the next step. Previous attempts to purify this compound by preparative HPLC had resulted in its complete degradation. ^{1}H NMR (400 MHz, CD_3OD)

δ 1.34 (s, 9H), 1.81 (quint, 2H), 2.57 (t, 2H), 2.76 (dd, 1H), 2.92 (dd, 1H), 3.38 (m, 2H), 4.11 and 4.22 (m, 1H), 7.50-7.80 (m, 4H).



16. A solution of **15** (100 mg, 0.23 mmol, 1 eq), HCl salt of **3** (112 mg, 2.81 mmol, 1.2 eq) and DIPEA (0.16 mL, 0.94 mmol, 4 eq) in dry DCM was cooled to 0 °C. EDC.HCl (90 mg, 0.47 mmol, 2 eq) was added, the reaction mixture was allowed to warm up to RT and stirred overnight at RT. Extensive TLC analysis (various eluents, normal phase and reverse phase, two-dimensional, analysis of spots by mass spectrometry) suggested that several spots contained the desired product (possibly esterified with MeOH). Test preparative TLC and filtration with reversed-phase silica were not successful. A part of the reaction mixture was purified by preparative HPLC (reversed-phase) to give a few mg of a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 1.33 (s, 9H), 1.79 (q, 2H), 2.54 (t, 2H), 2.64 (dd, 1H), 2.82 (dd, 1H), 3.23-3.60 (m, 14H + impurities), 4.12 (m, 1H), 7.23 (d, 2H), impurities.



Linker B. To **16** (unknown amount) was added 1 M HCl in Et_2O and the material did not dissolve. Some MeOH was added and the material dissolved. The solution was stirred at RT overnight. The reaction mixture, as analyzed by ESI-HRMS, contained the desired final compound.

4.5 High-resolution mass spectrometry

	Compound 12		Compound 13		Linker A	
	Calculated	**Measured**	Calculated	**Measured**	Calculated	**Measured**
M	488.1755		830.3058		608.2378	
Positive mode						
$M + H^+$	489.1828		831.3131		609.2450	**609.2442**
$M + Na^+$	511.1642		853.2945		631.2264	**631.2245**
$M + CH_2 + H^+$	503.1984		845.3288		623.2607	**623.2596**
$M + 2CH_2 + H^+$	517.2141		859.3444		637.2763	
$M + CH_2 + Na^+$	525.1798	**525.1794**	867.3102		645.2421	**645.2410**
$M + 2CH_2 + Na^+$	539.1955	**539.1957**	881.3258		659.2577	**659.2559**
Negative mode						
$M - H^+$	487.1682		829.2986		607.2305	
$M + Cl^-$	523.1449		865.2752		643.2072	
$M + CH_2 - H^+$	501.1839	**501.1827**	843.3142		621.2461	**621.2471**
$M + 2CH_2 - H^+$	515.1995	**515.1982**	857.3299		635.2618	**635.2629**
$M + CH_2 + Cl^-$	537.1605		879.2909	**879.2925**	657.2228	**657.2242**
$M + 2CH_2 + Cl^-$	551.1762		893.3065	**893.3077**	671.2385	**671.2394**

	Compound 15		Compound 16		Linker B	
	Calculated	**Measured**	Calculated	**Measured**	Calculated	**Measured**
Positive mode						
$M + H^+$	427.1705		769.3008		669.2484	
$M + Na^+$	449.1519	**449.1523**	791.2822		691.2298	**691.2314**
$M + CH_2 + H^+$	441.1861		783.3165		683.2641	**683.2655**
$M + 2CH_2 + H^+$	455.2018		797.3321		697.2797	**697.2808**
$M + CH_2 + Na^+$	463.1675	**463.1679**	805.2979	**805.2955**	705.2455	**705.2468**
$M + 2CH_2 + Na^+$	477.1832		819.3135	**819.3117**	719.2611	**719.2623**
Negative mode						
$M - H^+$	425.1559	**425.1554**	767.2863		667.2339	**667.2347**
$M + Cl^-$	461.1326		803.2630		703.2105	**703.2113**
$M + CH_2 - H^+$	439.1716	**439.1720**	781.3019	**781.3022**	681.2495	**681.2505**
$M + 2CH_2 - H^+$	453.1872	**453.1876**	795.3176	**795.3181**	695.2652	**695.2661**
$M + CH_2 + Cl^-$	475.1483		817.2786	**817.2795**	717.2262	**717.2276**
$M + 2CH_2 + Cl^-$	489.1639		831.2943	**831.2950**	731.2418	**731.2429**

Compound 11: no recognizable fragment was found in its mass spectra

5 Conclusion

Two linkers for the oriented and irreversible immobilization of antibodies were synthesized. They contained an amine group to react with an active ester on a surface, a boronic acid group to bind an antibody via its N-glycans in its Fc region, and a diazirine group to irreversibly stick the antibody to the surface while maintaining its orientation. However, when the final compounds were obtained as confirmed by high-resolution mass spectrometry, Adak et al. published an article describing a linker with the exact same functional groups (amine, boronic acid and trifluoromethylaryldiazirine), and they showed that an antibody that was immobilized on a surface via this linker had a superior antigen-binding ability.[18] Our work was therefore no longer publishable, as the antibody immobilization strategy that we had in mind had lost its novelty. Nevertheless, we would like to highlight two points from this chapter that may be useful in future research: 1) a novel building block, which consisted of a trifluoromethylaryldiazirine and a primary amine connected to each other by a flexible chain, was efficiently synthesized and purified, and our procedure may be used for other applications that require the attachment of a photoreactive and flexible molecule to an electrophile-containing scaffold; 2) boronic acid-containing intermediates were successfully isolated by means of diol-silica flash chromatography. On the basis of this we propose the use of diol-silica as an alternative to bare silica for the purification of boronic acids.

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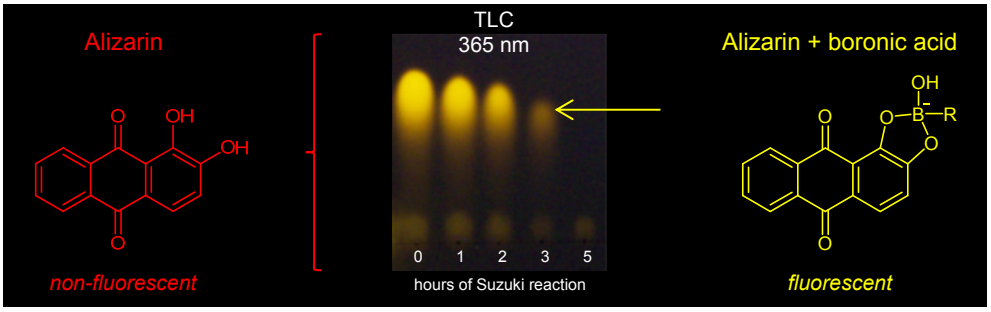
Chapter 4
Sensitive thin-layer chromatography detection of boronic acids using alizarin



A new method for the selective and sensitive detection of boronic acids on thin-layer chromatography plates is described. The plate is briefly dipped in an alizarin solution, allowed to dry in ambient air, and observed under 365 nm light. Alizarin emits a bright yellow fluorescence only in the presence of a boronic acid.

This chapter is a slightly modified version of the following publication: F. Duval, T.A. van Beek, H. Zuilhof, *Sensitive thin-layer chromatography detection of boronic acids using alizarin*, Synlett, 2012, **23**, 1751-1754.

1 Introduction

Boronic acids are used in a wide range of applications (see chapter 1). Before any use, these compounds first need to be synthesized. Easy detection of boronic acids during reactions, however, remains a challenge.

The conversion of boronic acids has been monitored in Suzuki-Miyaura reaction mixtures to which dihydroxycoumarins were added. Fluorescent complexes resulting from binding of these compounds with boronic acids were visualized under a hand-held UV lamp (365 nm).[1] TLC, however, is a more generally applicable and easier way to analyze a reaction mixture. The detection of boronic acids on TLC plates would enable the chemist to monitor not only the conversion of a boronic acid, but also the integrity of a boronic acid group during a reaction involving other functional groups. Moreover, it would not complicate the purification of the product as there would be no sensing molecule in the reaction mixture.

The natural anthraquinone alizarin, a weakly fluorescent compound in its free form, becomes highly fluorescent when bound to a boronic acid via its 1,2-diol (Figure 1).[2] This property has been more commonly exploited with the water-soluble derivative alizarin red S (ARS) (Figure 1), and the mechanism of the involved reaction has been studied in detail.[3] In particular, ARS has been used for the direct visualization of a boronic acid group on a cellulose surface[4] and on glass slides[5] by confocal microscopy.

During our synthetic work involving boronic acids (prior to the work described in chapter 3), we were interested to see whether the commercially available and inexpensive alizarin (not to be confused with alizarin Red S) would be of help in visualizing boronic acids on TLC plates. Alizarin has been used in combination with concentrated H_2SO_4 for the fluorescent detection of boric acid on spot plates.[6] Alizarin is also known as a TLC staining reagent for the detection of cations (e.g., Al(III)), which requires an after-treatment with ammonia.[7] In this chapter, we introduce alizarin as a TLC staining reagent for the detection of boronic acids.

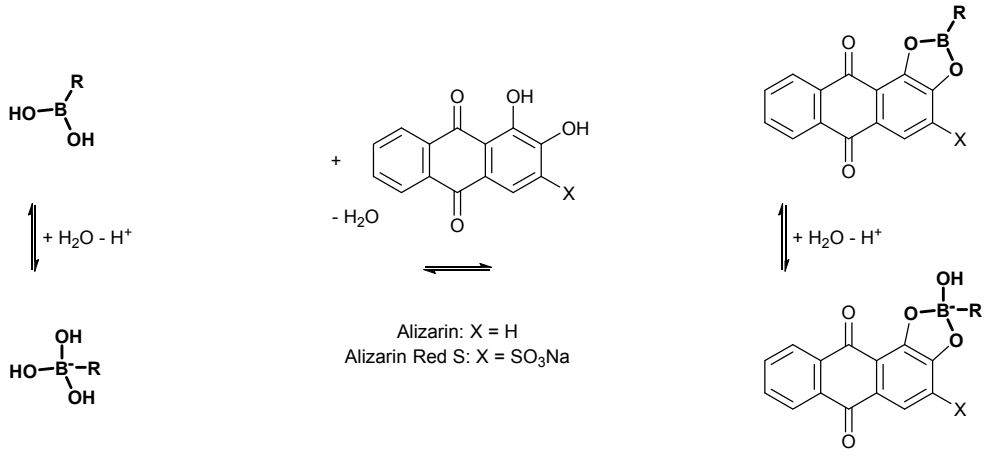


Figure 1. Complexation of alizarin and alizarin Red S with boronic acids.

2 Results and discussion

2.1 Method development

Preliminary tests looked promising: after brief immersion of a TLC plate in a saturated methanolic alizarin solution and spontaneous evaporation of the solvent, spots that contained phenylboronic acid (PBA) turned yellow, whereas the rest of the plate turned pink. Under 365 nm light, the spots showed an intense yellow fluorescence on a dark background, even though it is mentioned in the article of Barder and Buchwald that alizarin would require a significantly higher excitation wavelength (~550 nm).[1]

The detection of 2 µL spots of PBA solutions was optimized. A 50 mM solution of Na_2CO_3 in 50% aqueous MeOH has previously been used for measuring the affinity of alizarin for boronic acids attached to solid supports.[8] Solubility tests, moreover, showed that 1 mM was near the saturation concentration of alizarin in MeOH. The following solutions, therefore, were tested for the detection of PBA spots on a TLC plate: 1 mM alizarin in 1) 20 mM Na_2CO_3 in water/MeOH 20:80, 2) MeOH, 3) 20 mM HCl in water/MeOH 20:80. The most intense fluorescence was observed when pure MeOH was used (Figure 2A). Ethanol,

EtOAc, DCM, THF and acetone were also tested, and all solvents gave similar results in terms of sensitivity: 2 µL spots of PBA solutions at concentrations down to 0.1 mM (24 ng) could be easily detected (Figure 2A). Acetone was selected as the preferred solvent as it is inexpensive, relatively safe, and the solubility of alizarin was higher in acetone than in the other solvents (except THF). The detection of 2 µL spots of 1 mM PBA was subsequently tested with alizarin solutions at different concentrations in acetone: 10 mM, 1 mM, 0.1 mM, and 0.01 mM. The most intense fluorescence was observed with the initial concentration of 1 mM (Figure 2B). Staining the TLC plate using a piece of cotton, or dipping it in the alizarin solution for 1 s, 5 s or 10 s resulted in the same sensitivity. For unknown reasons, spraying the TLC plate with the alizarin solution did not result in any color change of the TLC plate.

The following method was therefore chosen for subsequent experiments:

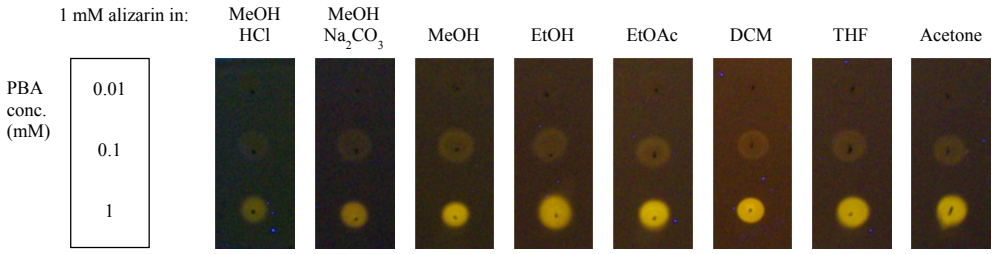
- Briefly dip the TLC plate in a 1 mM alizarin solution in acetone.

- Let dry and wait until the TLC plate is pink.

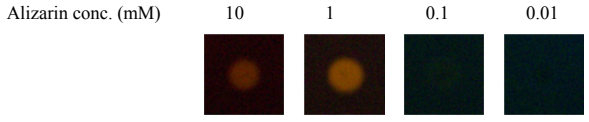
- Observe under 365 nm light.

After PBA was spotted in triplicate at concentrations ranging from 0.01 mM to 100 mM, staining using the chosen method indicated that the limit of detection of PBA was between 0.01 mM and 0.1 mM. Measurement of the mean spot brightness (using graphics editor GIMP) suggested that the developed method even enabled semi-quantification of PBA in the spotted solutions (Figure 2C).

A. Effect of acid, base and solvent used for the alizarin solution



B. Effect of the alizarin concentration in acetone



C. Semi-quantitative aspect

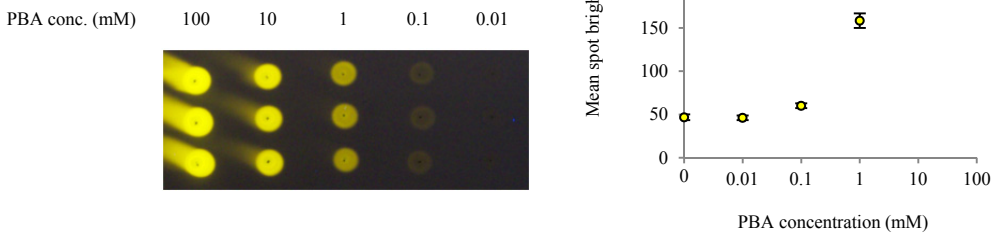


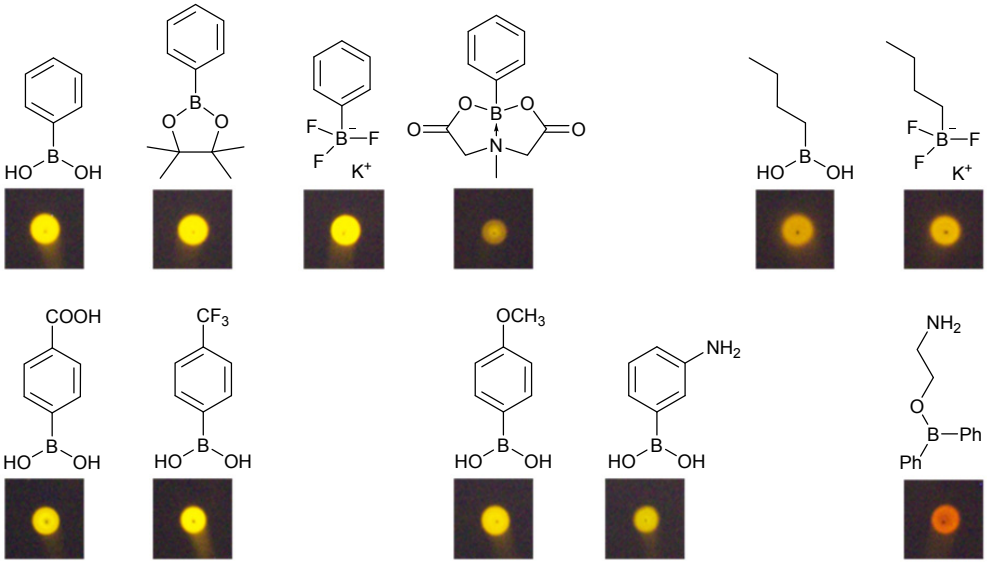
Figure 2. Development and semi-quantitative aspect of the method. Selections from original photographs of TLC plates spotted with PBA (2 µL, various concentrations), briefly immersed in alizarin solution (various compositions), left to dry and placed under 365 nm light.

2.2 Scope and selectivity

The ability of this method to detect diverse boronic acids and derivatives was tested (Figure 3A), and all compounds appeared yellow-orange. Electron-deficient boronic acids produced a slightly more intense color than electron-rich boronic acids, which is probably due to the pK_a dependence of the boronic acid-diol complexation.[9] PBA pinacol ester was revealed with the same fluorescence intensity as PBA, which can be explained by the much higher affinity of PBA for alizarin than for aliphatic diols,[10] resulting in displacement of pinacol by alizarin. Trifluoroborates were revealed with the same fluorescence intensity as their corresponding boronic acids. PBA N-methyliminodiacetate (MIDA) ester could also be detected, although it gave a much weaker fluorescence than PBA itself. The flavonoid reagent diphenylborinic acid 2-aminoethyl ester (DPBA) gave a distinct orange fluorescence upon contact with alizarin. Under neutral conditions the catechol group of alizarin, like the catechol group of some flavonoids (e.g., rutin), probably gives a negatively charged tetrahedral complex with DPBA after loss of ethanolamine.[11] The difference in fluorescence suggests that alizarin is able to distinguish between boronic and borinic esters.

The selectivity of this method towards boronic acids was tested by spotting compounds with various functional groups on a TLC plate (Figure 3B). The fluorescence from these compounds was very weak compared to the fluorescence from boronic acids at a 10-fold lower concentration, and none of the tested boron-free compounds resulted in the characteristic yellow spots observed in presence of the tested boron-containing compounds.

A. Boronic acids and derivatives: 2 µL, **10 mM**



B. Boron-free compounds: 2 µL, **100 mM**

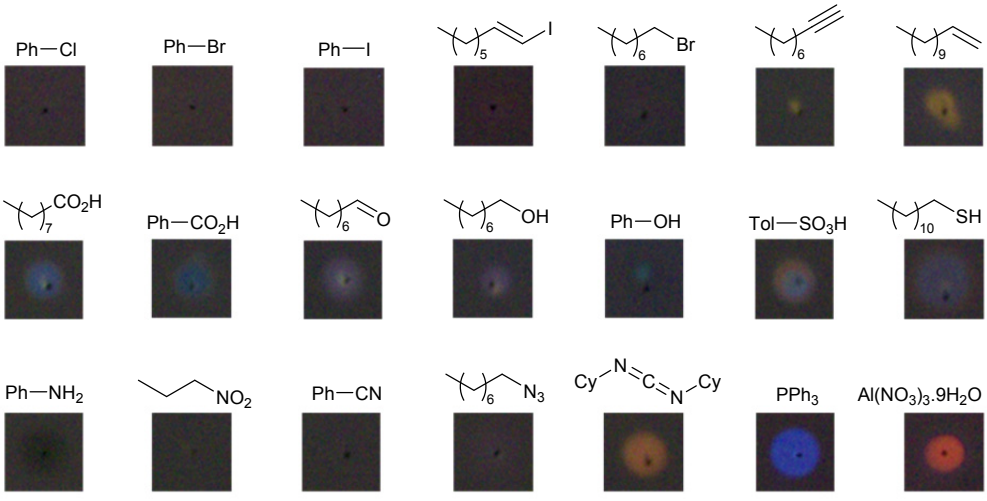


Figure 3. Scope and selectivity of the developed staining reagent. Selections from original photographs of TLC plates spotted with diverse boronic acids and derivatives (10 mM in MeOH*, 2 µL; *MIDA ester: dry THF) (**A**) and diverse boron-free compounds (100 mM in DCM, 2 µL) (**B**), briefly immersed in 1 mM alizarin solution in acetone, left to dry and placed under 365 nm light.

2.3 Applications

The applicability of this method to the analysis of reactions involving boronic acids was tested in 3 examples (Figure 4).

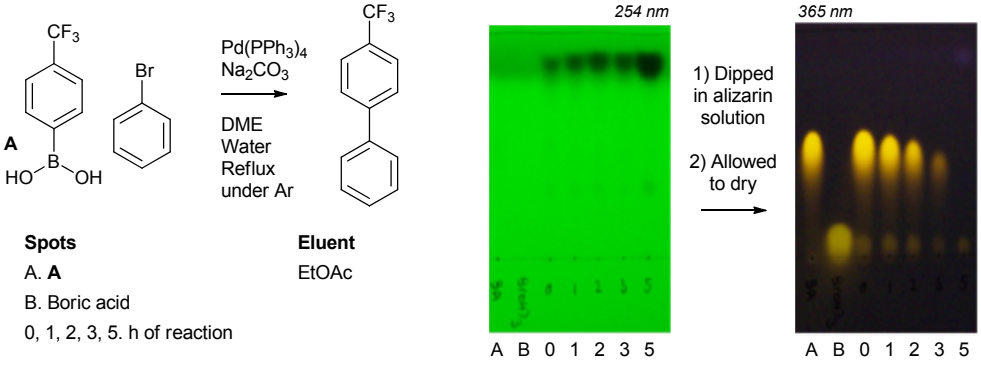
Example 1. Monitoring the consumption of a boronic acid during a classical Suzuki reaction. The reaction between 4-(trifluoromethyl)phenylboronic acid and bromobenzene was carried out, based on the procedure described by Yang et al.[12] A sample was spotted every hour on a TLC plate. TLC analysis using 254 nm light did not give any indication about the progress of the reaction. TLC analysis using the proposed method, however, showed a clear decrease of the concentration of boronic acid over time. After 5 h, the boronic acid was not detected anymore, which suggested that full conversion had been obtained.

Example 2. Checking the completion of a ligand-free Suzuki reaction. The reaction between PBA and 1-bromonaphthalene was carried out, using the modified procedure of Liu et al.[13] After 4 h of reaction, the reaction mixture was partitioned between DCM and brine, and both layers were analyzed by TLC. TLC analysis using 254 nm light was inconclusive. TLC analysis using the proposed method, however, showed that neither the DCM layer nor the brine layer contained any significant amount of PBA, and that full conversion had been obtained.

Example 3. Visualizing the transformation of a boronic acid into another boronic acid. The esterification of 4-carboxyphenylboronic acid in acidic ethanol was carried out, using the procedure of Davison et al.[14] After 1 h of reaction, the reaction mixture was analyzed by TLC. TLC analysis using 254 nm light showed only weak spots that corresponded to starting material and a new compound. TLC analysis using the proposed method, however, showed the same spots with an intense yellow fluorescence. Apart from a much easier observation, this also directly indicated that the new compound contained a boronic acid moiety.

An additional example is shown in chapter 3, page 45 (without UV).

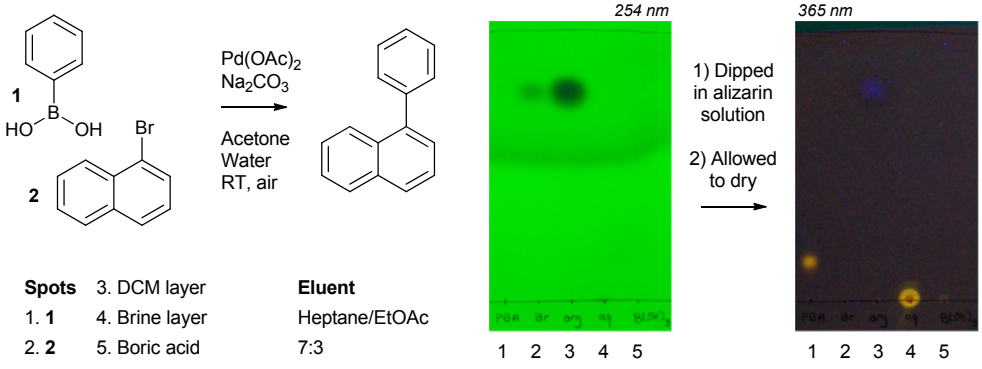
Example 1



Spots
A. **A**
B. Boric acid
0, 1, 2, 3, 5. h of reaction

Eluent
EtOAc

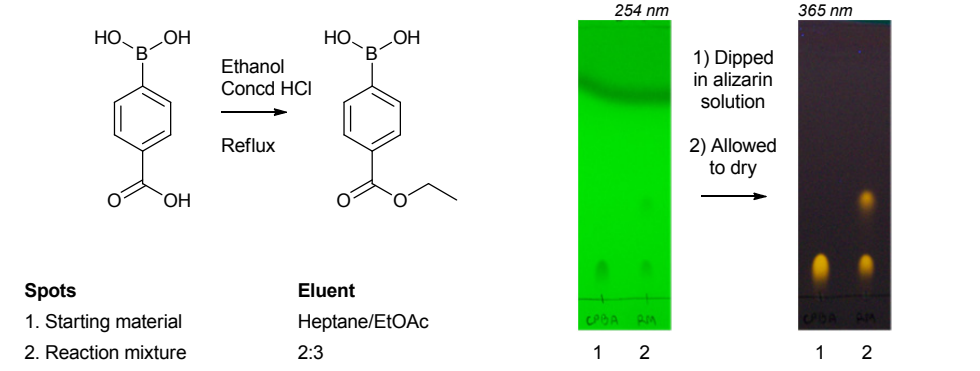
Example 2



Spots
1. **1**
2. **2**
3. DCM layer
4. Brine layer
5. Boric acid

Eluent
Heptane/EtOAc
7:3

Example 3



Spots
1. Starting material
2. Reaction mixture

Eluent
Heptane/EtOAc
2:3

Figure 4. Applications of the developed staining reagent. Selections from original photographs of TLC plates spotted with the indicated solutions, briefly immersed in 1 mM alizarin solution in acetone, left to dry and placed under 365 nm light.

3 Experimental section

Example 1. A mixture of 4-(trifluoromethyl)phenylboronic acid (190 mg, 1 mmol), bromobenzene (0.15 mL, 1.4 mmol) and Na_2CO_3 (318 mg, 3 mmol) in dimethoxyethane (6 mL) and water (1.6 mL) was degassed with Argon for 10 min and heated to reflux. $Pd(PPh_3)_4$ (58 mg, 0.05 mmol) was added and the reaction mixture was stirred under reflux. Immediately after adding the catalyst, and then after 1 h, 2 h, 3 h and 5 h, aliquots (~ 0.2 mL, except ~ 0.5 mL after 5 h) of the reaction mixture were partitioned between Et_2O (1 mL) and 0.1 M HCl (2 mL), and the Et_2O layer was spotted on a TLC plate.

Example 2. A mixture of phenylboronic acid (122 mg, 1 mmol), 1-bromonaphthalene (0.15 mL, 1.1 mmol), Na_2CO_3 (212 mg, 2 mmol) and $Pd(OAc)_2$ (~ 1 mg) in acetone (3 mL) and water (3.5 mL) was stirred at RT in air.

Example 3. 4-Carboxyphenylboronic acid (92 mg, 0.55 mmol) was dissolved in absolute ethanol (2.5 mL). A mixture of absolute ethanol (2.5 mL) and concentrated HCl (2 drops) was added, and the solution was heated to reflux.

4 Conclusion

The difficult monitoring of boronic acids during organic reactions can be simply overcome by briefly dipping TLC plates in a 1 mM alizarin solution, to reveal boronic acids on TLC plates in a selective and sensitive manner.

5 References

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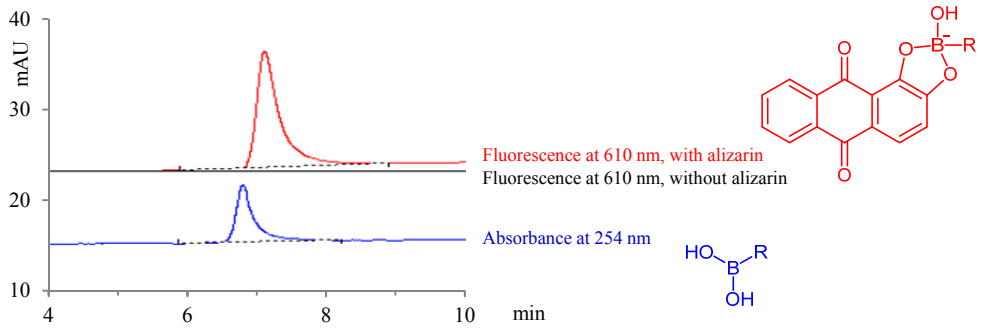
Chapter 5
Selective on-line detection of boronic acids and derivatives in HPLC eluates by post-column reaction with alizarin



An HPLC method for the rapid and selective detection of boronic acids in complex mixtures was developed. After optimization experiments at an HPLC flow rate of 0.40 mL/min, the HPLC-separated analytes were mixed post-column with a solution of 75 µM alizarin and 0.1% triethylamine in ACN, which was delivered at a flow rate of 0.60 mL/min. The reaction between alizarin and boronic acids occurred in a reaction coil of dimensions of 3.5 m × 0.25 mm at a temperature of 50 °C, resulting in fluorescent complexes that were detected as positive peaks by a fluorescence detector (λ_{exc} 469 nm and λ_{em} 610 nm). The method enabled the selective detection of various boronic acids and derivatives, with a limit of detection of phenylboronic acid of 1.2 ng or 1 µM. It could successfully monitor the progress of two organic reactions involving boronic acid-containing compounds, and provided useful insights into the course of the reactions.

This chapter is a slightly modified version of the following manuscript: F. Duval, P.A. Wardani, H. Zuilhof, T.A. van Beek, *Selective on-line detection of boronic acids and derivatives in high-performance liquid chromatography eluates by post-column reaction with alizarin*, Journal of Chromatography A, revision submitted.

1 Introduction

To facilitate the analysis of reaction mixtures that contain boronic acids, we developed a method to stain boronic acids on silica TLC plates in a selective and sensitive way, using the natural dye alizarin (chapter 4). Alizarin shows little fluorescence by itself, but when it interacts with a boronic acid in suitable conditions, a strongly fluorescent boronic ester is formed (chapter 4, Figure 1). When the alizarin-stained and dried TLC plate is placed under a UV lamp (365 nm), yellow fluorescent spots reveal the presence of boronic acids.

This method is limited, however, to TLC analysis on silica plates. TLC is a useful tool and the first line of approach in organic synthesis. However, its limited separation capacity renders it sometimes essential to analyze a complex reaction mixture by reversed-phase high-performance liquid chromatography (RP-HPLC). For the preparative separation of boronic acids, moreover, preparative RP-HPLC is superior to preparative TLC.

On-line HPLC post-column derivatization has received much attention, as it is a useful tool for the detection of specific analytes in complex mixtures.[1] In this approach an HPLC eluate and reagent solution are mixed together, and the resulting conversion of the analyte of interest allows its selective detection. Our group has developed different on-line HPLC detection methods for radical scavenging compounds.[2] Such compounds either react post-column with a stable free radical such as $DPPH^{\bullet}$ or $ABTS^{\bullet+}$,[3] or prevent the oxidation of a sensitive probe[4] resulting in a change in absorbance. Such methods proved versatile and could be used preparatively[5] and applied to complex mixtures.[6]

Our experience with TLC detection of boronic acids using alizarin and on-line HPLC detection based on post-column reactions led to the idea of combining both techniques with the aim of selectively detecting boronic acids in HPLC eluates to facilitate analysis or subsequent preparative HPLC of reaction mixtures containing boronic acids. In this chapter, the development and application of such on-line HPLC detection methodology for boronic acids are presented.

2 Experimental

2.1 Chemicals and their abbreviations

For the analytical experiments, the following chemicals were used: alizarin (97%) and triethylamine (TEA) (99%) from Acros Organics; acetonitrile (ACN) (HPLC-S) from Biosolve BV (Valkenswaard, The Netherlands); methanol (MeOH) (CHROMASOLV®, for HPLC, ≥99.9%), acetic acid (ACS Reagent ≥99.7%), phosphate-buffered saline pH 7.4 (PBS) (cat. no P3813), citric acid, 2-(cyclohexylamino)ethanesulfonic acid (CHES), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium hydroxide, alizarin Red S (ARS) (certified by Biological Stain Commission), phenylboronic acid (PBA) (purum, ≥97.0%), phenylboronic acid pinacol ester, phenylboronic acid N-methyliminodiacetic acid (MIDA) ester (97%), potassium phenyltrifluoroborate (95%), 4-trifluoromethyl-phenylboronic acid (≥95.0%), 4-carboxyphenylboronic acid, 4-methoxyphenylboronic acid (≥95.0%), 3-aminophenylboronic acid monohydrate (98%), butylboronic acid (97%), potassium butyltrifluoroborate (95%), benzoic acid (ACS reagent, ≥99.5%), nitrobenzene (ACS reagent, ≥99.0%), acetophenone (puriss. p.a., ≥99.0%), 4-hydroxybenzamide (98%), chlorobenzene (puriss. p.a., ACS reagent, ≥99.5%), bromobenzene (≥99.5%), p-toluenesulfonic acid monohydrate (ACS reagent, ≥98.5%), aniline (ACS reagent, ≥99.5%), phenol (unstabilized, ReagentPlus®, ≥99%) and 4-methoxybenzonitrile (99%) from Sigma-Aldrich; boric acid from an unknown source, sodium borate made in situ from boric acid and sodium hydroxide; benzaldehyde (≥98%) from Fisher Scientific and 4-fluorobenzaldehyde (98%) from VWR International. Ultrapure water (= "water") was produced using a Milli-Q Integral 3 system from Millipore (Molsheim, France). For the esterification reaction, the following chemicals were used: 4-carboxyphenylboronic acid (CPBA) from Sigma-Aldrich, absolute ethanol (AnalaR NORMAPUR® ACS) from VWR International and hydrochloric acid (37%) from Acros Organics. For the thiol-ene reaction, the following chemicals were used: (4-allylaminocarbonyl)phenylboronic acid (AACPBA) (97%) and N-α-t-butyloxycarbonyl-L-cysteine (Boc-Cys) (99%) from ABCR (Karlsruhe, Germany), 2,2-dimethoxy-2-phenylacetophenone (DMPA) (99%) from Sigma-Aldrich, HPLC-grade tetrahydrofuran (unstabilized, HPLC-S) from Biosolve BV.

2.2 Off-line fluorescence spectrometry

Solutions containing alizarin (or ARS) and/or boronic acids were placed in quartz cuvettes and analyzed with an Edinburgh Instruments FLS900 Fluorescence spectrometer. A Xe900 lamp was used for excitation and an R928 photomultiplier tube was used for detection. Excitation spectra were measured with λ_{em} 610 nm and emission spectra were measured with λ_{ex} 469 nm. Measurements were performed about 15 minutes after mixing alizarin (or ARS) with boronic acid solutions. Detailed composition of these solutions can be found in the corresponding figure captions.

2.3 On-line HPLC instrumental set-up

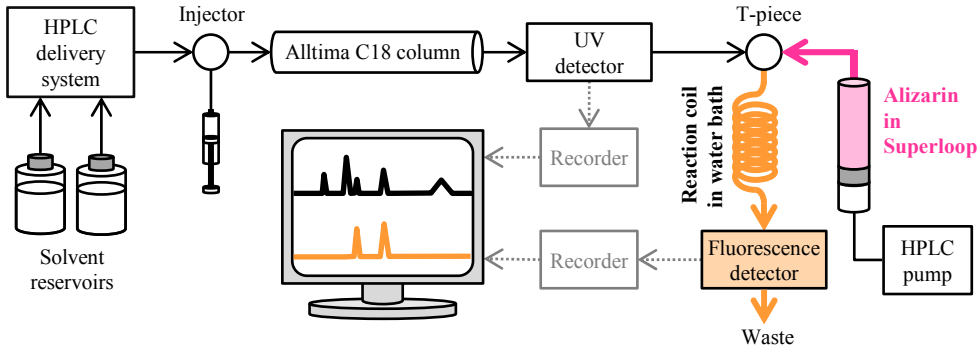


Figure 1. Instrumental set-up for HPLC on-line detection of boronic acids using alizarin.

The instrumental set-up is depicted in Figure 1. The HPLC delivery system consisted of a WellChrom HPLC Pump K-1001 and a WellChrom Solvent Organizer K-1500 (Knauer, Germany). The manual injector was a Multiport Streamswitch MUST HP 6 (Spark Holland, Emmen, the Netherlands) with a loop of 10 µL. Separations were carried out on an Alltima C18 HPLC column (5 µm, 150 mm × 3 mm i.d., pore size 100 Å, Alltech Associates Inc., Deerfield, IL), and the HPLC flow rate was set at 0.40 mL/min. UV detection was carried out using a 2487 Dual λ Absorbance Detector (Waters, Milford, MA) set at a detection wavelength of 254 nm. Post-column delivery of the alizarin solution was achieved as follows: the alizarin solution was displaced from a 150 mL Superloop™ (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) by infusing water into the opposite end of the

Superloop™ by means of a Gynkotek High Precision Pump Model 300. The flow rate of this HPLC pump is further described as "alizarin flow rate". The tested reaction coils were made of PEEK tubing (VICI AG International, Schenkon, Switzerland). Post-column fluorescence detection was done using an FP-1520 Intelligent Fluorescence Detector (JASCO, Tokyo, Japan), λ_{ex} 469 nm and λ_{em} 610 nm. Components of the set-up were connected with tubing of various materials and dimensions (details in appendix, Table 1).

2.4 Flow injection analysis: effect of coil temperature and length

2.4.1 Instrumental set-up

Flow injection analysis experiments were carried out with the same set-up as described in paragraph 2.3 and depicted in Figure 1 with one difference: the HPLC column was mounted before the injector, as it was used only for keeping the system at the desired pressure for proper functioning of the HPLC pump.

2.4.2 Operating conditions

The carrier flow consisted of ACN/water 1:1 (v/v) at a flow rate of 0.40 mL/min. The Superloop™ was filled with a solution of 3.13 µM alizarin and 0.10% TEA in ACN. The alizarin flow rate was set at 0.80 mL/min. 313 µM PBA in MeOH was injected at each new temperature or coil length.

2.4.3 Effect of coil temperature

The temperature of the reaction coil was changed by immersing the latter in a water bath on a heating plate equipped with a thermostat. Four different temperatures were tested for the detection of PBA: 22 °C (RT), 30 °C, 40 °C and 50 °C. The coil had dimensions of 3.50 m × 0.25 mm. All other conditions described in 2.4.2 were fixed.

2.4.4 Effect of coil length

Reaction coils of identical internal diameters (0.25 mm) and three different lengths (3.5, 6.0 and 10.0 m) were tested for the detection of PBA. All other conditions described in 2.4.2 were fixed.

2.5 On-line HPLC

2.5.1 Instrumental set-up

All experiments in this section were carried out using the set-up and conditions as described in 2.3 and depicted in Figure 1.

2.5.2 Effect of alizarin concentration on S/N ratio

The HPLC mobile phase consisted of ACN/water 2:3 (v/v). The Superloop™ was filled with a solution of alizarin and 0.10% TEA in ACN. The alizarin concentration ranged from 1 to 300 μM, and the alizarin flow rate was set at 0.60 mL/min. At each alizarin concentration, PBA solutions at concentrations ranging from 1 to 300 μM in MeOH were injected, in triplicate (unless stated otherwise). The S/N ratio was determined as follows: the signal S was defined as the difference between peak height and average signal height of the baseline, and the noise N was defined as 3× standard deviation of the baseline around the elution time of the analyte of interest for a blank injection (solvent used for dissolving the analyte).

2.5.3 Effect of alizarin flow rate on S/N ratio

The HPLC mobile phase consisted of ACN/water 2:3 (v/v). The Superloop™ was filled with a solution of 75 μM alizarin and 0.10% TEA in ACN. The alizarin flow rate was varied from 0.0 to 1.0 mL/min with increments of 0.10 mL/min. At each alizarin flow rate, 300 μM PBA in MeOH was injected, in triplicate. The S/N ratio was determined as in 2.5.2.

2.5.4 Proof-of-principle, scope and selectivity

The HPLC mobile phase consisted of a gradient of 20 to 50% ACN in water. The Superloop™ was filled with a solution of 75 µM alizarin and 0.10% TEA in ACN, and the alizarin flow rate was set to 0.60 mL/min. Solutions of various compounds in ACN/water 1:5 (see structures and concentrations in Figure 5) were injected in identical conditions (except compound concentration when a change was deemed necessary for sensitivity reasons). When it was necessary to check whether the compounds were autofluorescent, the same experiments were repeated without any alizarin flow. The S/N ratio was determined as in 2.5.2.

2.5.5 Estimation of the LOD

The limit of detection for PBA was calculated in two ways: (1) by finding the concentration in a calibration curve using peak heights giving a signal of 3× the SD of the average baseline of a blank injection at the elution time of PBA; (2) by injecting samples of 0.3, 1.0 and 3.0 µM of PBA and determining which concentration still gives a visible peak.

2.5.6 Applications

Acid-catalyzed esterification of CPBA

CPBA (92 mg, 0.55 mmol) was dissolved in absolute ethanol (2.5 mL). HCl 37% (2 drops) was diluted in absolute ethanol (2.5 mL) and added to the previous solution. The obtained solution was stirred and heated to reflux.

The HPLC mobile phase consisted of MeOH/water 2:3 (v/v) with 0.1% acetic acid. The Superloop™ was filled with a solution of 75 µM alizarin and 1% TEA in ACN, and the alizarin flow rate was set to 0.60 mL/min. Samples were taken from the reaction mixture just before reflux started, and 0.5, 1, 2, 3, 6 and 21 h after reflux started, diluted 100 times in MeOH/water 2:3 (v/v) and injected.

Thiol-ene reaction between AACPBA and Boc-Cys

AACPBA (125 mg, 0.61 mmol, 1 eq), Boc-Cys (675 mg, 3.05 mmol, 5 eq) and DMPA (156 mg, 0.61 mmol, 1 eq) were dissolved in THF (3 mL). The obtained solution was stirred under 365 nm irradiation (using a portable UV lamp as used for viewing of TLC plates, Camag, Switzerland).

The HPLC mobile phase consisted of a MeOH/water 1:1 (v/v) with 0.1% acetic acid. The Superloop™ was filled with a solution of 75 µM alizarin and 1% TEA in ACN, and the alizarin flow rate was set to 0.60 mL/min. Samples were taken from the reaction mixture before irradiation and 1, 2, 5, 10, 20, 40, 80 and 160 min after irradiation was started, diluted 100 times in MeOH/water 1:1 (v/v) and injected.

3 Results and discussion

3.1 Off-line fluorescence spectrometry

3.1.1 Choice of dye and solvent

The natural dye alizarin has been used for the TLC detection of boronic acids as described in chapter 4. However, the water-soluble derivative alizarin red S (ARS) is more widely used for its binding with boronic acids. To test the fluorescence detection of boronic acids in solution, preliminary experiments were therefore performed with ARS. Excitation and emission scans of ARS only and ARS/PBA mixtures (appendix, Figure 1) showed that 1) ARS had a significant autofluorescence, 2) the most efficient excitation wavelength for PBA detection was 469 nm (which was used in all subsequent experiments), and 3) subtraction of the ARS emission spectrum from the ARS/PBA emission spectrum resulted in a spectrum with a fluorescence intensity maximum at 610 nm (which was used in subsequent experiments that required the choice of an emission wavelength). Next, mixtures of ARS with various boronic acids were analyzed in the same way, and showed high variations in fluorescence intensity (appendix, Figure 2). The electron-deficient boronic acids resulted in high fluorescence intensities (e.g., 4-trifluoromethylphenylboronic

acid), and the electron-rich boronic acids resulted in low fluorescence intensities (e.g., 4-methoxyphenylboronic acid, MPBA). Based on these results, the immediate goal was to improve the detection of electron-rich boronic acids, and MPBA was taken as a model compound. Because we had positive experiences with alizarin, alizarin was compared with ARS for the detection of MPBA: ARS, alizarin, ARS/MPBA and alizarin/MPBA solutions were analyzed. The obtained fluorescence spectra (appendix, Figure 3) revealed that the detection of MPBA was improved when using alizarin. Alizarin, moreover, had a much lower autofluorescence than ARS. As this constituted a significant advantage in the on-line HPLC detection of boronic acids due to the much higher relative change in fluorescence intensity, **alizarin** was used in all further experiments.

Additional off-line experiments with alizarin showed that an increase of the organic solvent percentage resulted in an increase of the fluorescence intensity (appendix, Figure 4): the less water, the higher the intensity. Because HPLC eluates typically contain water, the final percentage of water after mixing eluate and alizarin solution was minimized by dissolving alizarin in 100% organic solvent. MeOH and ACN were both considered but **ACN was preferred** because 1) ACN is less viscous than MeOH, hence it causes a lower backpressure than MeOH does, and 2) MeOH is prone to give bubble formation upon mixing with a water-containing eluate, which might disturb the fluorescence detection after the low-pressure coil. The same would be true if water was to be mixed with a MeOH-containing eluate; this is an additional advantage of using alizarin instead of ARS, as ARS would have to be dissolved in water.

3.1.2 Effect of pH

To improve the composition of the alizarin solution, the effect of pH on the fluorescence intensity of alizarin/PBA mixtures was studied (a similar experiment has been published, where ARS was used instead of alizarin[7]), using an extended-range citrate/HEPES/CHES buffer system.[8] The largest difference in fluorescence intensity (= the highest sensitivity) between alizarin only and alizarin/PBA was obtained at pH values between 7 and 8 (appendix, Figure 5). Because ACN was chosen as solvent for the alizarin solution, it was not possible to make a buffer at a specific pH. Instead, the effect of triethylamine (TEA) on the fluorescence intensity of alizarin/PBA mixtures was explored. Conditions were chosen in such a way that they would roughly mimic on-line HPLC, and the analyzed solutions consisted of two parts of identical volumes: 1) "alizarin solution mimic": 20 μM alizarin solution in ACN that contained 0%, 0.1%, 1% or 10% TEA, and 2) "HPLC eluate mimic": 200 μM PBA in 50% water and 50% ACN or MeOH. Independent of using MeOH or ACN in the "HPLC eluate mimic", the highest sensitivity was achieved when the "alizarin solution mimic" contained 0.1% TEA (appendix, Figure 6).

The addition of small amounts of acid, e.g., acetic acid, to the mobile phase is often necessary for obtaining a good retention and separation of weakly acidic analytes. Thus, the same experiment as above was performed, with one difference: the "HPLC eluate mimic" was made acidic via the use of 0.1% acetic acid. When the "alizarin solution mimic" contained 0% or 0.1% TEA, the resulting fluorescence intensity was much weaker than in the previous experiment. However, when the "alizarin solution mimic" contained 1% TEA, the resulting fluorescence intensity could be brought back to the maximum observed in the previous experiment (appendix, Figure 7).

Based on the results of the off-line experiments, the following choices were made: the post-derivatization solution would consist of alizarin in ACN, to which 0.1% TEA (in case of a neutral eluate) or 1% TEA (in case of an eluate that contains small percentages of acidic modifiers) would be added.

3.2 Flow injection analysis: effect of coil temperature and length

Next, the efficacy of the above solution to enable on-line detection of boronic acids was tested by flow injection analysis. To ensure a proper operation (pulse dampening) of the HPLC pump providing the carrier flow, an HPLC column was mounted between the pump and the injector. Columns with diameters of 2.1 mm, 3.0 mm and 4.6 mm were available, with respective recommended flow rates of 0.20 mL/min, 0.40 mL/min and 1.0 mL/min. A flow rate of 0.40 mL/min was preferred, as the lowest flow rate may result in a poor detection sensitivity and significant peak broadening, and the highest flow rate would result in a too high pressure for the Superloop™ (maximum 10 bar) and a high consumption of HPLC solvents and alizarin solution. **A column of 3.0 mm diameter at a flow rate of 0.40 mL/min was therefore chosen**.

The sensitivity of the method depends on the conversion of alizarin and the specific boronic acid into a fluorescent alizarin-boronic acid complex. At given concentrations of alizarin and boronic acid, two major factors influencing this conversion are temperature and reaction time. First, the detection of PBA was tested with a coil of dimensions 3.5 m × 0.25 mm at four temperatures (22 °C to 50 °C). This showed that the peak height of PBA increased linearly with temperature (Appendix, Figure 8). **A coil temperature of 50 °C** was therefore used in all further experiments. A higher temperature was not considered because of problems associated with post-coil ACN volatility. Second, 0.25 mm i.d. coils of three different lengths (3.5, 6.0 and 10.0 m) were compared. Coil dimensions directly define the residence time, hence the time that alizarin and boronic acid have for reacting with each other. Upon increasing coil length, at 50 °C only a minor increase of PBA peak height was observed: for coil lengths of 3.5, 6.0 and 10.0 m (resulting in respective residence times of about 9, 15 and 25 s), respective peak heights were 8.3, 8.8 and 8.9 milli arbitrary units (mAU). Thus, to avoid (too) high backpressures in the Superloop™ as well as peak broadening, **coil dimensions of 3.5 m × 0.25 mm** were used in all subsequent experiments.

3.3 On-line HPLC

3.3.1 Effect of alizarin concentration on S/N ratio

The on-line detection of PBA is based on the S/N ratio of the produced fluorescent peak, which is directly affected by the alizarin concentration of the solution that mixes with the HPLC eluate. A too low concentration would result in a poor signal, and a too high concentration would result in too much noise. In order to find the optimum alizarin concentration, the S/N ratio for the detection of PBA (at concentrations from 1 to 300 μM) was determined at fixed flow rates: 0.40 mL/min for the HPLC mobile phase and 0.60 mL/min for the alizarin solution. 0.60 mL/min was selected as this flow was expected to be near the optimum on the basis of peak broadening, mixing, sensitivity and back pressure issues. The alizarin concentration was varied from 1 to 300 μM. As a result, **the highest S/N ratio was obtained with an alizarin concentration of 75 μM** (Figure 2A; see appendix, Figure 9 for lower PBA concentrations), which was used in all subsequent experiments.

3.3.2 Effect of alizarin flow rate on S/N ratio

An increase of the alizarin flow rate can give rise to two counteracting effects: (1) an increase of the alizarin concentration and of the percentage of organic solvent (ACN) when the alizarin solution and HPLC eluate are mixed, hence a possible increase in fluorescence intensity in presence of boronic acids; (2) a decrease of the boronic acid concentration and of the amount of time that is available for alizarin and boronic acid to form a fluorescent complex, hence a possible decrease in fluorescence intensity in presence of boronic acids. In addition, there are peak broadening issues (higher flows are better) and mixing issues (near-equal column and alizarin flows work best). In order to find the optimum alizarin flow rate, the latter was varied from 0.0 to 1.0 mL/min with increments of 0.1 mL/min at the optimized alizarin concentration of 75 μM. The S/N ratio was highest when an **alizarin flow rate of 0.6 mL/min** was used (Figure 2B), but flows of 0.5 or 0.7 mL/min are also usable.

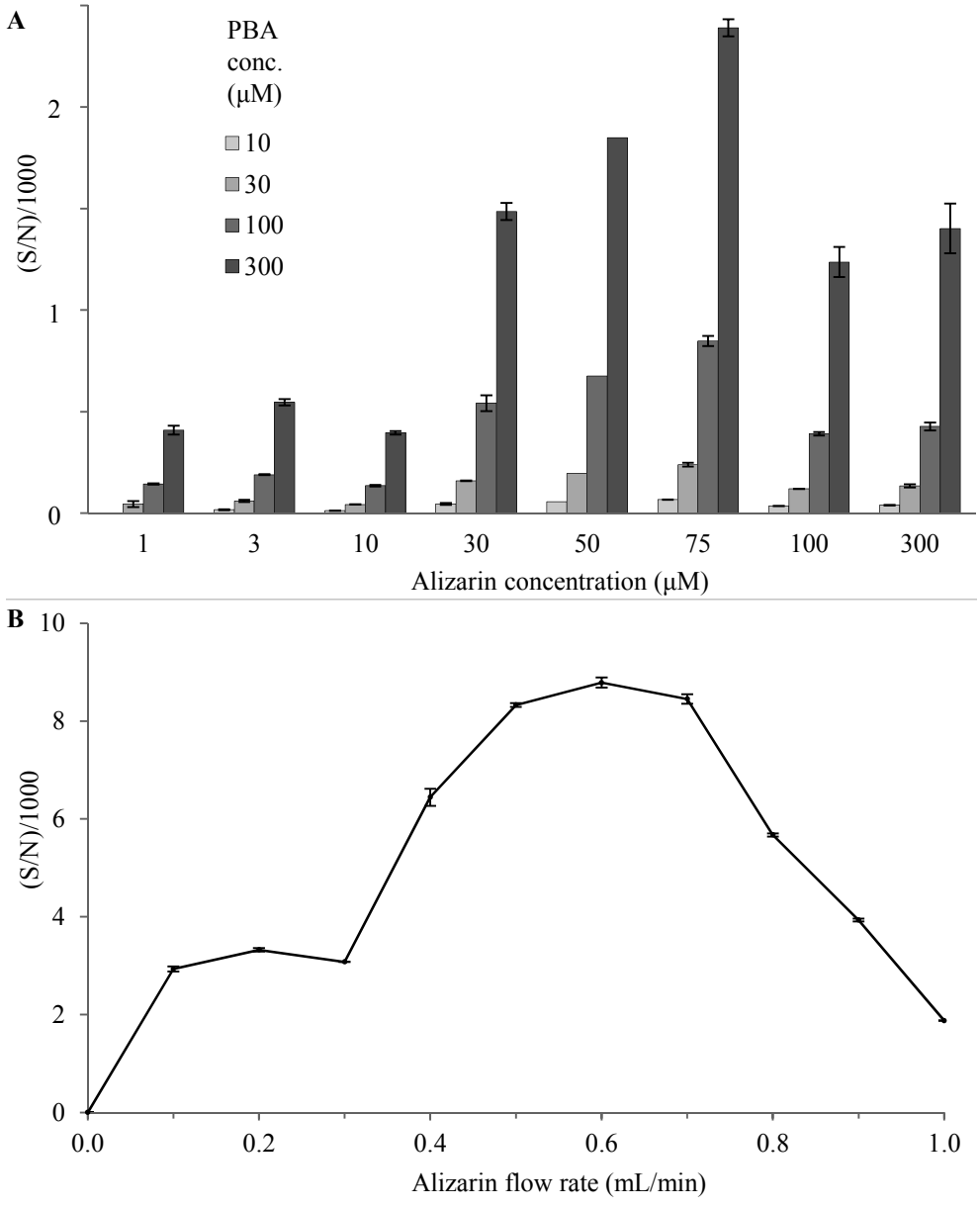


Figure 2. **A**. Effect of alizarin concentration on S/N ratio. At each alizarin concentration, PBA was injected at various concentrations and the signal-to-noise ratio (S/N) of each produced peak was determined. n = 3 except for 50 μM alizarin in which case n = 2. **B**. Effect of alizarin flow rate on S/N ratio. At each alizarin flow rate, phenylboronic acid (300 μM, 10 μL) was injected in triplicate and the signal-to-noise ratio (S/N) of the produced peak was determined.

3.3.3 Proof-of-principle and semi-quantitative aspect of the developed method

Based on the results that have been presented so far, the following conditions were fixed:

- The post-column derivatization solution consisted of 75 µM alizarin and 0.1% TEA in ACN and was delivered at a flow rate of 0.60 mL/min (at an HPLC flow rate of 0.40 mL/min).

- The reaction coil had dimensions of 3.5 m × 0.25 mm and was immersed in a water bath at a temperature of 50 °C.

- The fluorescence detector was set at λ_{exc} 469 nm, and λ_{em} 610 nm.

Figure 3 shows the proof-of-principle chromatograms using the conditions described above. PBA was injected, once with and once without post-column alizarin flow. The fluorescence was measured in both cases, and PBA was detected by fluorescence only in presence of alizarin. The on-line fluorescence detection resulted in an acceptable 35% peak broadening as compared to the UV detection.

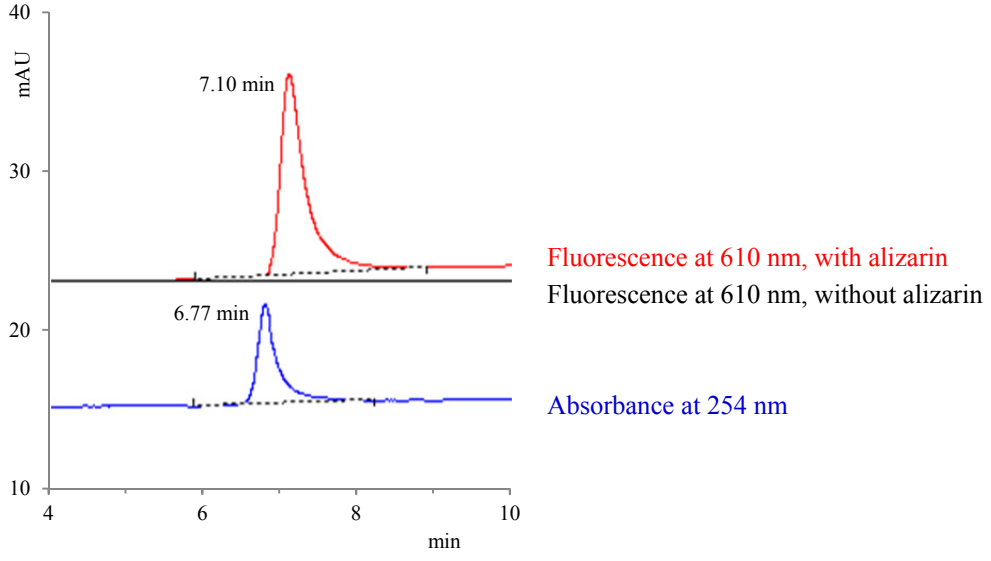


Figure 3. Proof-of-principle of the developed method. 300 µM PBA was injected, with (**red**) and without (**black**) alizarin delivery after the absorbance detector (**blue**).

The ability of the method to quantify PBA was evaluated using the chromatograms (appendix, Figure 10) obtained from the optimization of the alizarin concentration (3.3.1), when 75 µM alizarin was used. The peak area increased linearly with PBA concentration (appendix, Figure 11). Based on a peak height calibration curve (Figure 4), the LOD (3× SD of the noise) for PBA was estimated at ~ 0.15 µM. When solutions of 0.3, 1 and 3 µM of PBA were injected, the PBA peak corresponding with 1 µM was still visible, but not the one of 0.3 µM. Thus conservatively, the LOD is set at 1 µM in combination with a 10 µL loop. The minimal detectable amount of PBA is 1.2 ng, which gives the method ample sensitivity for the analysis of boronic acids in synthetic mixtures.

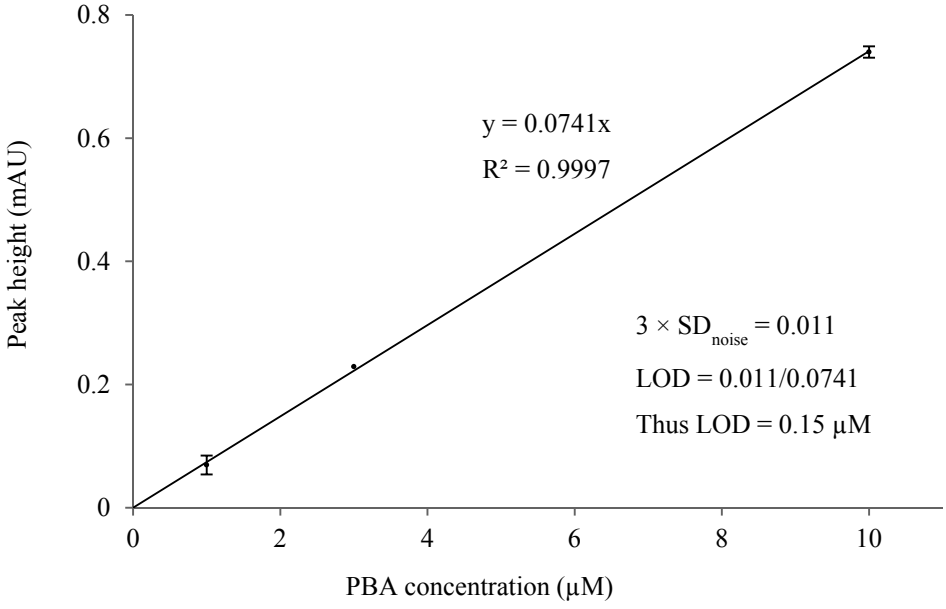


Figure 4. Peak height calibration curve and estimation of the limit of detection (LOD) of PBA. Peak heights taken from chromatograms in Figure S10 (+ replicates). n = 3 except for 3 µM PBA in which case n = 2.

3.3.4 Scope and selectivity

The final goal was the selective detection of all kinds of boronic acids and derivatives. To evaluate the scope and selectivity of the developed method, various boronic acids, boronic acid derivatives and boron-free compounds were injected. The S/N ratio observed for each compound can be found in Figure 5. The S/N ratios of boronic acids and derivatives were somewhat variable for the following reasons: (1) the complexation between boronic acid and alizarin depends on the pK_a of the boronic acid, which is affected by the presence of electron-donating or electron-withdrawing groups;[7] (2) the complexation between boronic acid and alizarin depends on the pH,[7] which can be affected by a basic or acidic group in the boronic acid molecule; (3) in one single case (4-methoxyphenylboronic acid), autofluorescence of the compound contributed to the signal (chromatograms of all analyzed boronic acids in appendix, Figure 12). Regardless of the differences in fluorescence intensities, all boronic acids and derivatives could be detected. They included compounds that could not be detected by UV (such as butylboronic acid and boric acid), showing an additional advantage of the developed method. In contrast, most boron-free compounds, which were injected at much higher concentrations, were not or hardly detected. The boron-free compounds that did give a signal (see Figure 5) were also injected in the absence of alizarin, and proved to be autofluorescent (chromatograms of autofluorescent compounds with and without alizarin in appendix, Figure 13). In other words, the false positives were not due to complexation with alizarin, but to the intrinsic fluorescent properties of the analyzed compounds.

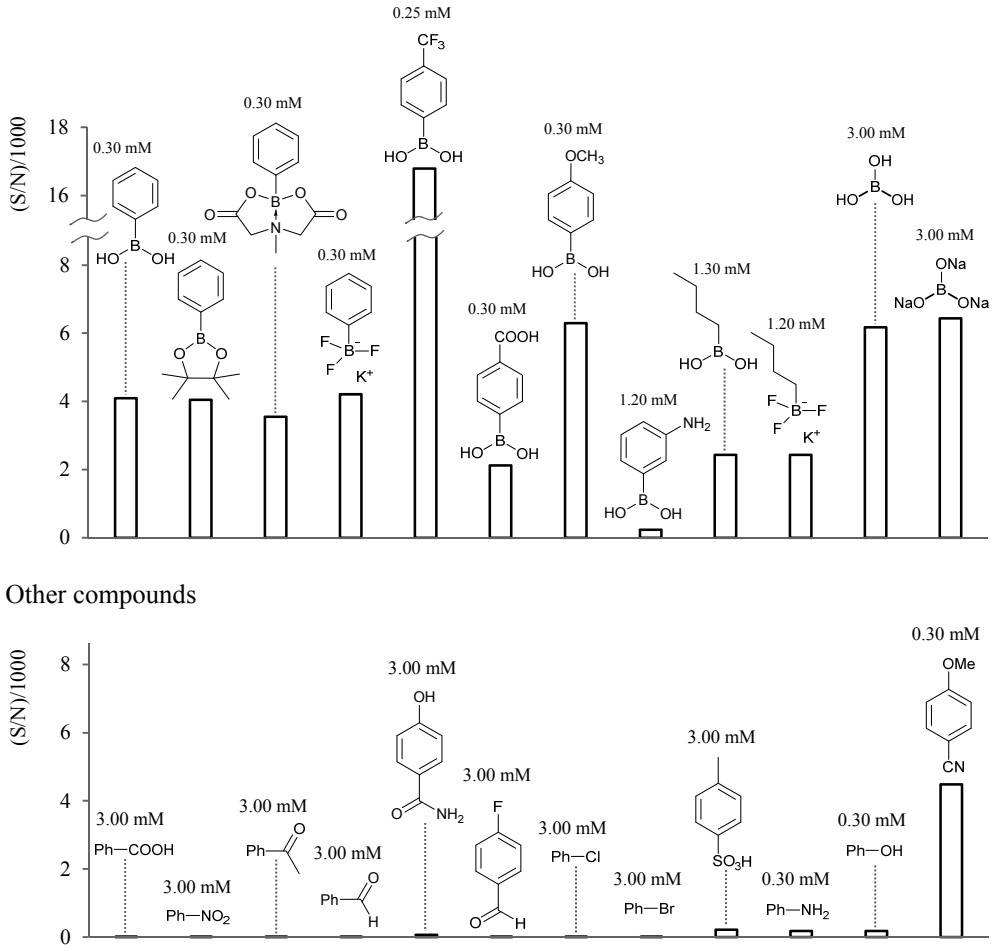


Figure 5. Scope and selectivity of the developed method. Various compounds were injected, and the signal-to-noise ratio (S/N) of each produced peak was determined.

3.3.5 Applications

The developed system was tested with real-life synthetic mixtures. The first example concerned the monitoring of the acid-catalyzed esterification of CPBA (Figure 6). The same experimental procedure as in chapter 4, in which this reaction was monitored by TLC, was used. Samples were taken from the reaction mixture after various reaction times, diluted and analyzed using the developed method. Figure 6 shows chromatograms at t = 0 and t = 21 h. At t = 0, only CPBA was detected, both by absorbance and fluorescence. At t = 21 h, CPBA had almost completely disappeared and two major new absorbance peaks appeared. The fluorescence chromatogram shows that only the second major peak (by retention time) may correspond to a boronic acid compound, most probably the expected product of the reaction. Figure 14 of the appendix shows the fluorescence peak heights of starting material and product against reaction time.

The second example is the monitoring of the thiol-ene reaction between AACPBA and Boc-Cys, as described in chapter 3, resulting in the formation of a new boronic acid-containing molecule (Figure 7). Samples were taken from the reaction mixture after various reaction times, diluted and analyzed using the developed method. Figure 7 shows parts of chromatograms at selected reaction times. In the UV 254 nm chromatograms, many new peaks appeared and it was hard to interpret the peak pattern. The new boronic acid-selective detection, however, led to more insight: the newly formed UV 254 nm peak at 8.1 min corresponded with a new fluorescence peak at 8.4 min in the post-column chromatogram (0.3 min between UV and fluorescence detectors), strongly indicating that it was the product of the reaction. The new UV 254 nm peak at 7.0 min, however, did not correspond with any fluorescence peak: this indicated that a by-product (among others) was formed, in which the boronic acid moiety had disappeared. Peak heights of starting material, product and by-product were plotted against reaction time, and suggested that the formed product (8.1 min in UV 254 nm chromatogram) degraded into the by-product (7.0 min in UV 254 nm chromatogram) (appendix, Figure 15).

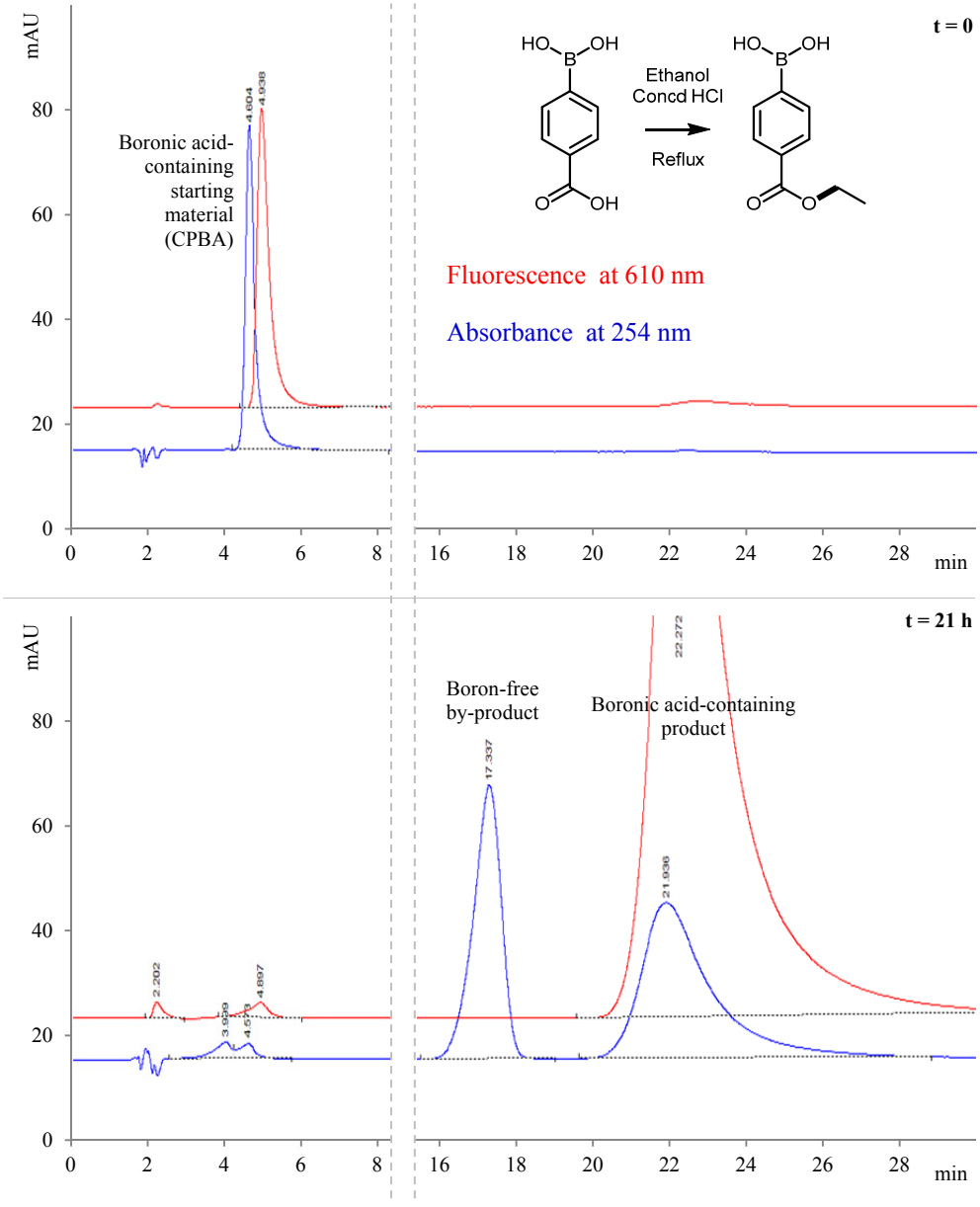


Figure 6. Monitoring of the acid-catalyzed esterification of CPBA. Samples were taken from the reaction mixture at various reaction times, diluted and injected. Parts of chromatograms for selected reaction times.

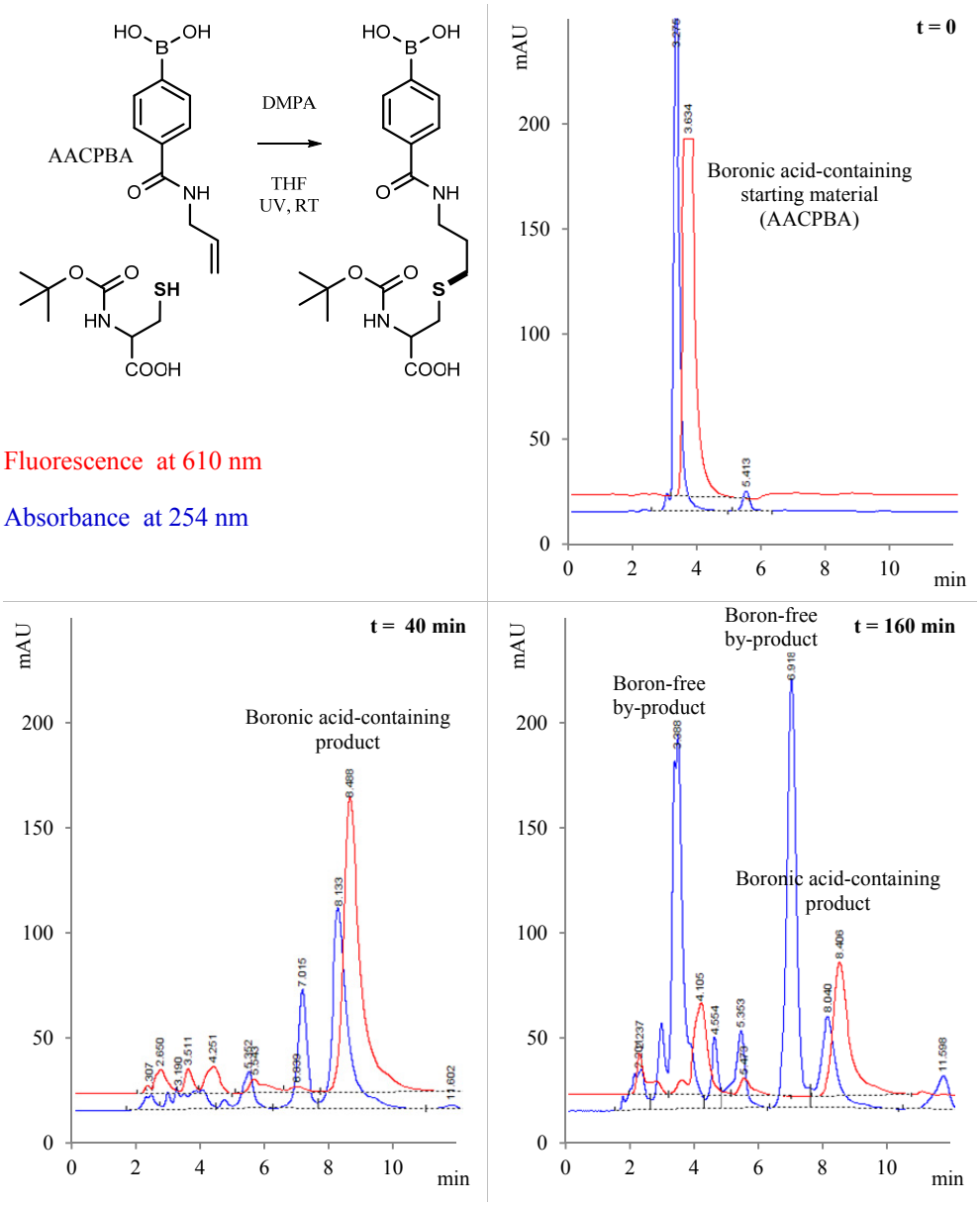


Figure 7. Monitoring of the thiol-ene reaction between AACPBA and Boc-Cys. Samples were taken from the reaction mixture at various reaction times, diluted and injected. Parts of chromatograms for selected reaction times.

4 Conclusion

An easy-to-use HPLC method was developed for the on-line detection by induced fluorescence of boronic acids and derivatives using alizarin. The method allows the specific detection of a wide range of boronic acids and derivatives, even in crude mixtures from organic synthesis. Boron-free compounds do not give rise to this induced fluorescence. Through optimization experiments, reasoning and limitations regarding the available instrumentation, choices were made for the set-up and conditions. Possibilities, however, are not limited to these choices. Alizarin proved better than ARS for the detection of MPBA, but ARS may be better in other cases. ACN may be replaced by another solvent or an aqueous buffer (in which case ARS is recommended), as long as it is compatible with the eluate. The presence of different acids or bases in the eluates may require a slight adjustment of the basicity of the alizarin solution (as was done when the eluate contained 0.1% acetic acid). Practical use, which does not always require the highest sensitivity, is not limited to the optimized parameters such as coil length, coil temperature and alizarin flow rate (in relation to HPLC flow rate). As such, the present method has shown good potential for pinpointing boronic acids and derivatives in complex mixtures: it can be of great help, for example, when one needs to get insight into the course of chemical reactions that involve boronic acids, or to select the right peak in preparative HPLC separations of boronic acid-containing mixtures.

5 References

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Chapter 6
General discussion

This chapter provides a reflexion about the work presented in this thesis, suggestions for future research, and a general conclusion.

1 Immobilization of antibodies using boronic acids: what next?

The oriented immobilization of antibodies using boronic acids, as discussed in chapter 2, is promising but not yet straightforward. The sugars in the Fc chain of the antibody (N-glycans) need to expose diols that have enough affinity with boronic acids. The binding conditions need to be chosen in such a way that the antibody is immobilized on the surface via the reaction between boronic acid and diol, and not via other types of interactions. The surface needs to bear long hydrophilic chains to which suitable boronic acids are attached, for strong and selective binding with the N-glycans. When all these requirements are met, problems caused by the reversibility of the reaction between boronic acid and diol, thus release of the antibody from the surface, may still be encountered. In chapter 3, the idea of making the antibody immobilization irreversible is developed, and it has also been published by Adak et al.[1] A molecule containing an amine, a boronic acid and a diazirine is attached to the surface via its amine. The antibody binds to the boronic acid via a diol in its N-glycans, the sample is irradiated with UV light (365 nm) which causes transformation of the diazirine into a carbene, and this carbene inserts into a C-C, C-H or X-H (X being a heteroatom) bond in its vicinity, resulting in oriented and irreversible antibody immobilization.

This novel antibody immobilization strategy looks promising, and it may further be optimized in future research. In the article of Adak et al., only one trifunctional linker was tested. However, we expect that the antibody orientation and density on the surface strongly depend on the structure of the linker. The whole linker (as well as the spacer between linker and surface) needs to be hydrophilic enough to prevent hydrophobic interactions with the antibody, and preferably electrically neutral (at the desired pH) to minimize electrostatic interactions with the antibody (although electrostatic interactions may facilitate antibody orientation in some cases). In order to choose the most suitable boronic acid structure, stability of the ester resulting from the binding between boronic acid and diol is no longer the dominant issue (as opposed to immobilization without diazirine) but most importantly, the boronic acid needs to bind specifically to a diol in the antibody N-glycans. Secondary interactions between boronic acid and antibody must be avoided.

Once the antibody is bound to the surface via the boronic acid, and the sample is irradiated with UV light, a carbene is formed, which is supposed to react with a part of the antibody in the vicinity of the N-glycans. The length and flexibility of the spacer between boronic acid and diazirine play an important role: if the spacer is too short or too stiff, then the carbene may not be able to access any C-C, C-H or X-H bond of the antibody. If the spacer is flexible but too long, then the carbene may react with a part of the antibody that is far away from the N-glycans, resulting in a random orientation, or even with another antibody. Optimization of the trifunctional linker structure, therefore, may provide even better results than reported so far.

2 Synthesis of boronic acid-containing compounds

2.1 Boronic acid: to protect or not to protect?

In previous work, before the synthesis of the linkers reported in chapter 3, we protected 4-carboxyphenylboronic acid (CPBA) with pinacol and 2D-TLC revealed that the obtained CPBA-pinacol ester degraded on silica. This suggests that synthesized boronate esters cannot be purified by silica-based chromatography. Moreover, it has been reported that the boronic acid protecting groups often had to be removed in harsh conditions,[2] or were difficult to separate from the free boronic acids.[3] As we had also seen in a literature survey that unprotected 3-aminophenylboronic acid (APBA) had been used more often than protected APBA in coupling reactions with carboxylic acids, we decided to work without protecting groups. Whether this was a good idea is debatable. On the one hand, the use of free boronic acids saved two steps in the synthetic route (protection and deprotection), potential problems getting rid of the protecting diol were avoided, and it proved possible to isolate boronic acid-containing compounds by diol-silica flash chromatography. On the other hand, a general survey in the Reaxys database (this time, not with APBA only, but with all boronic acids) showed that boronic esters were used much more often than boronic acids in coupling reactions between primary amines and carboxylic acids, and in Boc removal reactions. It is also mentioned in the literature that boronic acids are incompatible

with most synthetic reagents,[2] and that their purification and characterization is easier when they are protected.[4] In order to protect boronic acids for a better compatibility with chemical reagents, and isolate them easily, it may be a good option to use a solid support that bears diols, as reported by several groups.[5-7] Solid-phase synthesis presents the disadvantage that the reaction has to be clean and complete, but after optimization of the reaction conditions, this approach can be promising for the synthesis of boronic acid-containing compounds.

2.2 Boronic acid and thiol-ene reaction: a good combination?

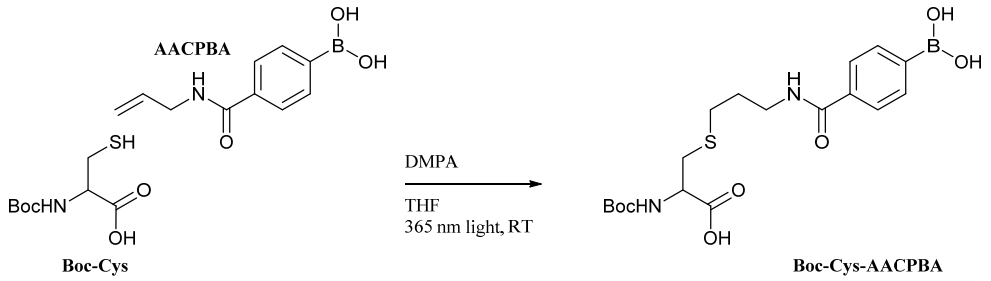

Figure 1. Thiol-ene reaction between Boc-Cys and AACPBA, as performed in chapters 3 and 5.

The reaction between Boc-Cys and AACPBA in the presence of DMPA (Figure 1) was reported in chapters 3 and 5. In chapter 3, the reaction was first monitored by TLC, which suggested complete conversion of AACPBA after 45 min. In subsequent experiments, the reaction was run for 2 h to ensure complete conversion of AACPBA. When the material was purified once, by diol-silica chromatography (without water), Boc-Cys-AACPBA was obtained in 88% yield. When the material went through several purification steps by reversed-phase chromatography (with water), ESI-HRMS suggested that the desired product had been oxidized: a phenol instead of a boronic acid, and a sulfoxide instead of a thioether were observed. In chapter 5, the same reaction was monitored by HPLC using a detection method that differentiates boronic acids from other compounds. The peak corresponding to AACPBA decreased over time until it disappeared after about 40 min (which is in accordance with previous TLC monitoring), while a new peak corresponding to a boronic acid (most probably Boc-Cys-AACPBA) increased. Whereas this observation

was in line with our expectations, from the moment that AACPBA was completely consumed, the concentration of Boc-Cys-AACPBA started decreasing again and meanwhile, a boron-free compound increased dramatically in concentration. Putting all these observations together, we propose that the following happened: the thiol-ene reaction took place as expected (in pure and dry THF), and when a sample from the reaction mixture was diluted 100 times in MeOH/water 1:1 for HPLC injection, Boc-Cys-AACPBA started being oxidized, to an extent that depended on the concentration of alkene (AACPBA) present. The reason for this is unclear, but it may have to do with radicals present in the reaction mixture, which first react with the alkene, and second cause the transformation of water into hydrogen peroxide, which in turn causes oxidation of the boronic acid into a phenol[4] and oxidation of the thioether into a sulfoxide. Other factors may play a role, as no thiol-ene reaction in the presence of boronic acids or esters can be found in the Reaxys database (at the time of writing). However, we showed that this type of reaction is possible, as we were able to obtain Boc-Cys-AACPBA in a satisfying yield and purity. The yield and purity may be improved further by stopping the reaction immediately after complete consumption of the alkene (AACPBA), and avoiding contact with water as much as possible.

3 Detection of boronic acids using alizarin

3.1 TLC staining reagents for boronic acids: which one is best?

A staining reagent for the detection of boronic acids on silica TLC plates was developed, as reported in chapter 4. It is based on the reaction between boronic acid and alizarin to form a fluorescent boronate ester, which can be detected under ambient light (yellow spot on pink background) or 365 nm light (fluorescent yellow spot on dark background). The staining reagent simply consists of 1 mM alizarin in acetone, it is selective for boronic acids and derivatives and detects all of them. This alizarin solution has been used extensively in our labs, and proved very useful. Less than a month after acceptance of our paper, Lawrence et al. published another method for the TLC detection of boronic acids.[8] TLC plates are

dipped into an acidic curcumin solution in ethanol, and then heated with a heat-gun. Complexation of yellow curcumin with boronic acids forms red/orange spots. However, we think that our alizarin-based TLC stain is better than the curcumin-based one: 1) the stain solution is simpler and safer as it does not require 2 M HCl; 2) the stain solution is approximately 1000 times cheaper in terms of dye use; 3) it does not require any heating; 4) the color difference is more obvious (Figure 2); 5) last but not least, it is approximately 10 times more sensitive for the detection of phenylboronic acid (PBA) (Figure 2). The following year, Aronoff et al. reported a new TLC staining reagent for boronic acids, based on the binding between 10-hydroxybenzo[h]quinolone and boronic acids.[9] An apparent comparison between the authors' method and ours is reported, and from these results, the authors claim their method to be more sensitive than ours. However, our method was actually not reproduced, as alizarin red S (ARS) was used instead of alizarin. In organic solvents such as acetone, ARS is poorly soluble and performs poorly, unlike alizarin. By looking at the real comparison between both methods (Figure 2), it seems that they are equivalent in terms of sensitivity. However, the alizarin staining reagent presents the advantages that it is approximately ten times cheaper in terms of dye use and does not require any heating. We were pleased to see that our method was reported in a book chapter by Spangenberg on specific TLC staining reagents (in German),[10] and used by Marques et al. for monitoring the conversion of aryl halides into boronic esters.[11]

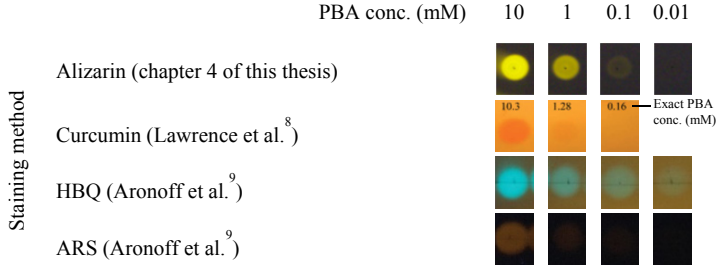

Figure 2. Detection of phenylboronic acid using different staining methods. In part from ref. 8 with permission of The Royal Society of Chemistry and from ref. 9 with permission of the American Chemical Society.

3.2 TLC staining reagents for boronic acids: other TLC plates?

After our article was published, we realized that the alizarin staining reagent had not been tested on other supports than silica plates. In the course of synthetic work, it turned out that 1 mM alizarin in acetone performed very poorly for the detection of boronic acids on reversed-phase (C_{18}) TLC plates. However, while developing the method for the HPLC on-line detection of boronic acids using alizarin (as reported in chapter 5), we found that the addition of 0.1% triethylamine (TEA) to 1 mM alizarin in ACN dramatically improved the detection of PBA in solution. These results gave the idea of an additional TLC experiment: solutions of 1 mM alizarin in acetone without and with 0.1% TEA were compared for the detection of PBA on various TLC plates (Figure 3). The presence of TEA did not have much effect with silica plates, but it considerably improved the detection of PBA on reversed-phase (C_{18} and C_8) plates and chromatography paper. Thus, a simple adjustment of the alizarin solution makes it usable for other TLC plates than silica (except alumina Al_2O_3), and fine-tuning of the TEA concentration may further improve the sensitivity of the method.

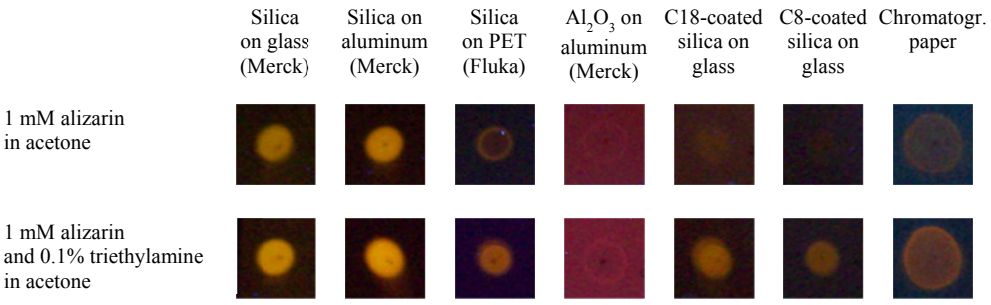

Figure 3. Effect of triethylamine on the detection of PBA. Selections from the original photograph of various TLC plates spotted with PBA (10 mM in MeOH, 2 µL), briefly immersed in 1 mM alizarin solution in acetone (without or with 0.1% triethylamine), left to dry and placed under 365 nm light.

3.3 HPLC: why not alizarin red S?

In chapter 5, the development of a method for the HPLC on-line detection of boronic acids using alizarin was shown. In preliminary off-line experiments, ARS was used because it is much more commonly used than alizarin. When the detection of various boronic acids was tested with ARS, very weak signals were observed in the case of electron-donating boronic acids, especially 4-methoxyphenylboronic acid (MPBA). Therefore, ARS and alizarin were compared for the detection of MPBA, and the change in fluorescence intensity was much higher with alizarin than with ARS. From this result, we decided to continue developing the method with alizarin.

After our work on the HPLC detection of boronic acids, we thought it might be interesting to compare alizarin and ARS in a more systematic way. Our results had shown that alizarin was better than ARS for the detection of MPBA, but a literature survey had shown that ARS had almost always been preferred over alizarin. A comparison of ARS and alizarin may, therefore, promote the use of alizarin for the detection of boronic acids. Alizarin is known to be "insoluble" in water, but at a concentration of ~ 10 µM in PBS, solubility did not cause a problem in our hands. Solutions of alizarin or ARS (10 µM) in PBS pH 7.4, with or without boronic acid (100 µM), were analyzed by fluorescence spectroscopy. Various boronic acids were analyzed in this way. After subtracting the background fluorescence of alizarin and ARS themselves from the excitation and emission spectra of the mixtures, it was confirmed that alizarin performed better than ARS for the detection of MPBA. However, the obtained spectra revealed that all the other tested boronic acids resulted in higher fluorescence intensities with ARS than with alizarin. It turned out that MPBA was a very particular case on which we had, by chance, based our choice of alizarin over ARS for the development of the HPLC method.

Further comparative experiments consisted of the detection of 100 µM PBA by 10 µM alizarin or ARS in various conditions, again by fluorescence spectroscopy. When the pH was varied from 4 to 10, without any organic solvent, the optimum pH was between 7 and 8 in both cases, but ARS performed slightly better than alizarin in terms of PBA detection. In PBS without organic solvent, or with 1% MeOH, acetone, THF, ACN, DMF or DMSO,

ARS still performed slightly better than alizarin. However, in PBS with 50% of the same solvents, alizarin performed slightly better than ARS. To further investigate the effect of the percentage of organic solvent, the detection of 100 µM PBA by 10 µM alizarin or ARS was tested in PBS/MeOH and PBS/ACN mixtures with MeOH or ACN percentages of 10%, 50% and 90%. As a result, as the fraction of organic solvent increased, the fluorescence intensity increased when alizarin was used and decreased when ARS was used. With 90% organic solvent (and also 50% in the case of ACN), alizarin proved better than ARS for the detection of PBA.

To summarize, our comparative study has shown the following: 1) ARS results in higher changes in fluorescence than alizarin for the detection of boronic acids in aqueous solutions (possibly with a small percentage of organic solvent), except for the detection of MPBA; 2) alizarin results in higher changes in fluorescence than ARS for the detection of PBA (and *probably* other boronic acids) in solutions with high organic solvent content.

To come back to our work on the on-line HPLC detection of boronic acids as described in chapter 5, the question is: what is better, alizarin or ARS? We used a solution of 75 µM alizarin and 0.1% triethylamine in ACN. Because we made the choice of alizarin over ARS in preliminary off-line experiments, ARS was not tested on-line. Considering the experiments described above, a buffered aqueous solution of ARS at pH 7-8 may result in a higher sensitivity for the on-line detection of boronic acids. However, mixing the HPLC eluate with the ARS solution may cause problems: salts from the buffered aqueous solution of ARS may precipitate when mixing with an organic solvent from the eluate, organic molecules in the eluate may precipitate when mixing with the aqueous buffer, and bubbles may be formed upon mixing of MeOH from the eluate and water from the ARS solution.

In conclusion of this section, both alizarin and ARS may be considered for the HPLC on-line detection of boronic acids, depending on the experimental conditions. If the HPLC eluate contains no or little organic solvent, then ARS in an aqueous buffer may be preferred. If the HPLC eluate contains a high proportion of organic solvent, alizarin in an organic solvent is the way to go.

4 Separation of boronic acids by diol-silica chromatography

The isolation of boronic acid-containing compounds by diol-silica flash chromatography was reported for the first time (to the best of our knowledge) in chapter 3. Commercially available diol-silica (structure page 43) was used in the same way as bare silica in a traditional flash chromatography setup. The eluent was a gradient of MeOH (and acetic acid in one case) in EtOAc which enabled, first, the elution of boronic acid-free compounds and second, the elution of boronic acid-containing compounds. Only two compounds were purified in this way, and this separation method deserves further investigation.

Separation modes. Diol-silica has previously been used, as discussed in chapter 3, for separations based on polarity (normal-phase and reversed-phase chromatography) or hydrophilicity (hydrophilic interaction chromatography) of the analytes. In addition, diol-silica may be used for separations based on the reversible reaction between diols of the stationary phase and boronic acid groups of the analytes. The separation of boronic acids may be based on one or several of these separation modes (polarity, hydrophilicity and boronic acid-diol interaction), depending on the structure of these compounds and the composition of the mobile phase. Thus, it would be interesting to investigate the influence of these factors on the separation of boronic acids.

Scope. Until now, only two boronic acids were isolated using diol-silica. The results look promising, but they are just a starting point as they cannot tell whether this purification method is generally applicable. Further investigations, therefore, may include the purification of a wide range of boronic acids.

Diol-silica HPLC. The separation of boronic acids by a diol-silica stationary phase may further be investigated by HPLC, using a commercially available diol-silica HPLC column. HPLC would enable rapid screening of boronic acids and mobile phases, provide information more clearly and more efficiently than “manual” column chromatography about scope and separation modes, and expand the applicability of this unexplored separation strategy. Moreover, HPLC analysis would be a first step towards the separation

of boronic acids by preparative diol-silica HPLC, which may be a solution to the difficult purification of unprotected boronic acids.

5 General conclusion

The initial goal of the PhD project was to functionalize sensor chips in such a way that biomarkers could be detected at low concentrations (~ 1 pM) in urine. This would help to enable an objective and reliable diagnosis of depression. Antibodies would be immobilized on the chips in an oriented way, with their antigen binding sites pointing away from the surface. With this goal in mind, the immobilization of antibodies via boronic acids was explored. A strategy for the oriented and irreversible immobilization of antibodies was devised, and relevant boronic acid-containing linkers were synthesized. This strategy and its proof-of-principle were published by another group at the moment our linkers were obtained. Eventually, the initial goal of the PhD project was not achieved within the available time, for several reasons that were out of our hands. However, the challenges encountered during our work with boronic acids gave rise to valuable findings. Some purification experiments suggested that boronic acids can be isolated by means of diol-silica chromatography (chapter 3). Boronic acids can be detected on TLC plates by dipping these plates in an alizarin solution and visualizing them under a UV lamp (365 nm), as shown in chapter 4. This is the first published TLC staining method for boronic acids. It is selective, sensitive, simple, safe and inexpensive, and by these combined advantages, we find it better than alternative, even more recently published, TLC staining methods for boronic acids. Moreover, as shown in chapter 5, boronic acids can also be detected on-line after HPLC separation, again by using alizarin. The HPLC eluate mixes with an alizarin solution, and fluorescent complexes are produced when boronic acids are present. This facilitates the pinpointing of boronic acids in complex mixtures, and enables the optimization of conditions for the isolation of boronic acids by preparative HPLC. As such, the synthetic potential of boronic acids may be further developed, as detection of these compounds and derivatives thereof is significantly facilitated by the work presented in this thesis.

6 References

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Appendix
Information supplementing chapter 5

Position	Material	o.d. (mm)	i.d. (mm)	Length (cm)	Supplier	Part number
From solvent reservoirs to HPLC delivery system	PTFE		2	90	Gilson	3645357
From HPLC delivery system to injector	PEEK		0.13	20	Vici	JR-T-6007
From injector to column				-		-
From column to absorbance detector	Stainless steel	1.59	0.25	30	Grace	2106936
From absorbance detector to T-piece	PEEK	1.59	0.25	15	Vici	JR-T-6009
From Superloop to T-piece	PTFE	1.59	0.76	15	Grace	35670
From HPLC pump to Superloop	PTFE	1.59	0.76	85	Grace	35670
From T-piece to fluorescence detector	PEEK		0.25	Var.*	Vici	JR-T-6009
From fluorescence detector to waste	PTFE	1.59	0.76	100	Grace	35670

Table 1. Tubing used in the on-line HPLC instrumental set-up.*Several reaction coil lengths were tested, and a length of 350 cm was selected.

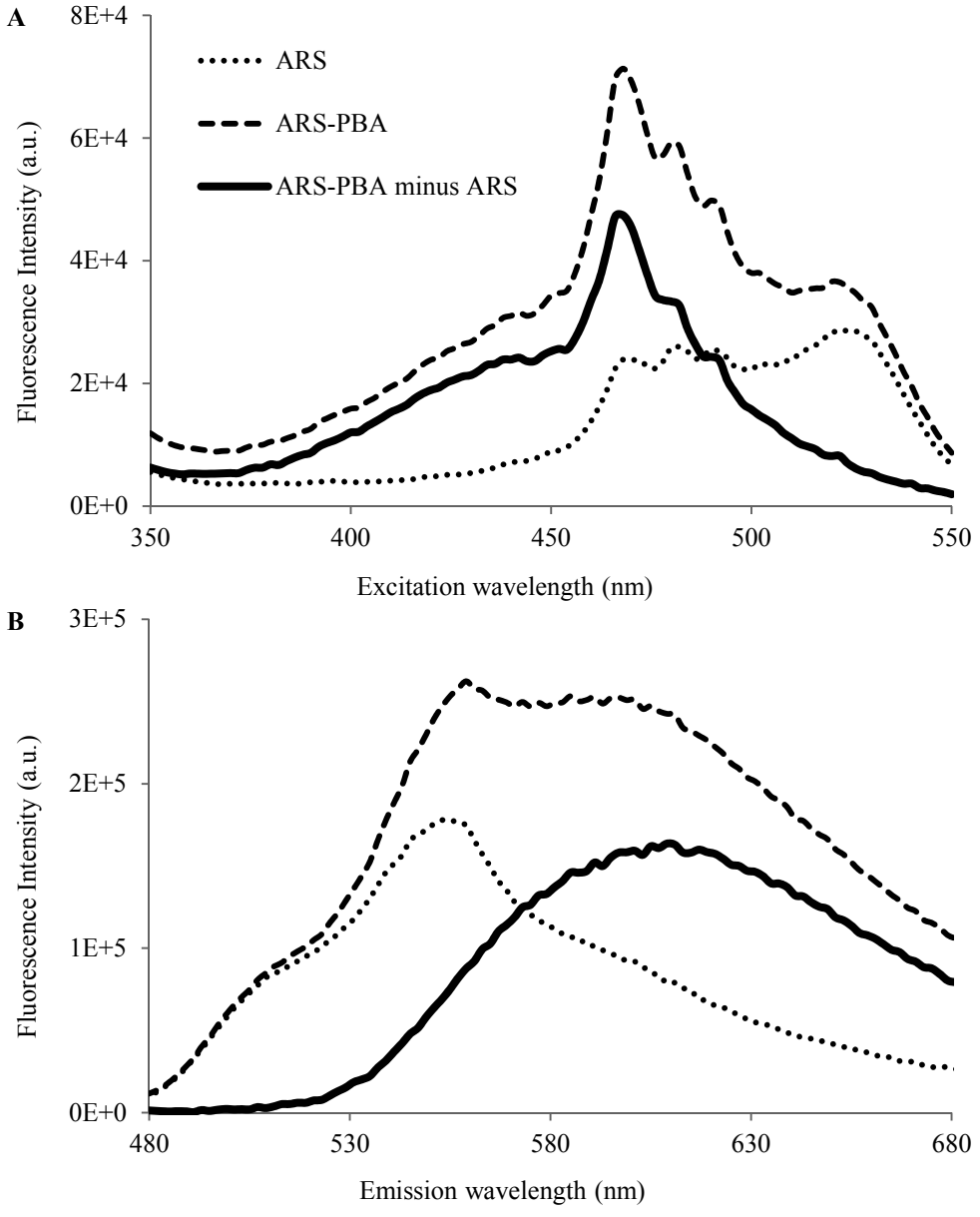

Figure 1. Analysis of 3.13 µM ARS and 3.13 µM ARS + 313 µM PBA solutions in PBS pH 7.4. **A**. Excitation spectra, λ_{em} 610 nm, and subtracted spectrum (difference between fluorescence intensities of ARS and ARS-PBA). **B**. Emission spectra, λ_{ex} 469 nm, and subtracted spectrum.

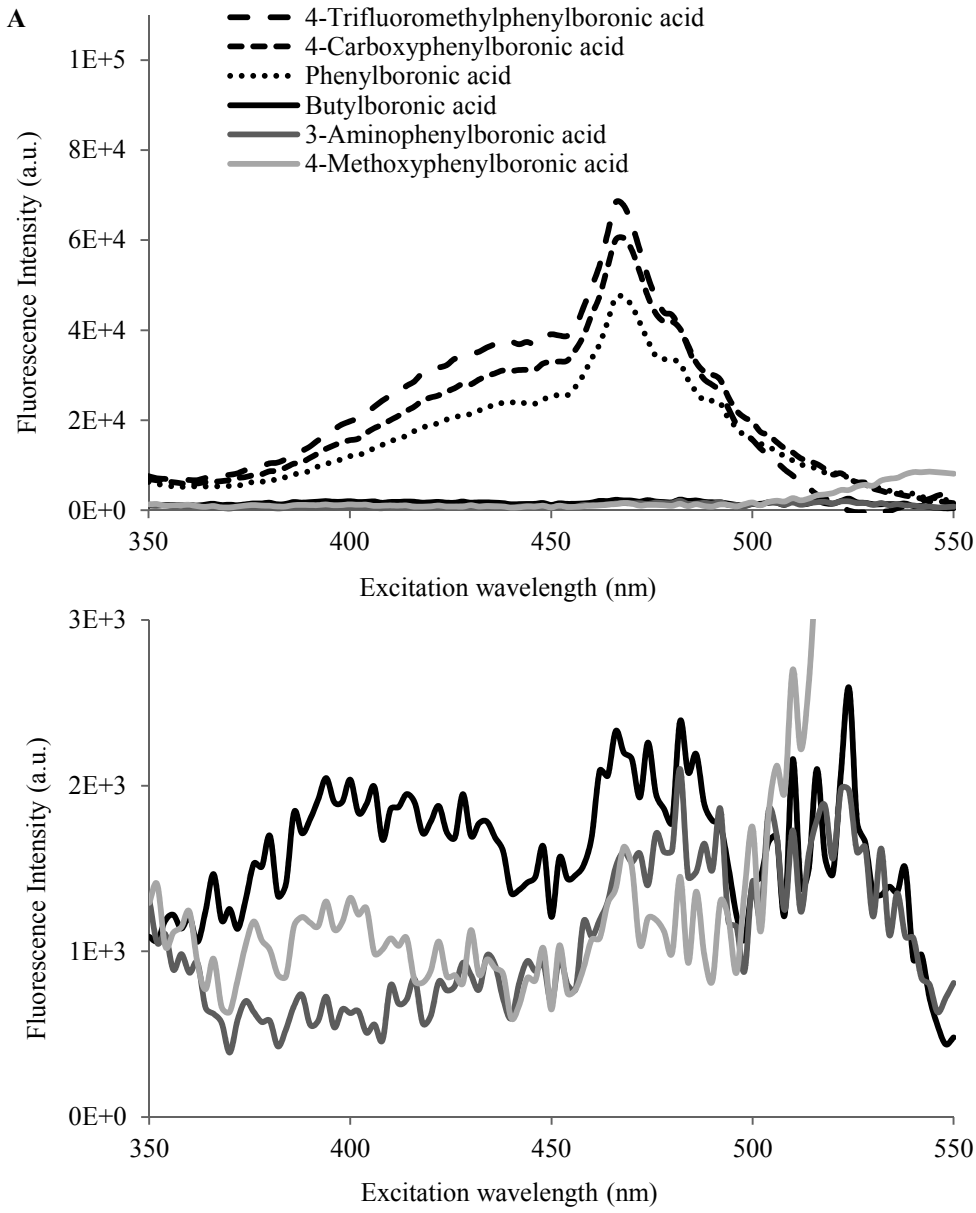

Figure 2. Analysis of 3.13 μM ARS and 3.13 μM ARS + 313 μM solutions of various boronic acids in PBS pH 7.4. **A**. Subtracted (see explanation in Figure 1) excitation spectra, λ_{em} 610 nm. **B**. Subtracted emission spectra, λ_{ex} 469 nm.

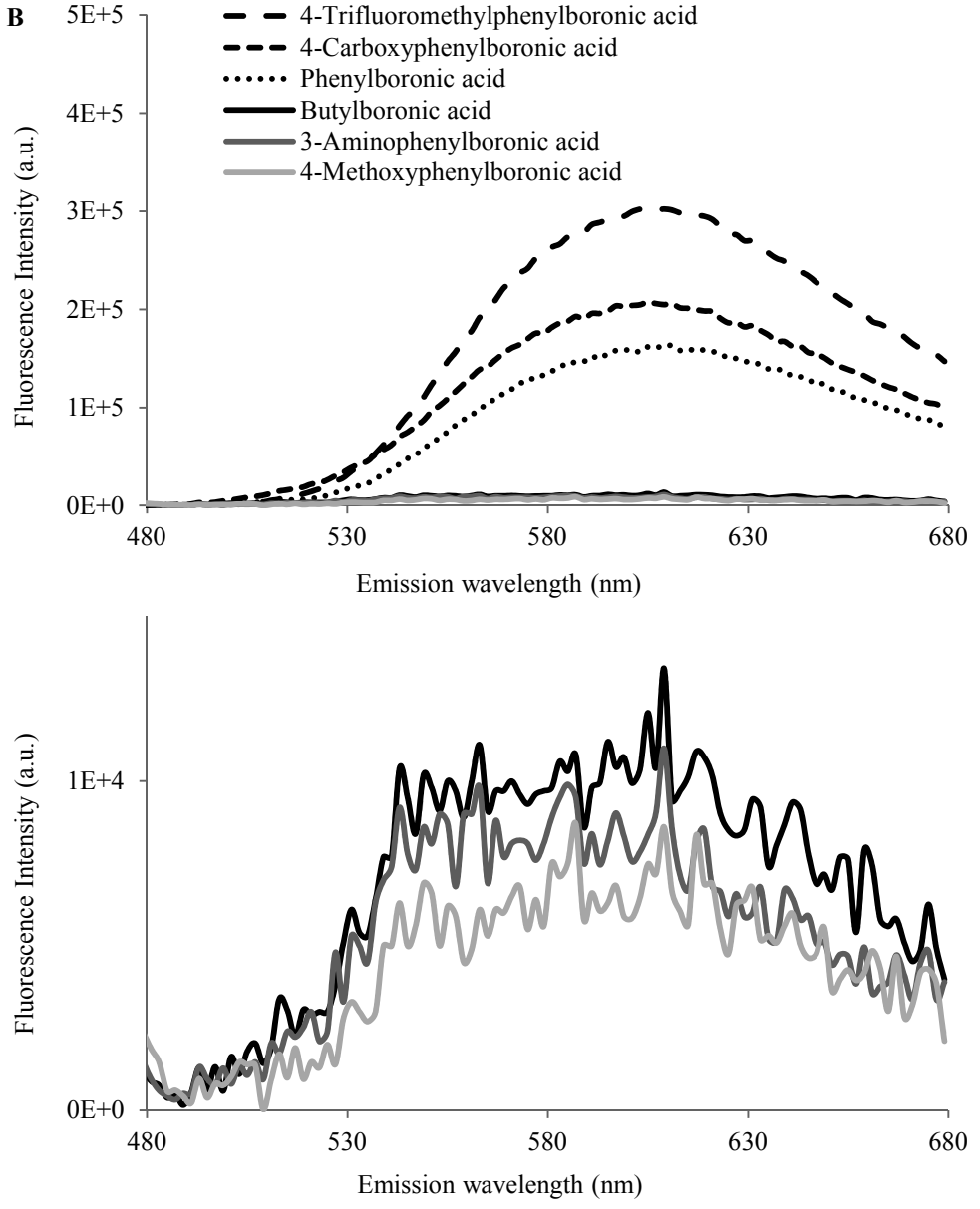

Figure 2. (*Continued)*.

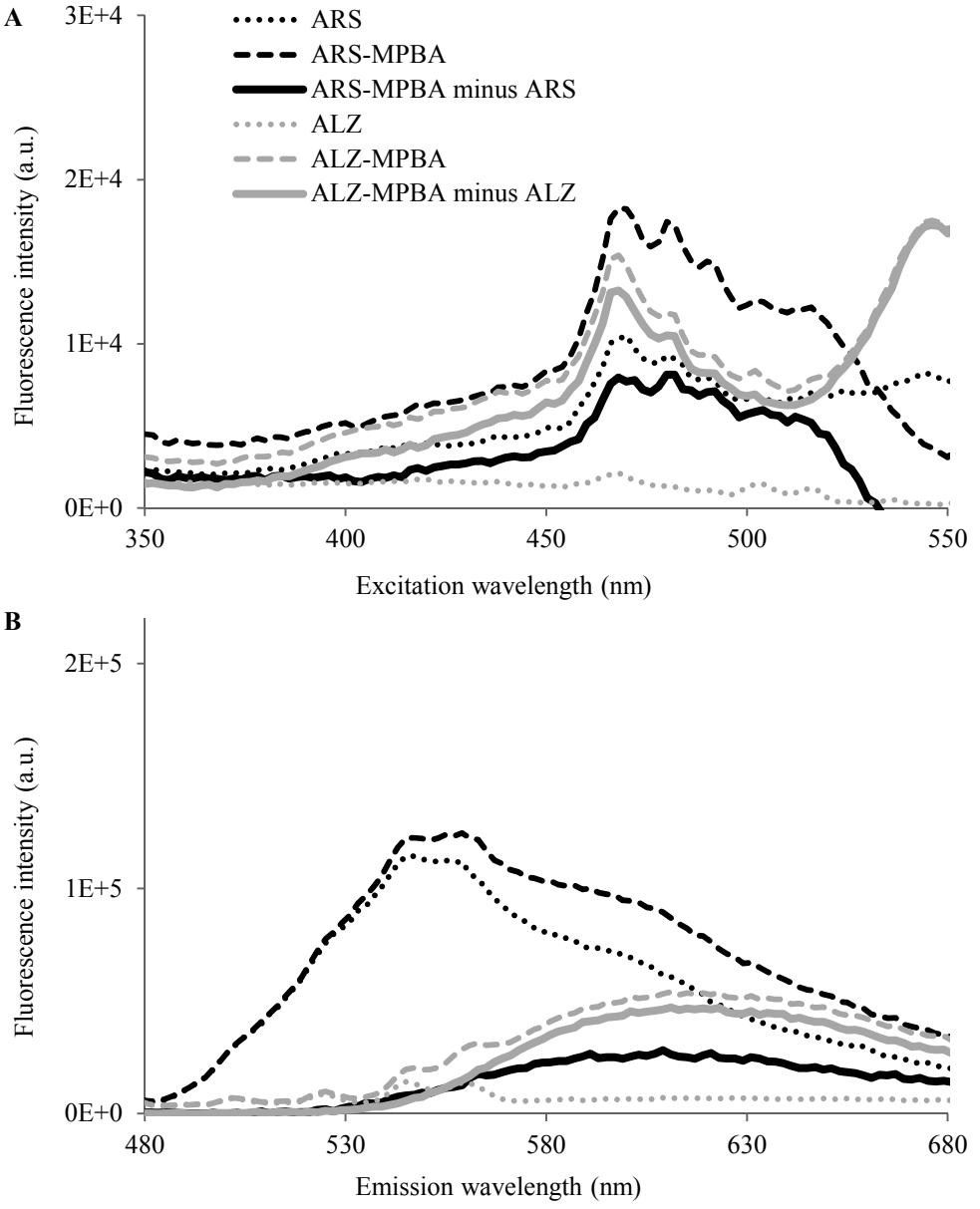

Figure 3. Comparison of 3.13 µM ARS and 3.13 µM alizarin for the detection of 313 µM PBA in ACN/water 1:1. **A**. Excitation spectra, λem 610 nm, and subtracted spectrum (difference between fluorescence intensities of ARS and ARS-MPBA or ALZ and ALZ-MPBA) . **B**. Emission spectra, λ_{ex} 469 nm, and subtracted spectra.

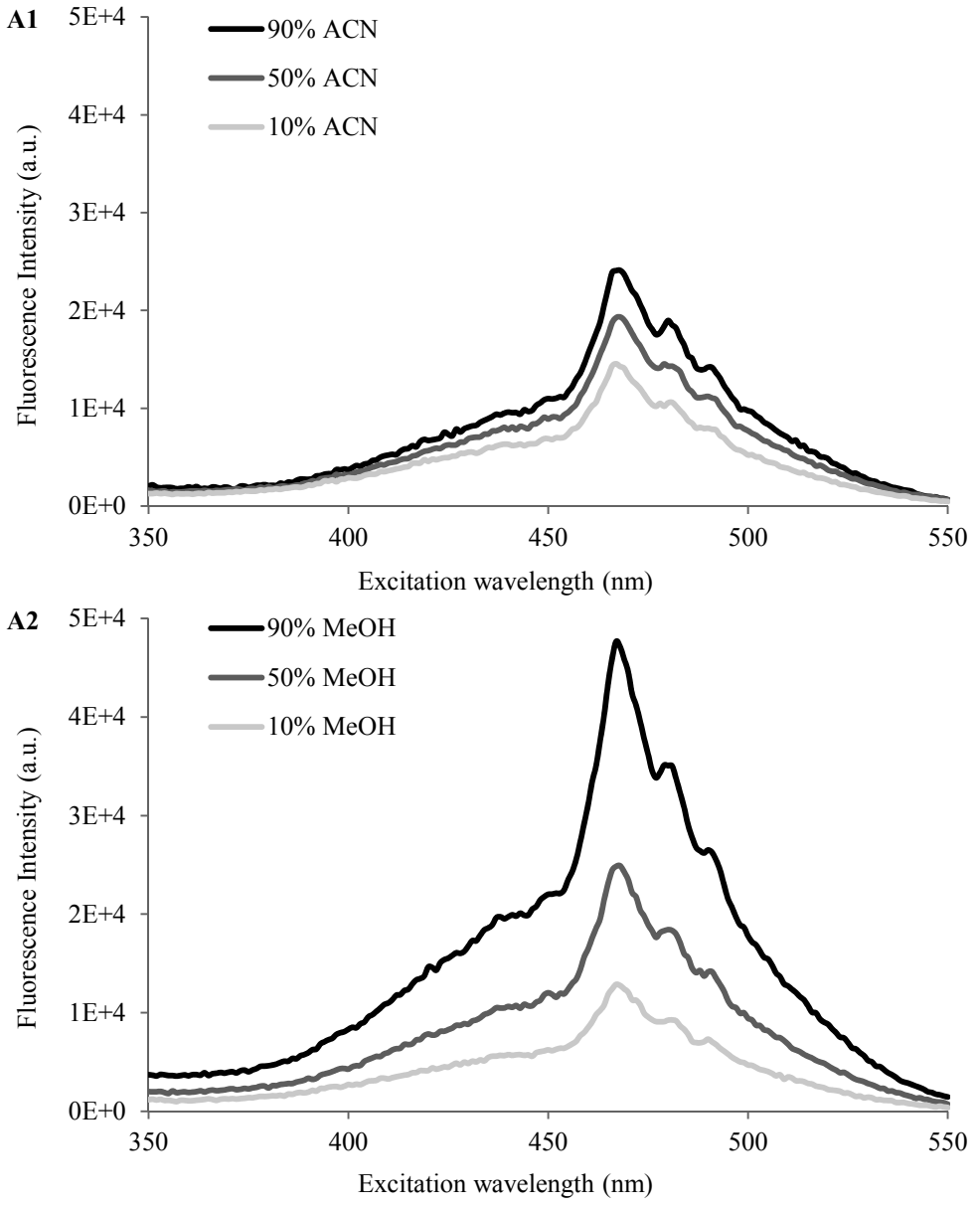

Figure 4. Effect of solvent composition on the detection of 100 µM PBA using 10 µM alizarin. Various percentages of ACN (**1**) or MeOH (**2**) were used. The remaining volumes consisted of phosphate buffered saline pH 7.4. **A**. Subtracted (see explanation in Figure 1) excitation spectra, λ_{em} 610 nm. **B**. Subtracted emission spectra, λ_{ex} 469 nm.

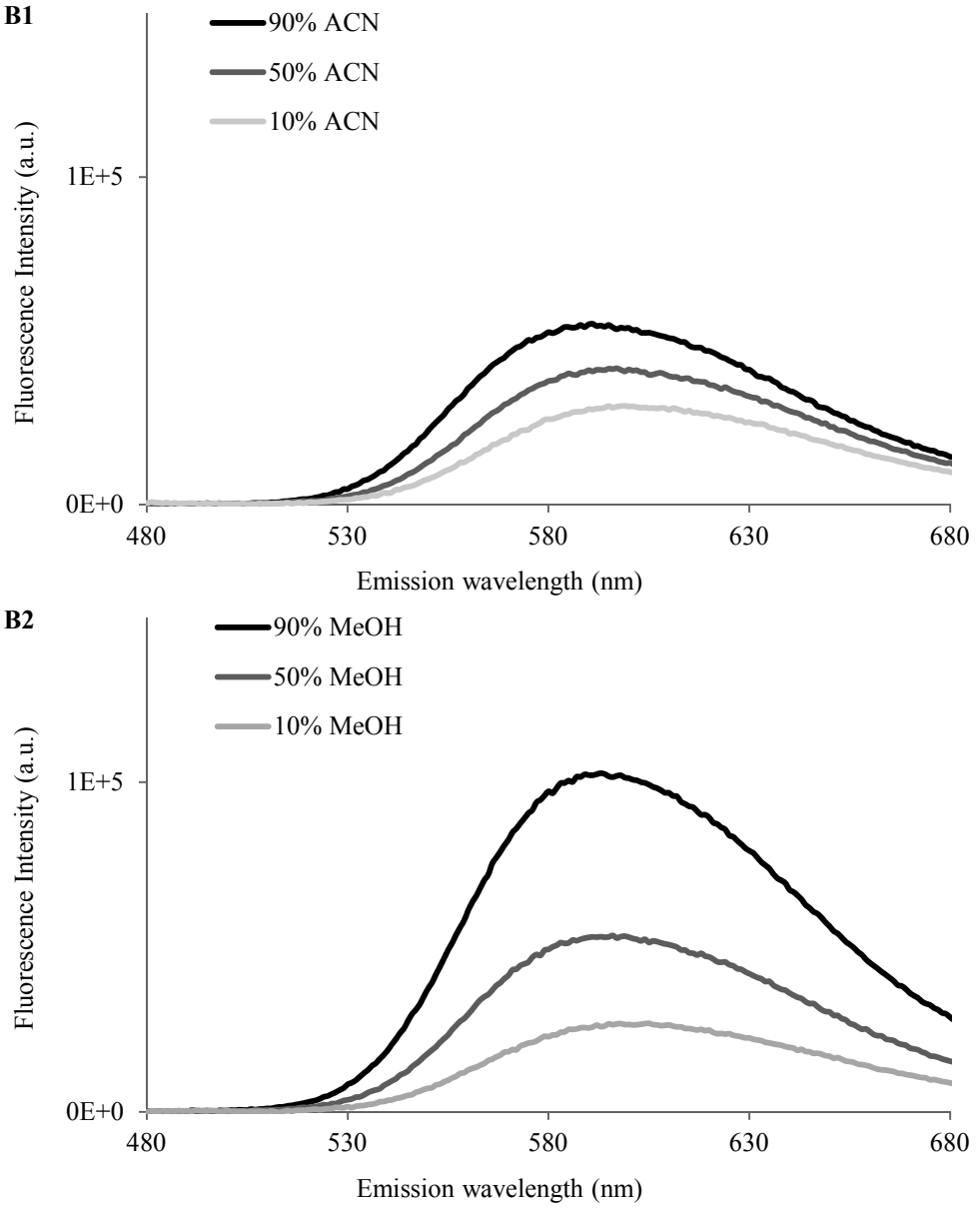

Figure 4. (*Continued).*

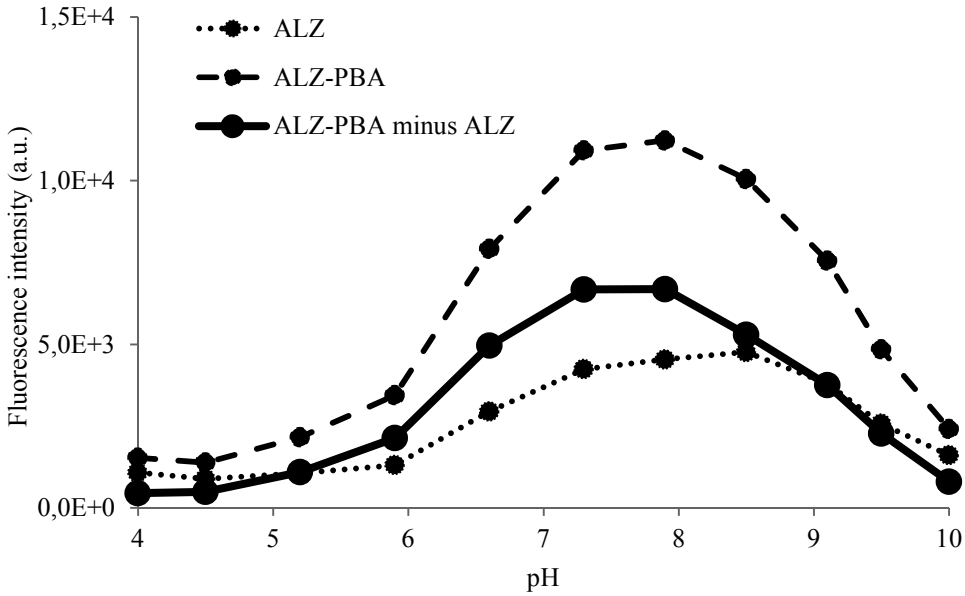

Figure 5. Effect of pH on the detection of 100 μM PBA using 10 μM alizarin, in 20 mM citrate/30 mM HEPES/40 mM CHES buffer at various pH values and identical organic compositions. λ_{ex} 469 nm, λ_{em} 610 nm.

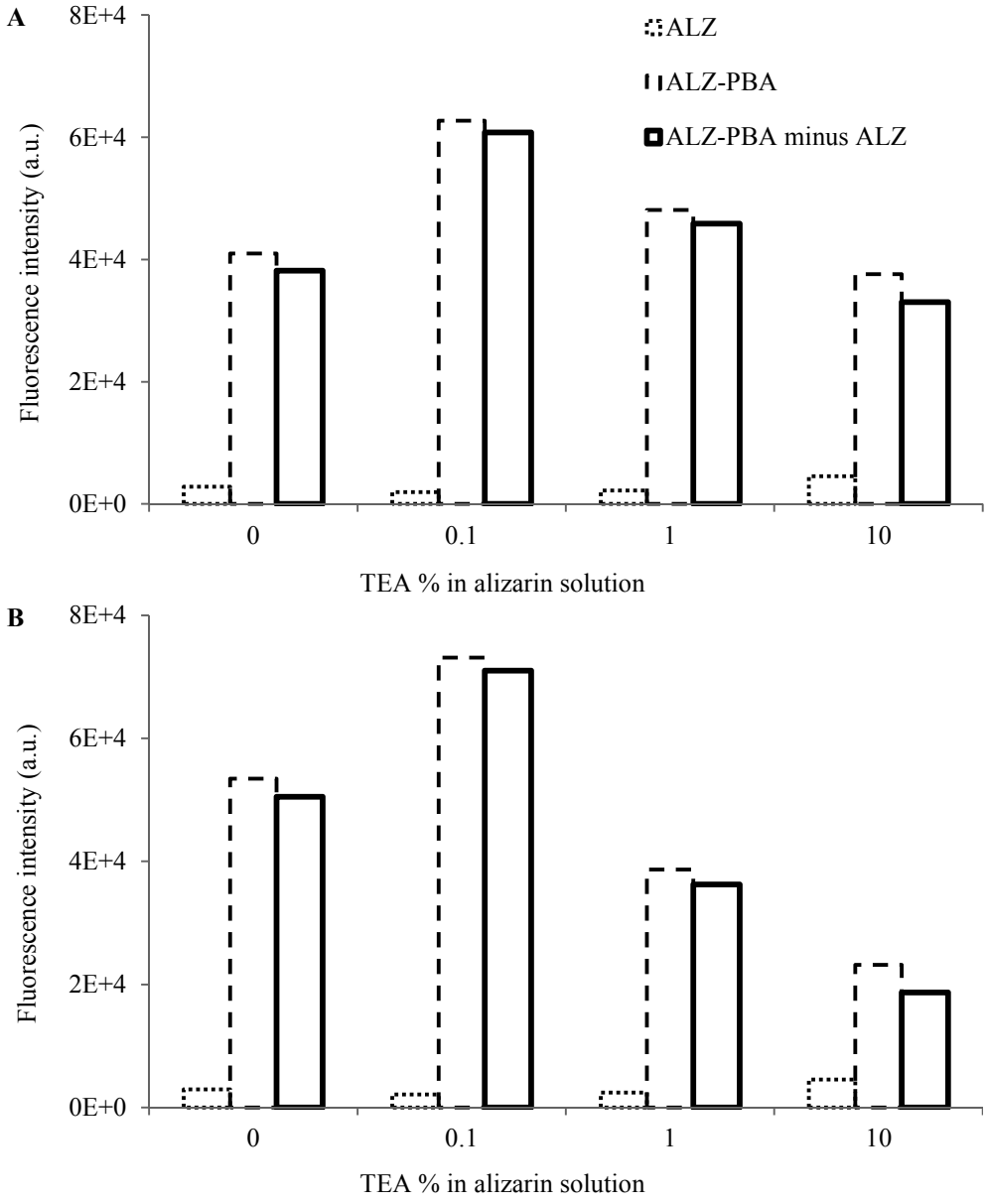

Figure 6. Effect of TEA on the detection of 100 µM PBA using 10 µM alizarin. Solutions consisted of two parts of equal volumes: 1) "alizarin solution mimic", 20 µM alizarin solution in ACN that contained 0%, 0.1%, 1% or 10% TEA, 2) "HPLC eluate mimic", 200 µM PBA in 50% water and 50% of ACN (**A**) or MeOH (**B**). λ_{ex} 469 nm, λ_{em} 610 nm.

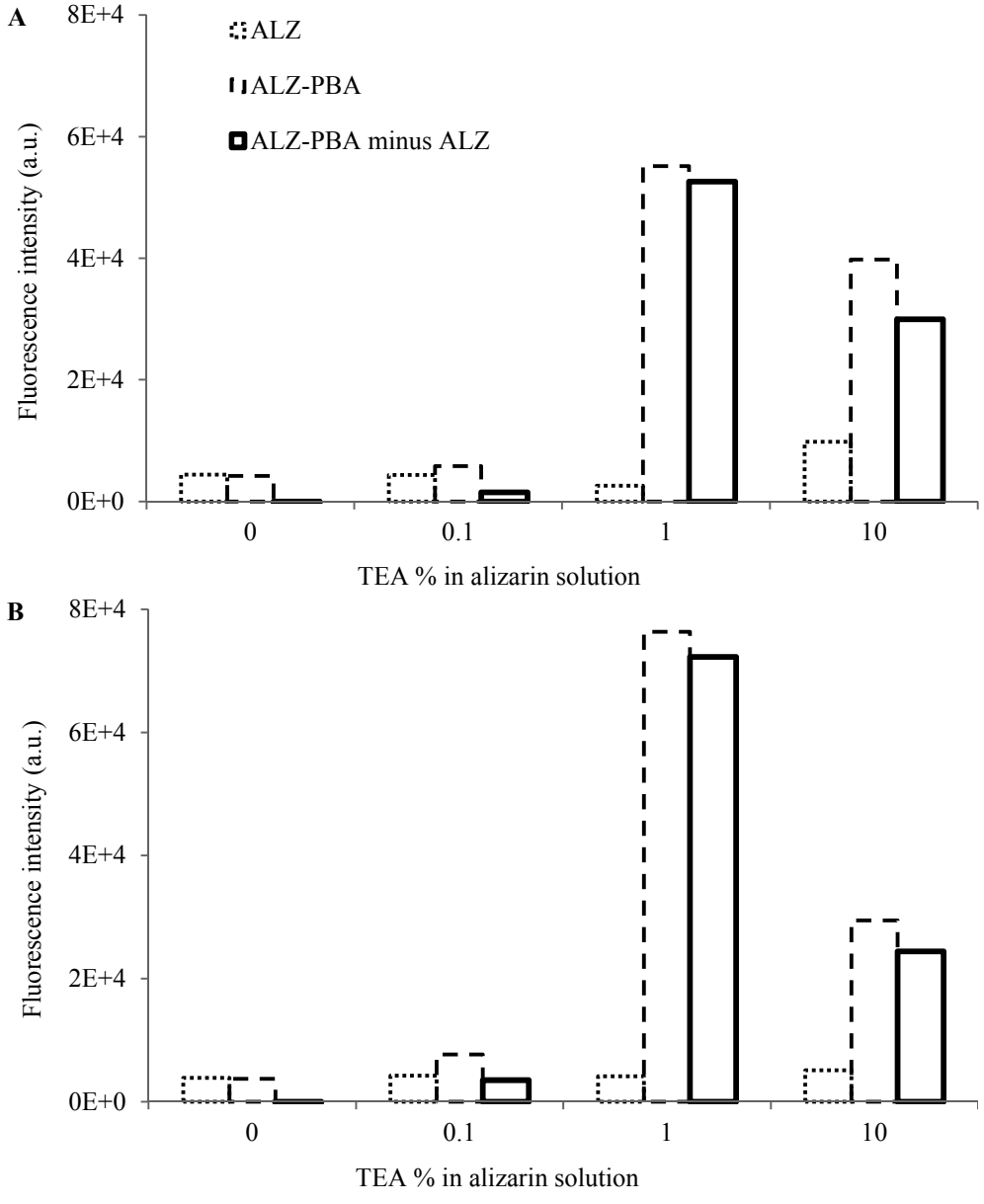

Figure 7. Effect of TEA on the detection of 100 µM PBA using 10 µM alizarin, **in presence of acetic acid**. Solutions consisted of two parts of equal volumes: 1) "alizarin solution mimic", 20 µM alizarin solution in ACN that contained 0%, 0.1%, 1% or 10% TEA, 2) "HPLC eluate mimic", 200 µM PBA and 0.1% acetic acid in 50% water and 50% of ACN (**A**) or MeOH (**B**). λ_{ex} 469 nm, λ_{em} 610 nm.

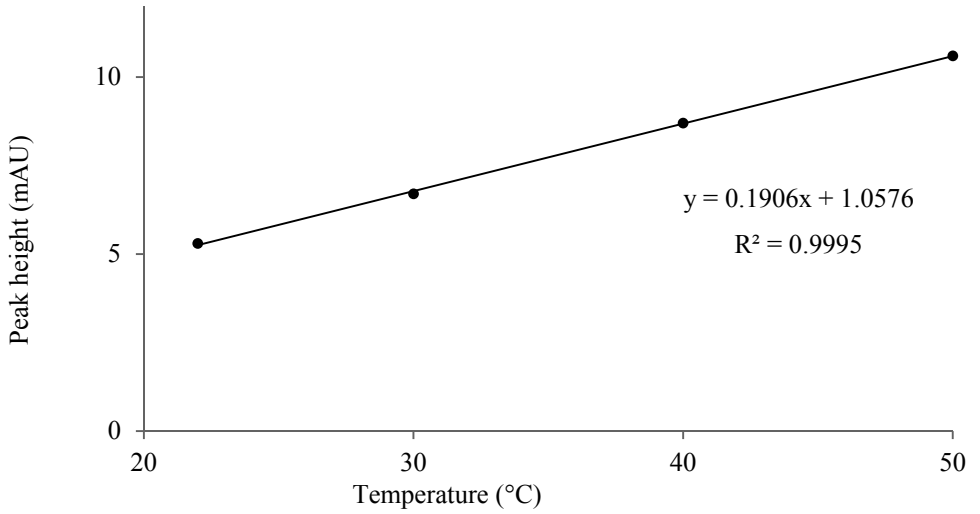

Figure 8. Effect of the coil temperature on the height of the fluorescent peak produced by the reaction between alizarin and the injected PBA.

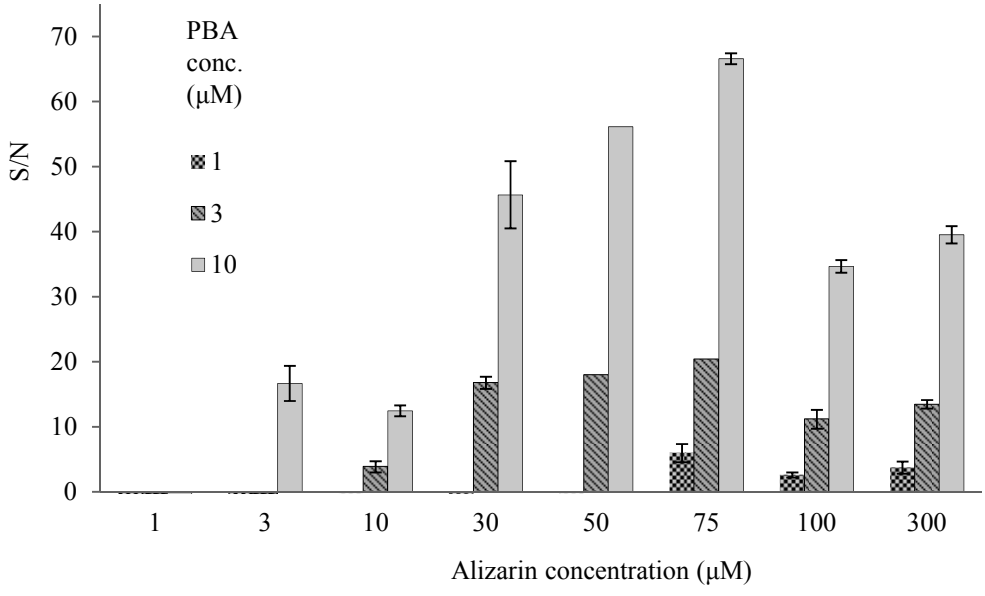

Figure 9. Effect of the alizarin concentration on the signal-to-noise ratio of the peaks produced by the reaction between alizarin and the three lowest concentrations of injected PBA. n = 3 except for 50 µM alizarin/all PBA concentrations and 75 µM alizarin/3 µM PBA in which case n = 2.

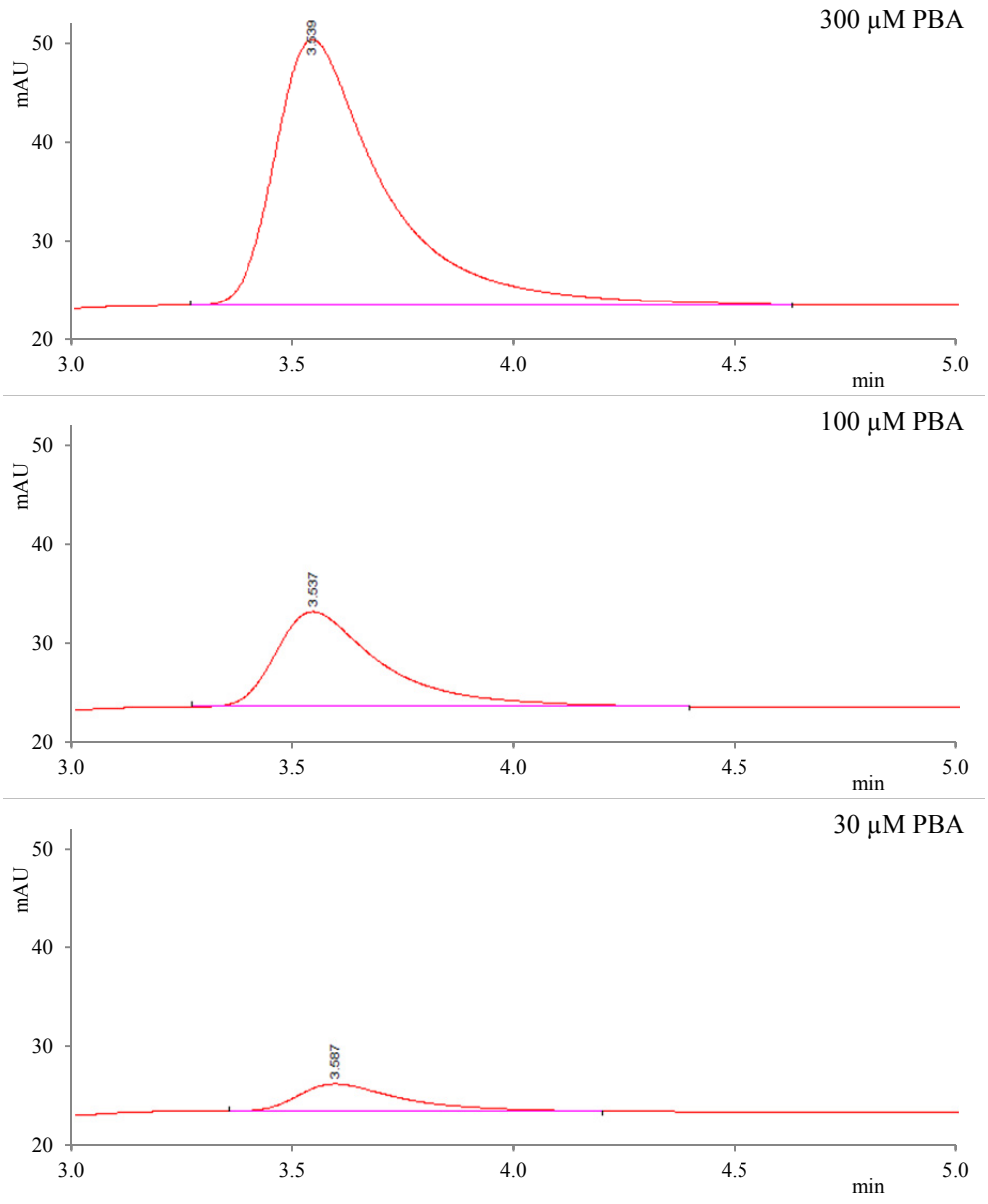

Figure 10. Chromatograms for the detection of various concentrations of PBA using the developed method. Results from paragraph 3.3.1 in chapter 5, for an alizarin concentration of 75 µM.

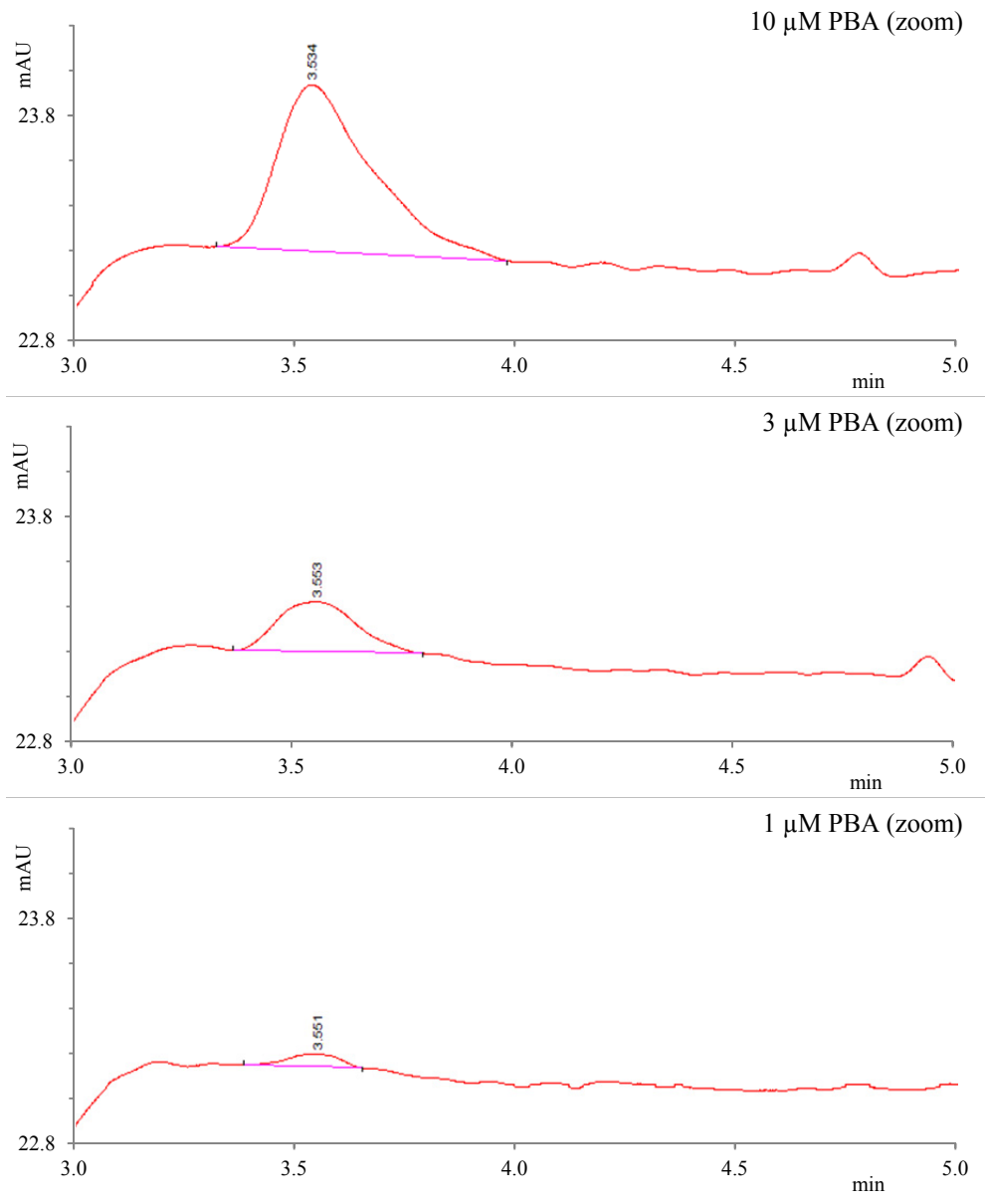

Figure 10. (*Continued).*

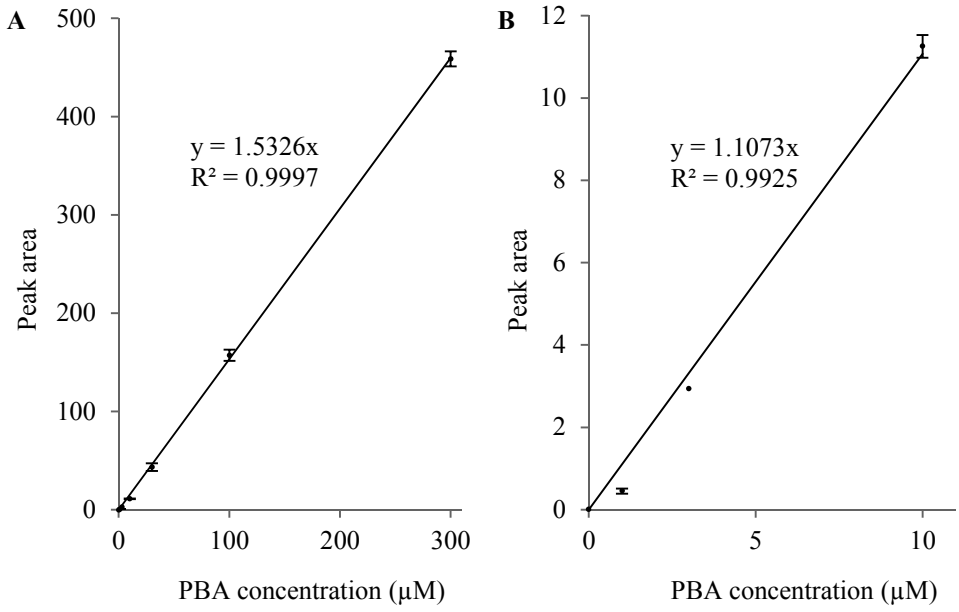

Figure 11. Ability of the developed method to quantify PBA. Peak areas taken from chromatograms in Figure S10 (+ replicates). n = 3 except for 3 µM PBA in which case n = 2. **A**. Peak area for all PBA concentrations. **B**. Peak areas for lower PBA concentrations (1, 3 and 10 µM).

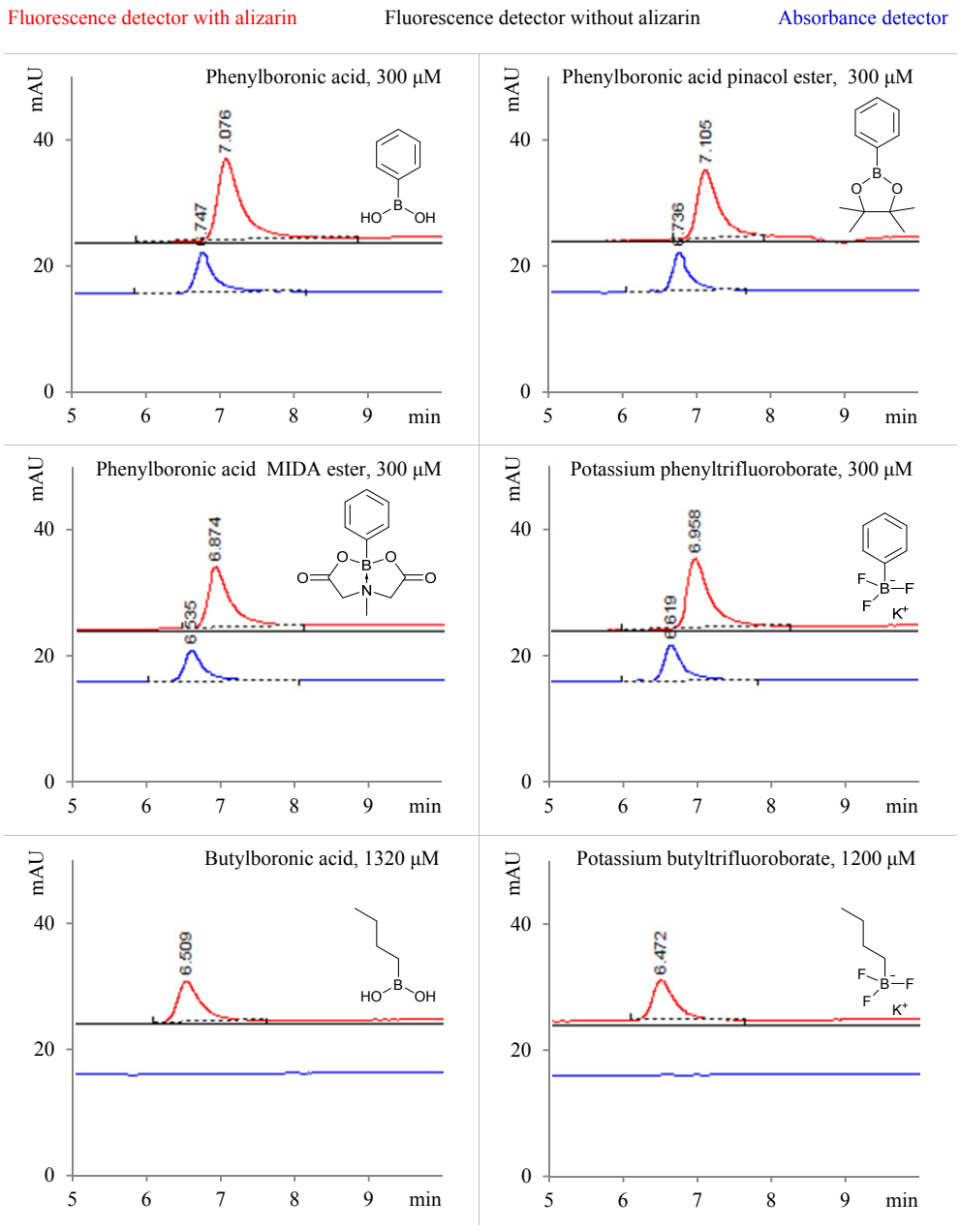

Figure 12. Detection of various boronic acids using the developed method.

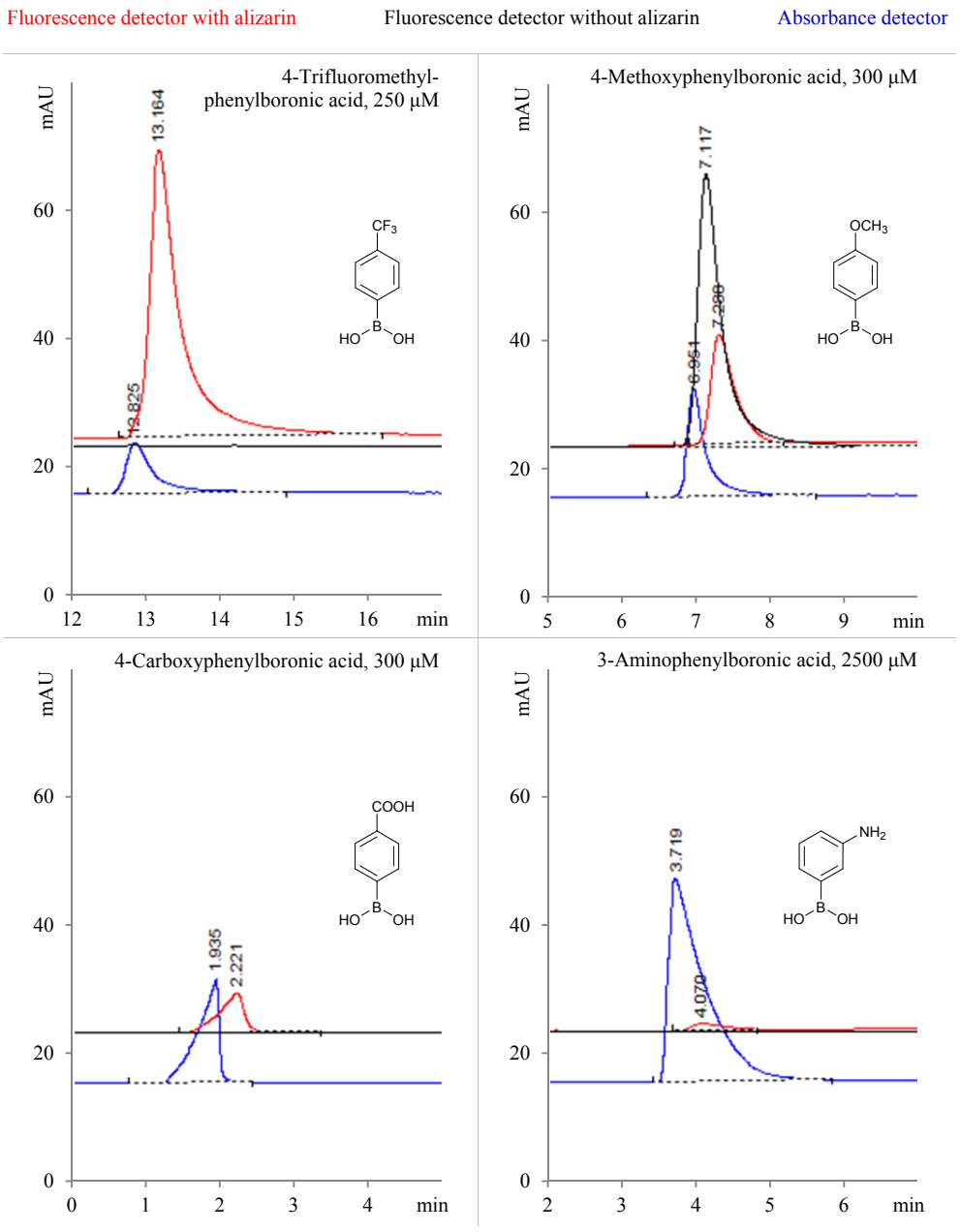

Figure 12. (*Continued).*

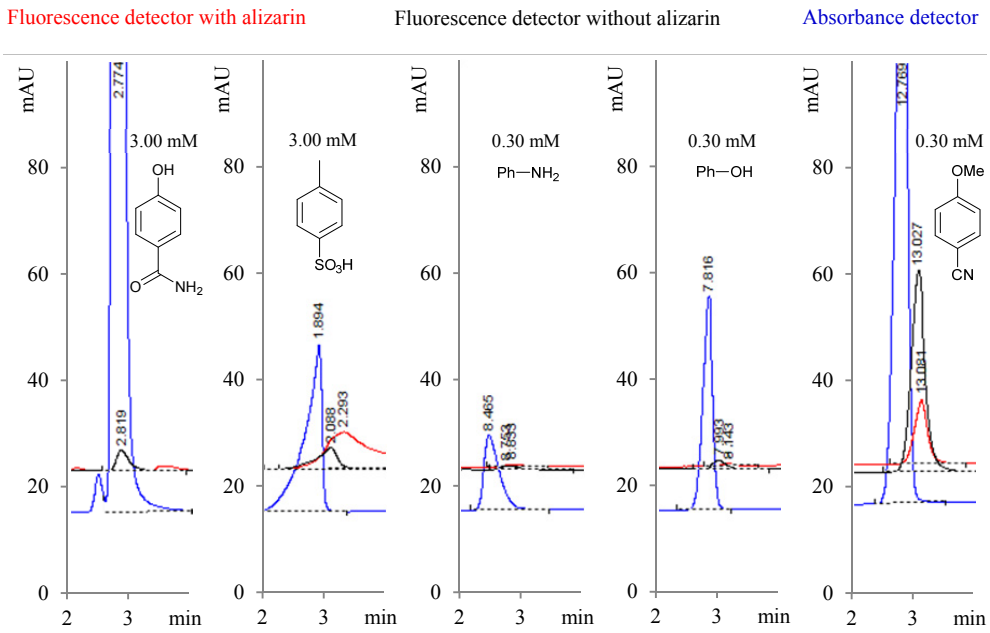

Figure 13. Detection of the autofluorescent boron-free compounds using the developed method.

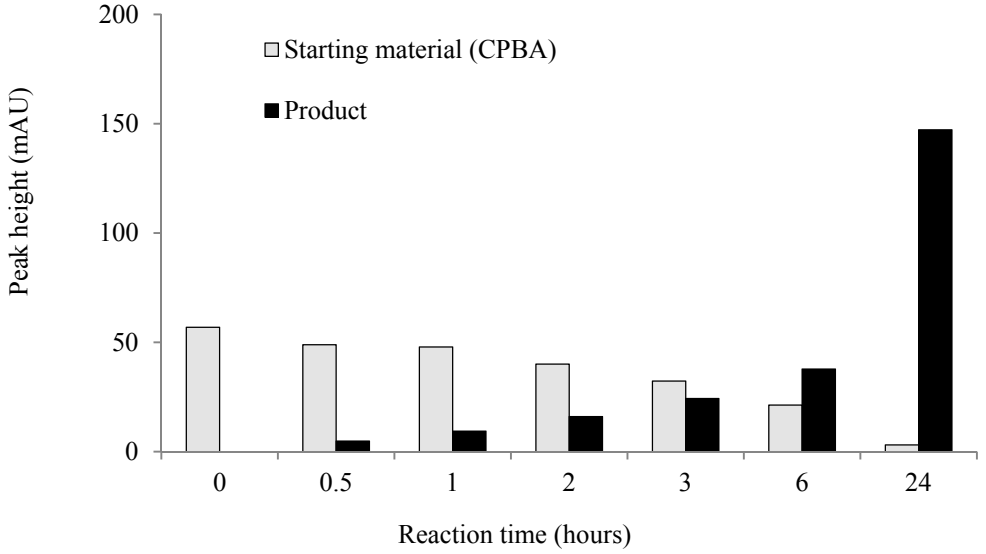

Figure 14. Monitoring of the acid-catalyzed esterification of CPBA. At each reaction time, fluorescence peak heights that correspond to starting material CPBA (4.6 min) and product (22.6 min) were determined.

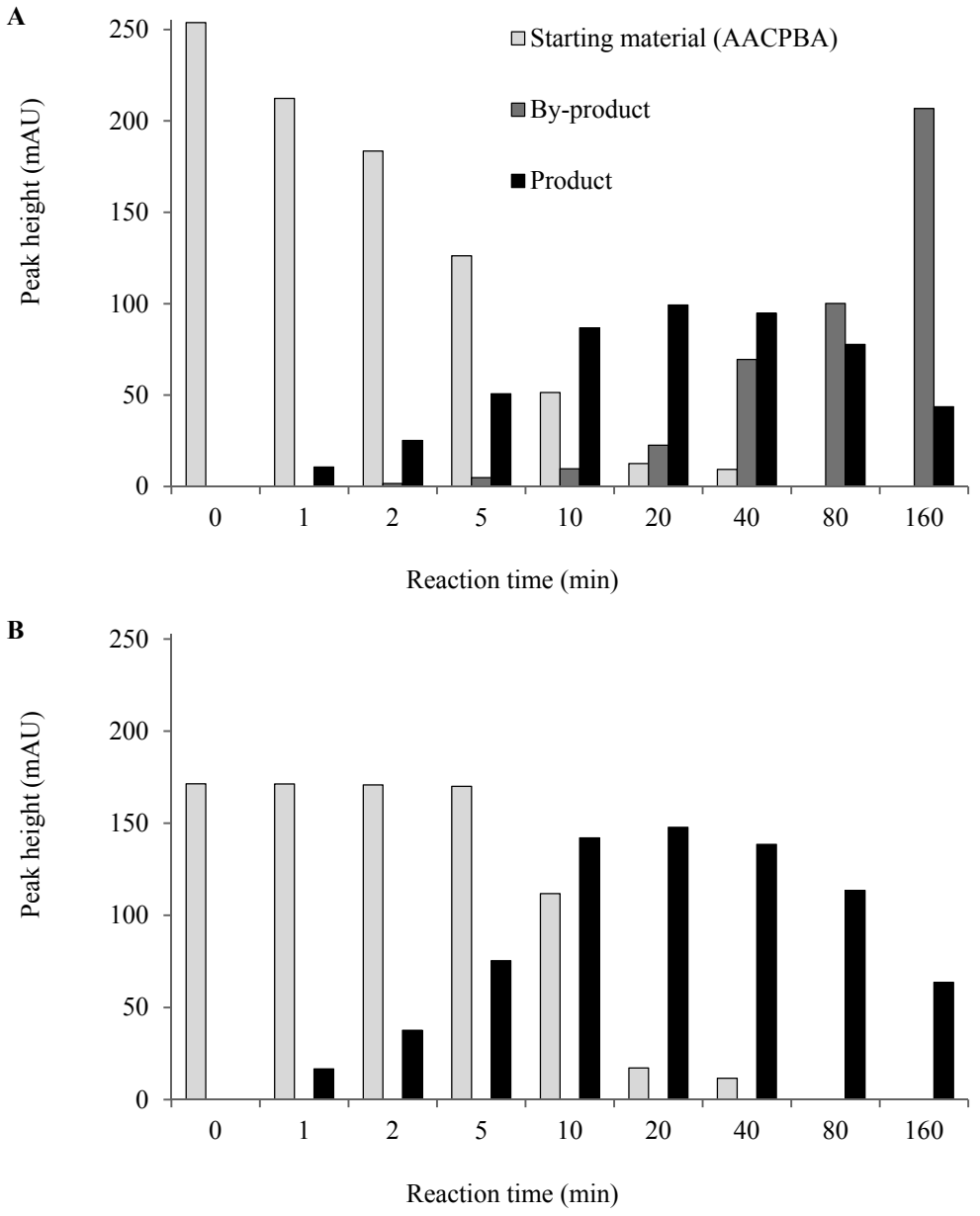

Figure 15. Monitoring of the thiol-ene reaction between AACPBA and Boc-Cys. At each reaction time, peak heights that correspond to starting material (3.2 min by absorbance), by-product (7.0 min by absorbance) and product (8.1 min by absorbance) were determined. **A**: absorbance detection at 254 nm. **B**: fluorescence detection at 610 nm.

Summary

Chapter 1 introduces the theory and known applications of the interaction between boronic acids and diols, and explains the context of this thesis. Diagnosis of depression was the initial goal of this multidisciplinary project. The focus of the PhD project was the development of a strategy to immobilize antibodies on the surface of a chip in such a way that very low concentrations (~ 1 pM) of biomarkers for depression could be detected in urine. To achieve this, the immobilization of antibodies using boronic acids seemed promising.

However, preliminary experiments and further insights revealed the many challenges that this immobilization strategy faces, giving rise to **Chapter 2**. This chapter discusses several important points that need to be taken into account when one plans to immobilize antibodies via boronic acids: choice of the boronic acid structure and spacer to attach it to the surface, use of an antifouling polymer, choice of an antibody with suitable glycosylation, optimization of the conditions for antibody immobilization...

One big issue for antibody immobilization using boronic acids is the reversibility of the reaction between boronic acids and diols, hence the possible release of the antibody from the surface. **Chapter 3** describes the design and synthesis of boronic acid-containing linkers that would enable the oriented *and* irreversible immobilization of antibodies. Two linkers were designed with an amine for surface attachment, a boronic acid for capturing antibodies via the N-glycans in their Fc chain, and a diazirine for irreversible immobilization upon UV irradiation while maintaining antibody orientation. From a diazirine building-block that was obtained in three steps, the first linker was synthesized in four steps and the second linker was synthesized in three steps. Diol-functionalized silica was used for the chromatography of two boronic acid-containing intermediates, this method being novel (to the best of our knowledge) and likely based on boronic acid-diol interactions. High-resolution mass spectrometry, through matching exact masses, matching isotope patterns and observation of species corresponding to the esterification of boronic acids with MeOH, confirmed that both linkers were synthesized successfully.

During the synthesis of boronic acid-containing linkers, it was difficult to see which spots on TLC plates corresponded to boronic acids. To solve this problem, a new TLC staining method based on the reaction between boronic acids and alizarin was developed. **Chapter 4** presents this work in detail. After optimization experiments, 1 mM alizarin in acetone was shown to be the preferred staining solution. When the TLC plate was briefly dipped in this solution, allowed to dry in ambient air and observed under 365 nm light, bright yellow fluorescent spots were observed where boronic acids were present. Phenylboronic acid was detected at a concentration as low as 0.1 mM. A range of boronic acids and derivatives was successfully detected, and boron-free compounds resulted in no or very weak fluorescence. The staining method was further tested in the monitoring of three reactions involving boronic acids, and provided clear information about the consumption or formation of boronic acid-containing compounds.

Although TLC is useful to synthetic chemists, analysis of reaction mixtures by HPLC is sometimes necessary for obtaining more accurate information or for optimization of preparative HPLC conditions. **Chapter 5** presents the development and applicability of a method for the on-line HPLC detection of boronic acids using alizarin. After optimization experiments at an HPLC flow rate of 0.40 mL/min, the HPLC-separated analytes were mixed post-column with a solution of 75 μM alizarin and 0.1% triethylamine in ACN, which was delivered at a flow rate of 0.60 mL/min. The reaction between alizarin and boronic acids occurred in a reaction coil of dimensions of 3.5 m × 0.25 mm at a temperature of 50 °C, resulting in fluorescent complexes that were detected as positive peaks by a fluorescence detector (λ_{exc} 469 nm and λ_{em} 610 nm). The method enabled the selective detection of various boronic acids and derivatives, with a limit of detection of phenylboronic acid of 1.2 ng or 1 μM. It could successfully monitor the progress of two organic reactions involving boronic acid-containing compounds, and provided useful insights into the course of the reactions.

Chapter 6 provides a reflexion about the work presented in this thesis, suggestions for future research, and a general conclusion.

Acknowledgements

Teris van Beek, **Han** Zuilhof, **Alexandre** Villela, **Radostina** Manova, **Elly** Geurtsen, **Elbert** van der Klift, **Cees** van de Haar, **Denis** Dorokhin, **Barend** van Lagen**, Hendra** Willemen, **Frank** Claassen, Sidharam (**Sidhu**) Pujari, **Ai** Nguyen, **Nagendra** Bhairamadgi, **Marcel** Giesbers, **Jacob** Baggerman, **Anke** Trilling, **Satesh** Gangarapu, **Ronald** de Bruin, **Willem** van Heugten, **Sourav** Bhattacharjee, **Nagesh** Wagdare, **Ton** Marcelis, **Aleida** Ruisch, **Umesh** Chinnaswamy Panchatcharam, **Loes** Ruizendaal, **Jaime** Garcia Hartjes, **Jos Paulusse**, **Carel** Weijers, **Saurabh** Srivastava, **Tin** Doan, **Maurice** Franssen, **Wouter** Biesta, **Erik** van Rozendaal, **Mabel** Caipa Campos, **Anne-Marie** Franssen, **Kim** van Gessel, **Maarten** Posthumus, **Michel** Nielen, **Cees** van Rijn, **Bart** Rijksen, **Dani** Lucas-Barbosa, **Tom** Wennekes, **Willem** Haasnoot, **Suzanne** Jansze, **Aart** van Amerongen, **Truus** Posthuma-Trumpie, **Peter** Steunenberg, **Esther** van Andel, **Quirijn** van Dierendonck, **Luc** Scheres, **Yao** Shen, **Aline** Debrassi, **Michiel** Uiterweerd, **Bas** van den Berg, **Esther** Roeven, **Aljosha** Filippov, **Geert** Noordzij, **Wilco** Duvivier, **Sweccha** Joshi, **Martine** Tiddens, **Jorin** Hoogenboom, Jose Maria (**Txema**) Alonso Carnicero, **Rickdeb** Sen, **Tjerk** Sminia, **Christie** Nguyen, **Zhanhua** Wang, **Carole** Messerli, **Hanna** de Jong, **Jelmer** van der Rijst, **Iris** van Marwijk, **Bram** Bielen, **Maarten** Smulders, **Sjoerd** Slagman, **Puspita** Wardani, **Farida** Kurniawati, **Medea** Kosian, **Pepijn** Geutjes, **Adriaan** van der Meer, **Sven** Krabbenborg, **Digvijay** Gahtory, **Laura** Schijven, **Judith** Firet, **Fred** van Geenen, **Fátima** García Melo, **Jorge** Escorihuela Fuentes, **Linda** Kaster and **Stefanie** Lange (in approximate order of appearance in the story behind this thesis), you have all made contributions to my PhD project, ranging from answering simple questions to getting into the core of my work. Moreover, I really enjoyed getting to know you and sharing nice moments with you. Each one of you, thank you!

In particular, I would like to highlight some important contributions to my PhD project, inside and outside the work described in this thesis. **Han**, you believed in me from the start to the end. You encouraged me with your positive attitude, supported and enriched my ideas. Thank you! **Teris**, we sat together for many hours, having in-depth discussions on our work, and you helped me with patience and eye for detail. Thank you! **Aart**, **Truus** and

Laura, thank you for your commitment to our antibody-related projects. For reasons that were out of our hands, the obtained results were not satisfying, but many of these results provided insights that made chapter 2 possible. **Txema**, **Jorge**, **Laura**, **Tjerk** and **Bas**, thank you for revising my manuscript for chapter 2. **Suzanne**, **Martine**, **Hanna** and **Iris**, thank you for your hard work on the synthesis of boronic acid-containing linkers for antibody immobilization. Together we learned how to handle these difficult boronic acids, giving rise to chapters 3 and 4. **Tom**, thank you for your help and suggestions with synthetic issues and your original ideas. **Puspita**, thank you for your devotion to our HPLC project, almost one year long. Your family was at the other side of the world, you went through tough times, but your hard work made chapter 5 possible. **Alexandre**, thank you for all our meaningful conversations. From my very first day at ORC, I have learned from you in many aspects, such as theoretical and practical analytical chemistry, time management, and especially looking at the positive side of things! **Radostina**, thank you for answering my countless questions, especially when I had just started my PhD. **Jaime**, thank you for the extensive answers to my questions related to finishing my PhD. **Elly** and **Aleida**, thank you for your help with the administrative tasks. **Elbert**, thank you for your multidisciplinary technical help, from urgent product order to special tubing and from software installation to broken freezer. **Barend** and **Pepijn**, thank you for your help with NMR, UV-vis and fluorescence spectroscopy. **Frank** and **Wilco**, thank you for your help with mass spectrometric issues. **Luc**, thank you for your practical help with surface modifications. **Sidhu**, thank you for analyzing many of my samples by XPS. **Bas**, you helped me with thiol-ene chemistry, computer issues and microparticles, just to name a few things. Also, you accepted to be my paranymph although my defense is just a few weeks before the end of your contract at ORC. Thank you! **Ai**, you helped me with questions about antibodies, other proteins, silane versus alkene chemistry, slide holders and other things, and you accepted to be my paranymph, although you are a busy mom... thank you!

My Dutch and French families, thank you for your support, and especially **Martijn**, my true love, thank you for your patience!

About the author's chemical pathway

Florine Duval was born on 10th December 1985 in Toulouse, France. She grew up happily in a quiet village with her parents and brother. When she was 10, she knew she wanted to become a scientist. At the age of 15, she knew she wanted to become a chemist and took a scientific direction at school. Two years later, organic chemistry already was her subject of choice. After obtaining a scientific high-school diploma with honors in 2003, she went for hands-on experience and followed a two-year practical training in chemistry in Université Paul Sabatier (Castres, France). This training included an internship in Université de Montréal (Montréal, Canada), where she took her first steps in organic synthesis outside practical courses. She obtained her "Diplôme Universitaire de Technologie" (DUT) in 2005. This would have enabled her to work as a technician, but she wanted to learn more theory, entered the "Ecole Nationale Supérieure de Chimie de Montpellier" (ENSCM, Montpellier, France) and followed a three-year intensive training for future executives in chemistry. Between the second and third years, she carried out an internship at Pfizer (Sandwich, UK) for one year, where she synthesized medicinal targets and investigated regioselective O-alkylations of indazolinone. The third year of training (fifth academic year) included a six-month project at Mercachem (Nijmegen, The Netherlands), where she synthesized molecules for a client in pharmaceutical research. After obtaining her "Diplôme d'Ingénieur Chimiste" and MSc in biomolecular chemistry in 2009, she continued working at Mercachem for one year, as a "Senior Research Chemist". In 2010, she started her PhD under the supervision of Teris van Beek and Han Zuilhof, in the Laboratory of Organic Chemistry, Wageningen University. The successful results are presented in this thesis.

Publications

A. Randall and F. Duval, *Regioselective O-alkylations of indazolinone using (cyanomethylene) triphenylphosphorane*, Synlett, 2009, **16**, 2673-2675.

F. Duval, T.A. van Beek and H. Zuilhof, *Sensitive thin-layer chromatography detection of boronic acids using alizarin*, Synlett, 2012, **23**, 1751-1754.

F. Duval, T.A. van Beek and H. Zuilhof, *Key steps towards the oriented immobilization of antibodies using boronic acids*, Analyst, 2015, DOI: 10.1039/C5AN00589B.

F. Duval, P.A. Wardani, H. Zuilhof and T.A. van Beek, *Selective on-line detection of boronic acids and derivatives in high-performance liquid chromatography eluates by post-column reaction with alizarin*, Journal of Chromatography A, revision submitted.

Overview of completed training activities

Name of the activity	Graduate school/institute	Year
Discipline-specific activities		
Advanced Organic Chemistry 1&2	VLAG	2010-2014
NWO analytical chemistry conference, Lunteren (2×) (poster)		2010-2012
NWO organic chemistry conference, Lunteren (3×) (poster)		2010-2013
Lab-on-a-Chip European Congress, Hamburg, Germany (poster)		2011
NWO CHAINS conference, Maarssen		2011
NanoSensEU symposium on Biosensor Development, Hasselt, Belgium		2012
KNCV organic chemistry conference, Wageningen		2013
General courses		
VLAG PhD week	VLAG	2011
Advanced course guide to scientific artwork	WUR Library	2011
Techniques for writing and presenting a scientific paper	Language Centre	2012
Career perspectives	WGS	2013
ACS on Campus event, Utrecht		2013
Effective behaviour in your professional surroundings	WGS	2014
Mobilizing your network in 2.5 hours	Young AFSG	2014
Optionals		
Preparation of PhD proposal		2010
Colloquia	ORC	2010-2014
Group meetings	ORC	2010-2014
PhD trips: UK in 2011, Germany and Switzerland in 2013	ORC	2011-2013

The research presented in this thesis was financially supported by Pieken in de Delta Oost-Nederland.

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Design and layout by Florine Duval

Printed by GVO drukkers & vormgevers B.V. | Ponsen & Looijen, Ede, The Netherlands