High-Performance Thin-Layer Chromatography of Wood Derived Sugars:

Method Development and Validation

Masterarbeit

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Kurzfassung

Der Einsatz von Bioraffinerien zur Gewinnung von biobasierten Chemikalien gewinnt zunehmend an Bedeutung. Um die chemischen und technischen Prozesse einer Lignocellulosen-Bioraffinerie optimal steuern zu können, bedarf es einer schnellen und zuverlässigen Methode zur Analyse von Zuckergemischen aus industriellen Prozessen. In dieser Arbeit wird die Hochleistungsdünnschichtchromatographie (HPTLC) herangezogen, um Kohlenhydrate in komplexen Gemischen aus hydrolysierter Lignocellulose zu analysieren.

Die entwickelte Methode bietet die Möglichkeit, Monosaccharide schnell, präzise und genau zu untersuchen. Diese wurde sorgfältig entwickelt und erlaubt eine vollständige Trennung der Zucker Glucose, Galactose, Arabinose, Mannose, Xylose und Cellobiose. Die Validierung erfolgte hinsichtlich Selektivität, Genauigkeit, Präzision, Robustheit, Nachweis- und Bestimmungsgrenze. In einem weiteren Schritt wurden Industrieproben einer qualitativen und quantitativen Analyse unterzogen. Darüber hinaus wurde das Verfahren der Säurehydrolyse von Lignocellulose optimiert, da aktuelle Standardmethoden keinen vollständigen Abbau der Lignocellulose in die monomeren Bestandteile erlauben oder eine zu hohe Salzfracht die Anwendung auf der HPTLC unmöglich machen.

Diese Arbeit zeigt, dass Hochleistungsdünnschichtchromatographie nicht nur eine Ergänzung zu anderen chromatographischen Methoden ist, sondern vielmehr eine zuverlässige, funktionierende und eigenständige Methode darstellt, um Monosaccharide qualitativ und quantitativ zu bestimmen.

Schlagworte: *Säurehydrolyse, Zellstoff, Papier, Cellulose, Bioraffinerie, HPTLC, Kohlenhydrate, Monosaccharide, Quantifizierung, Imprägnierung*

Abstract

Great efforts are put in the replacement of petrochemicals with bio-based ones gained in biorefineries. For an optimal process control, lignocellulosic biorefinery concepts require fast and accurate analytical methods for mixtures of carbohydrates derived from industrial processes. In this work high-performance thin-layer chromatography (HPTLC) was tested as an alternative system to analyse carbohydrates in more complex mixtures of hydrolysed lignocelluloses.

The method presented in this work provides a useful tool of analysing carbohydrates in a rapid, precise and accurate way. It was carefully developed allowing a complete separation of glucose, galactose, mannose, arabinose, xylose and cellobiose. The method was validated regarding selectivity, accuracy, precision, robustness, limit of detection and limit of quantification. In a third step, industrial samples were applied showing a reliable qualitative and quantitative analysis. The procedure of acid hydrolysis was modified to achieve a complete degradation of lignocellulose, as current standard methods applicable to HPTLC do not fully convert oligosaccharides into its monomeric compounds.

This work showed that high-performance thin-layer chromatography does not only complement other chromatographic methods, but is a reliable and fully functional analytical stand-alone method of detecting carbohydrates both in a qualitative and quantitative way.

Keywords: *acid hydrolysis, pulp, paper, cellulose, biorefinery, HPTLC, carbohydrates, monosaccharides, quantification, impregnation*

Contents

1 Introduction

1.1 The concept of a lignocellulosic biorefinery

The following chapters shall give an overview on the importance of carbohydrates for the concept of lignocellulosic biorefineries and the possibilities for their analysis. Finally, the reader shall get an idea, why there might be a need for an improved chromatographic method for the qualitative and quantitative analysis of sugars.

Since the industrial revolution, the global economy is mainly driven by non-renewable resources. Despite big efforts in green technologies, most of the global energy consumption as well as the feedstock for the chemical industry is based on crude oil, natural gas and coal. Big parts of our life rest upon petrochemicals. No matter if we talk about the clothes we wear, the groceries we carry home in plastic bags, lots of medications we are using, the fertilizers on our fields or composite materials in cars, it is impossible to imagine our life without petrochemicals. However, crude oil is a finite resource, therefore it is achievable to replace this feedstock by renewable ones. The facilities, where biomass derived chemicals are gained, are known as biorefineries.

According to the IEA Bioenergy [1, p. 5], a "*biorefinery is the sustainable processing of biomass into a spectrum of marketable products and energy*". The flow scheme is similar to a crude oil refinery, where one starting material is used to produce multiple products such as, chemicals, fuels, heat or electricity using biomass as feedstock [2]. The products might be intermediates or final ones, used in the food, feed or chemical industry. As the definition is quite comprehensive, many established industries, such as sugar and starch factories or paper mills can be considered as a biorefinery [1]. These facilities are examples of the First Generation of biorefineries, where one resource is used to produce only one product. In the Second Generation, several products are gained from one feedstock resource and in the Third Generation of biorefineries, several feedstock resources are used to produce several products [3].

Cherubini and his co-workers are describing several types of biorefineries, classified either by their feedstock, the final products, the processes used or by the platform chemicals that are produced. Some examples for the first one are the green biorefinery that is using wet biomass like grass, the whole crop biorefinery using corn or wheat or

the marine biorefinery, where the feedstock is algae. Finally, there is the lignocellulosic biorefinery [4]. The concept was often criticized for using food crops as a feedstock, which is not acceptable from an ethical point of view. When it comes to whole crop biorefinery, this accusation might be true to some extent. However, a lignocellulosic one is using resources and waste material from forestry or agricultural lignocellulosic waste. A competition with the food industry may derive only from changes in land use, e.g. plantations of wood fuel instead of crop production, but not from the feedstock itself.

The second variant that was descried by Cherubni and his co-workers [4] provides the production of platform chemicals. A platform is an intermediate product. It is gained from the feedstock and allows the production of several final products. According to Cherubni, some of the most common platforms are biogas, syngas, hydrogen, lignin, C5 and C6 sugars. C5 sugars are composed of 5 carbons and therefore known as pentoses. Typical pentoses are xylose or arabinose. These sugars are gained by hydrolysing hemicelluloses. C6 sugars are called hexoses, as they are consisting of 6 carbons. Glucose, galactose and mannose are three representatives of this group. By hydrolysing sucrose, starch, cellulose and hemicellulose, hexoses are gained as platform chemicals [4]. In this thesis, the concept of a lignocellulosic biorefinery is considered. Therefore, the feedstock for the platforms of pentoses and hexoses is lignocellulosic biomass, including wood.

Fujita and Harata described wood as a very complex material that consists of cellulose, hemicellulose, lignin and extractives. Cellulose with a percentage of about 40 – 45 % is framing the other substances. Cellulose consists of fibrils with parallel chain orientation in the native state. These structural hierarchy is responsible for the chemical and physical characteristic of wood. Hemicellulose is the substance closely associated with cellulose. Its percentage is about 10 to 35 %. The amount of lignin with 18 to 35 % is quite diverse as well. Lignin is also responsible for the strength of wood [5].

Cellulose is a linear polymer that consists of glucose or more specifically anhydroglucose (AGU). The D-glucopyranoses are connected with β-1,4-glycosidic bonds. According to Chen [6], the molecule consists of several hundreds or even

thousands of AGU units. Hemicellulose is much more diverse. The main chain of the molecule might be a homopolymer composed of only one type of monosaccharide or a heteropolymer that consists of different monosaccharides. Connected to the main chain, no matter if it is a homo- or heteropolymer, are different groups of monosaccharides. The most common components are xylose, mannose, galactose and arabinose. Finally, lignin is a more complex macromolecule. The composition depends on the type of plant, and lots of other factors such as stress situations are of importance. It is a nonlinear molecule that is mainly composed of polymerized and hence cross-linked coumaryl, coniferyl and sinapyl alcohol [6].

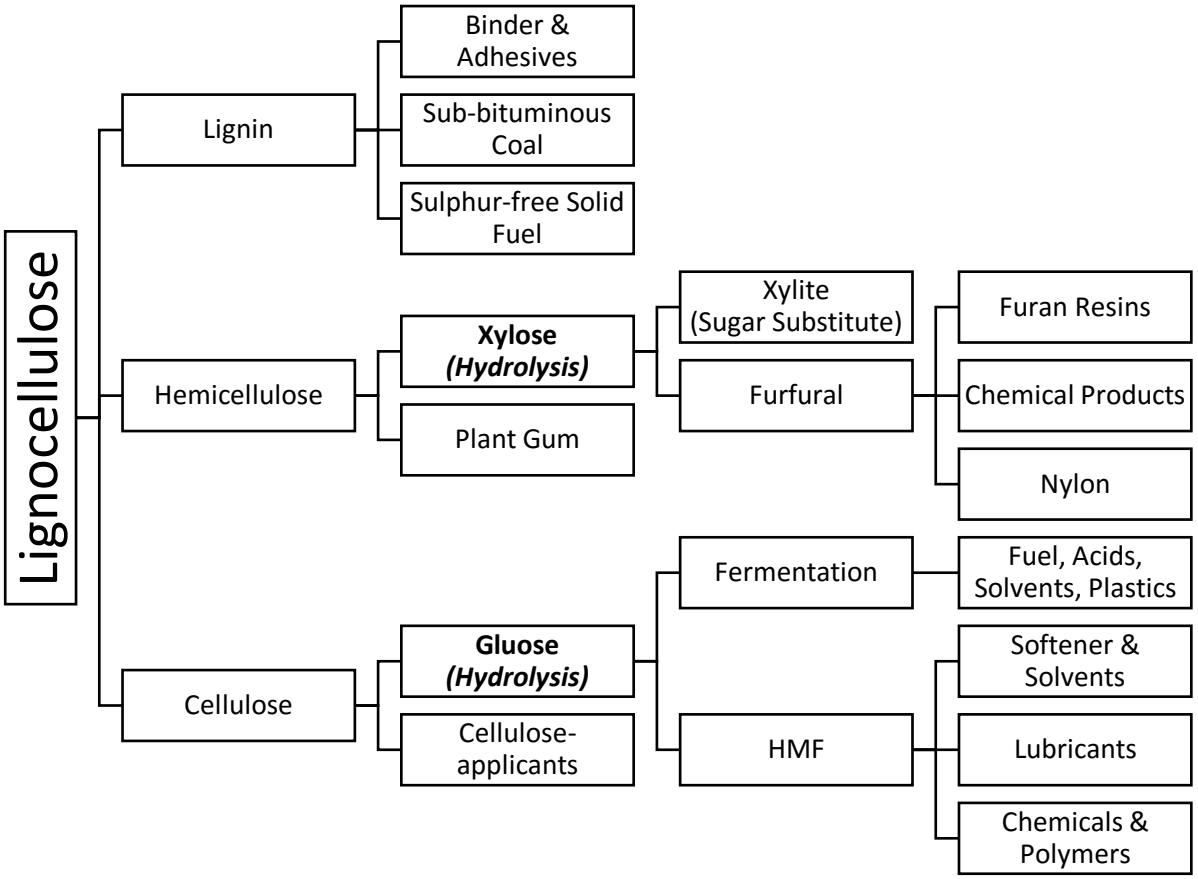

Figure 1: Products of lignocellulose in a lignocellulosic biorefinery concept; adapted from [7, p. 25]

Figure 1 is showing potential products obtained from lignocellulosic biorefinery. It shows that each component of lignocellulose provides its own portfolio of products.

If we imagine all the waste material derived from biomass, this is a huge feedstock worldwide. Zamani describes the potential purpose of use for lignocellulosic biomass in his work: Some products can be gained directly from cellulose and hemicellulose. The fibres might be used as paper, in composite materials, as textiles or in medical applications [8].

For other products cellulose and hemicellulose must be broke down into their building blocks first. The lignocellulose is pre-treated in order to remove the lignin and loosen the compact structure. Then, an enzymatic or chemical hydrolysis is performed to gain pentoses and hexoses. Fermentation processes allow a wide range of products. First of all, biofuels of the second generation can be produced using low cost waste material to replace petrol by ethanol, butanol, hydrogen or methane. Second, organic acids such as citric, lactic or gluconic acids are produced by microorganisms. Other promising products are furfural and HMF. Furfural is produced mainly from pentoses found in hemicelluloses. It is used for the production of different chemicals like furfuryl alcohol or solvents. Hydroxymethyl Furfural (HMF), derived from the C6 sugars, allows the production of fuels, solvents, lubricants and materials used in the pharmacy [8].

1.2 Total hydrolysis and modern analysis of carbohydrates with respect to lignocellulosic biomass

As described above, lignocellulosic biomass has to be converted into the building blocks in order to gain biofuels, organic acids and chemicals or to produce bioplastics. According to Fan and his co-workers, cellulose is degraded by adding a water molecule by the help of hydronium ions of sufficient activity, which is randomly splitting the β-1,4-glycosidic bonds of the polymer. This process is called hydrolysis. The reaction is intensified by using catalysts, mainly enzymes or acids as mentioned above. While the enzymatic hydrolysis is working very specifically producing no by-products, the acid hydrolysis has got short reaction times and needs pre-treatment procedures that are less expensive [9].

According to Wertz and co-workers, the enzymatic hydrolysis is the biological degradation of cellulose that occurs in nature as well. Microorganisms such as bacteria, fungi or protozoa produce enzymes, called cellulases that are catalysing the degradation process. In nature, this is part of the carbon cycle, where dead plant matter is decomposed into its polymeric and monomeric constituents. In this way, nutrients are made available. The enzymatic hydrolysis has enormous potential due to the possibility of very specific degradation as there are various enzymes known. Non-biological degradation processes are using acid, alkali, oxidants or even thermal and mechanical energy to break down cellulose. The acid hydrolysis is used predominantly due to the high yield gained in a short time. A distinction is made between the diluted and concentrated acid hydrolysis, which is considered in [10].

For analytical purposes, the total hydrolysis is of interest as well. All β-1,4-glycosidic bonds have to be hydrolysed to obtain a quantitative yield of monomeric compounds. At the same time, side reactions of the monomers have to be avoided. These opposing requirements are solved by using two-stage approaches of hydrolysis. The total hydrolysis usually is performed using 72% sulfuric acid in the first stage. In the following steps, the acid is diluted and the temperature raised. Introducing mechanical energy like stirring is reported to have a positive effect on the yield of monomers [11].

For industrial processes, the applied kind of hydrolysis depends on the desired end-products. The decomposition products of the hydrolysates differs a lot as there might be oligosaccharides, monosaccharides or derivatives thereof. Furthermore, the composition of the feedstock is not stable throughout a production period. To gain high quality products it is essential for further processing to have a clear idea of the structure of the respective batch. It is not only important to know the components, but also their quantity. For the separation, identification and quantification of carbohydrates, appropriate analytical methods are needed that are fast, reliable and easy to handle.

According to El Ziad, gas chromatography (GC) was one of the first chromatographic systems that allowed a reliable quantification of monosaccharides. After the development of highly efficient capillary columns and the combination with mass spectrometry (MS), it was possible to separate even complex mixtures of carbohydrates. El Ziad was reporting the capability of simultaneously identifying and

quantifying up to 30 substances including mono- and oligosaccharides. The disadvantage of GC is the need for volatile substances. Otherwise, the substance must be transformed into a volatile state in order to be applied on GC. Still this system is widely used in analytical chemistry [12].

In the 1970s, the high-performance liquid chromatography (HPLC) was spreading very fast. Soon it was becoming the most widely used method to analyse sugars, while paper chromatography (PC) was more and more dwindling [13]. According to Fallon and co-workers, HPLC is providing a variety of different techniques specialized on different kind of substances, e.g. adsorption chromatography, ion-exchange chromatography or size exclusion chromatography. In most applications there is no need for derivatization as the compounds of interest can be analysed with an UV-detector. Only in some cases, i.e. low concentrations, a derivatization is needed in order to detect the substances. This circumstance was named by Fallon as one of the major advantages of HPLC over other techniques. However, when mono- and oligosaccharides are applied on HPLC, a derivatization is needed as well, as they do not contain any chromophores [14].

GC and HPLC are reliable techniques to identify and quantify even complex mixtures of carbohydrates. Both systems are predominantly used today and there is a lot of effort to still improve these methods. Still they have one crucial disadvantage: Samples derived from industrial processes with a complex matrix and impurities may cause problems when applied on GC or HPLC. A solution to this problem might be found in the technique of the time-honoured paper chromatography (PC). Already decades ago it was fully replaced by thin-layer chromatography (TLC), which is using silica gel, aluminium oxide or cellulose as stationary phase instead of chromatography paper. Even though TLC became a niche method, it was further developed, evolving into high-performance thin-layer chromatography (HPTLC). Some steps were fully automated and the modern chromatographic plates enhanced the sensitivity and reliability of the method.

1.3 High-Performance Thin-Layer Chromatography

To get a profound idea of HPTLC, the work of Srivastava [15] is a valuable source of information. The basic principle of separating substances is the same for HPTLC as for TLC. A sample is applied on a stationary phase and transported by a mobile phase. If the interaction between stationary and mobile phase is ideal, the sample will be separated into different spots or bands. Thin-layer chromatography is a manual technique. According to Srivastava, it is difficult to control the size of the application volume with TLC and to perform a quantitative analysis is very difficult. Whereas HPTLC is an instrumental technique, providing an automation and offering scanning and more selective detection. This allows a reliable quantification of the investigated substances. The introduction of HPTLC-plates in the 1970's was a big milestone in the implementation of this method. The particle size of silica gel got smaller, which was improving the precision and reducing the running time [15].

Srivastava was outlining the advantages of HPTLC compared to other chromatographic systems: low costs, minimal sample preparation, possibility to analyse substances with poor detection or different nature and the possibility to combine HPTLC with other analytical methods, such as mass spectrometry [15].

Morlock and Sabir were comparing the quantification of sugars in food on HPTLC and HPLC. It was found out that the applied specific method of HPTLC allows a cost-effective analysis. For HPLC costs are approximately € 1.74 per sample, but it is only € 0.37 per sample when HPTLC is used. According to these results, HPLC was 5 times more expensive than high-performance thin-layer chromatography. Even the time of analysis per sample was showing a factor 5 between the two chromatographic techniques. One sample took 18 minutes to analyse using HPLC, but only 3.8 minutes using HPTLC [16].

When it comes to process control in industrial facilities, one of the most important advantages of HPTLC is the possibility to apply samples with impurities and a high matrix. Basically, there is no need for sample preparation, as the chromatographic plates are eventually discarded. In HPLC, impurities could clog the columns causing high costs. Furthermore, it is possible to apply samples containing substances of very

different nature simultaneously. Different derivatization techniques and a variety of derivatization reagents allow to visualize these substances. The operator is getting a fingerprint of a sample, providing important information for any further analysis.

Recapitulating, we can say that high-performance thin-layer chromatography is a simple and fast method with low running costs and providing a high sensitivity. It is flexible to impurities, complex matrices and high salt contents. Finally, it gives you a fingerprint of a sample with an unknown composition. These properties might be advantageous for any future development in the concept of a biorefinery.

1.4 Historical review of analysing carbohydrates on planar chromatography

As carbohydrates are a major substance class, their analysis plays an important role in various fields. Therefore, there are a lot of methods to determine the presence of mono- and disaccharides, such as refractometry or colorimetry. However, these methods cannot determine individual sugars, which was first possible with paper chromatography [17]. This chapter shall give an idea about the research achievements of planar chromatography between the first attempts of analysing sugars in a qualitative way towards the quantification of individual carbohydrates using high-performance thin-layer chromatography today.

In 1945, the U.S. Department of Agriculture publishing the paper “Analysis of Wood Sugars” stating that big progress was achieved in the saccharification of wood, but that there is need for an appropriate way of analysing the sugars and alcohols gained during this process. During this project, not only a single eluent was found to separate a complex mixture of sugars because of the high diversity of carbohydrates. It was only possible to separate them in groups with similar characteristics [18].

Lew and co-workers have been more successful. They were establishing a chromatographic method in 1946 to separate a mixture of D-glucose and sorbitol. The spots of these sugars were even quantitatively analysed. It was possible as well to separate D-mannitol from dulcitol and sucrose from raffinose [19].

In 1960, Stahl and Kaltenbach were using TLC to separate amino acids successfully and first steps in separating hydrophilic sugars were done. Their tests might be seen as a major step in TLC-analysis of carbohydrates, as it was possible for the first time to separate glucose and galactose. For the tests, the chromatographic plates were prepared manually, even though the first prefabricated plates were already available on the market [20].

Adachi was trying to quantify sugars in milk, primarily tagatose, lactulose and sucrose in 1964. The author stated that none of the existing methods for TLC was able to separate these sugars. A new method was investigated by applying sodium bisulphite on the chromatographic plates by mixing it with silica gel. The tests were showing a separation not only of the sugars mentioned above, but also of ribose, xylose, glucose and galactose [21].

In 1967, Lombard and his co-workers reported the use of a silica gel impregnated with a phosphate buffer giving satisfying results for the separation of D-ribose, D-xylose, D-arabinose and D-glucose [22].

One year later, Lato and co-workers reported a dwindling acceptance of TLC methods for analysing sugars [23]. Thinking about today's requirements, the selectivity at that time was not satisfactory, a detection of sugars often was based on the colour of the spots only. This might be an explanation, why other chromatographic methods such as GC and HPLC started to dominate this research field. Still Lato and co-workers were investigating in a major research project for the separation of eleven different carbohydrates on silica gel [24].

In 1975, a successful separation and quantification of mannose, glucose and galactose on a standard TLC plate was reported by Pruden and co-worker [25]. Ghebregzabher et al. improved the separation of sugars in their experiments by using phenylboronic and boric acid-containing eluents. With this method, it was possible to use non-impregnated layers to separate complex solutions of sugars [26].

In the 1980s, Puri and Anand reported, that HPLC became the most promising technique as it was superseding GC in analysing samples from the pulp and paper

industry. Thin-layer chromatography was seen as time consuming and not reliable enough, as the sensitivity was not satisfying at all [27]. Still there were some new developments. In 1983 the thermal-UV method for the detection of carbohydrates in TLC was reported by Alperin. Different temperatures allowed to distinguish mono- and disaccharides [28].

In 1992, Batisse and co-workers reported a method for the quantification of monosaccharides including galactose, glucose, mannose, arabinose, xylose, rhamnose and sugar acids, such as galacturonic and glucuronic acid. Plastic sheets precoated with silica gel were used as stationary phase, impregnated with a phosphate buffer (0.2 M, pH=6.8). The three developments were performed with acetonitrile, 1-pentanol, water (60:20:20, v/v/v) and N-(1-naphtyl)ethylenediamine dihydrochloride was used as derivatization reagent. The results yields a successful separation and quantification of the sugars and the acidic sugars simultaneously, which is not possible with GC in one run [29].

In 1998, Han and Robyt reported a method for the separation of alditols from their parental aldoses (xylose, ribose, arabinose, mannose, glucose, galactose and maltose). A silica gel TLC plate was used as stationary phase. Two developments were performed with acetonitrile, ethyl acetate, 1-propanol, water (85:20:20:15, v/v/v/v). A solution of silver nitrate and acetone served as derivatization reagent. The separation of alditols from their parental aldoses was successful [30].

In the 1990's, thin-layer chromatography was attracting attention as an alternative to other analytical methods, as the separation got more accurate. HPTLC allowed an automated procedure of applying and developing samples and better derivatization techniques led to better quantification results.

Uremovic and co-workers were comparing the quantification of lignocellulosic sugars (glucose, galactose, mannose, arabinose and xylose) on three chromatographic methods: borate complex anion exchange chromatography, anion exchange chromatography in NaOH medium and high-performance thin-layer chromatography. Precoated HPTLC-plates were used impregnated with 0.2 M sodiumdihydrogen-ethanol (1:1). Three developments were performed with ethyl acetate, pyridine, water

(8:2:1). HPTLC was showing the worst results for reproducibility, but was superior in separating the sugars, while the other methods were showing problems to separate e.g. mannose and xylose. [31].

Morlock and Sabir were comparing HPTLC and HPLC for the quantification of the sugars fructose, glucose, galactose, maltose-1-hydrate, mannose, lactose-1-hydrat and sucrose. They were using HPTLC silica gel plates, developed with n-butanol, i-propanol, acetic acid, boric acid (6:14:1:3, v/v/v/v). The derivatization was performed with p-aminobenzoic acid. Morlock was reporting, that HPTLC is a reliable method with mean difference of only 1.5 % compared to HPLC [16].

This thesis is a contribution to previous investigations in developing a chromatographic method that allows a qualitative and quantitative analysis of lignocellulosic sugars.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

All chemicals used can be found in table 1:

Table 1: Chemicals used including the formula, purity, CAS-number and supplier

Substance	Formula	Purity	CAS	Supplier*
• Acetic acid glacial	$C_2H_4O_2$	100 %	64-19-7	Merck
• Acetonitrile	C_2H_3N	≥ 99.9 %	26809-02-9	Merck
• Aniline	C_6H_7N	≥ 99.5 %	62-53-3	Sigma-Aldrich
• L-(+)-Arabinose	$C_5H_{10}O_5$		5328-37-0	VWP Internat.
• Barium hydroxide octahydrate	$Ba(OH)_2$ * 8 H_20	≥ 98 %	12230-71-6	Merck
• 1-Butanol	$C_4H_{10}O$	-	71-36-3	Merck
• D-(+)-Cellobiose	$C_{28}H_{38}O_{19}$	≥ 96.0 %	5346-90-7	Sigma-Aldrich
• Diphenylamine	$C_{12}H_{11}N$	99 %	122-39-4	Sigma-Aldrich
• Disodium hydrogen phosphate	HNa_2O_4P	≥ 99.0 %	7558-79-4	Sigma-Aldrich
• D-(+)-Galactose	$C_6H_{12}O_6$	98 %	59-23-4	Alfa Aesar
• D-(+)-Glucose	$C_6H_{12}O_6$		50-99-7	Sigma-Aldrich
• D-(+)-Mannose	$C_6H_{12}O_6$	≥ 99 %	3458-28-4	Sigma-Aldrich
• Methanol for HPLC	CH_4OH	100 %	67-56-1	VWP Internat.
• N-(1-Naphtyl)-ethylendiamine	$C_{12}H_{14}N_2$ * OHCl	-	1465-25-4	Merck
• Ninhydrin	$C_9H_6O_4$	-	485-47-2	Merck
• Ortho-phosphoric acid 85 %	H_3O_4P	85 %	7664-38-2	Carl Roth
• 1-Pentanol	$C_5H_{12}O$	98 %	71-41-0	Carl Roth
• Sodium dihydrogen phosphate	H_2NaO_4P * H_20	≥ 99.0 %	10049-21-5	Sigma-Aldrich
• Sulfuric acid	H_2SO_4	95 - 97 %	7664-93-9	Merck
• Water for HPTLC	H_20	-	7732-18-6	Sigma-Aldrich
• D-(+)-Xylose	$C_5H_{10}O_5$	> 99 %	58-86-6	Carl Roth

*Supplier:
Merck KGaA, D-64271 Darmstadt
Carl Roth GmbH + Co KG, Schoemperlenstraße 3-5, D-76185 Karlsruhe
Sigma-Aldrich Chemie GmbH, Riedstraße 2, D-89555 Steinheim
VWP International S.A.S., 201 Rue Carnot, F-94126 Fontenay-Sous-Bois
Alfa Aesar GmbH & Co KG, Zeppelinstraße 7b, D-76185 Karlsruhe

2.1.2 Samples and standards

For the development and validation of the HPTLC-method, sample solutions were obtained by dissolving cellobiose, galactose, glucose, mannose, arabinose and xylose in water.

The method was tested with samples derived from industrial processes. Whether the concentration nor the composition was known. The samples had the following labels: A, B, C, D, E, F, G, H and I.

To gain lignocellulosic hydrolysates, Cotton Linters (CL), Beech dissolving Pulp (BKZ), Thermomechanical Pulp (TMP), Paper Rag Modern (Pap) and Rag Paper (Rag) as well as Xylane (Xyl) were used as basic raw material.

2.1.3 Plates

In this study, two different layers were tested. A Silica gel 60 F_{254} AMD extra thin (hereinafter called AMD plate) from Merck Millipore with a size of 20 x 10 cm and a layer thickness of 100 µm was used. The second plate was a LiChrospher® HPTLC Silica gel 60 RP-18 $WF_{254}s$ plate (Merck Millipore) with a size of 20 x 10 cm and a layer thickness of approximately 180 µm.

2.1.4 Equipment and accessories for HPTLC

Sample application was performed with an Automatic TLC Sampler 4 (ATS 4) from CAMAG (Muttenz, Switzerland). For the developing process, the Automatic Developing Chamber 2 (ADC 2) was used (CAMAG). The chromatographic layers were developed in Twin Trough Chambers from CAMAG. These chambers were used for prewashing and impregnation as well. The layers were dried on the TLC heating plate Ceran 500 from LHG (Karlsruhe, Germany). For the evaluation, the TLC Visualizer (CAMAG) was used for taking pictures of the plate. The detection was performed with the TLC Scanner 3 (CAMAG). Two software systems were used, visionCATS and winCATS from CAMAG. During the acid hydrolysis, heating/stirring modules were used (Pierce Reacti Therm III).

2.2 Methods

2.2.1 Sample and Standard Preparation

A stock solution was prepared containing 1 mg ml^{-1} of each sugar dissolved in water. Standard mixtures were obtained by diluting the stock solution. For the calibration, the following concentrations were used [ng μl^{-1}]: 50, 100, 400, 700 and 1000.

For the development and validation of the HPTLC-method, sample solutions were prepared by dissolving cellobiose, galactose, gluclose, mannose, arabinose and xylose in water. For these tests, a concentration of 1 mg ml^{-1} water was used.

As the developed HPTLC method was aimed at analysing pulp and paper hydrolysates, a total hydrolysis of different pulp and paper was performed. All pulp and paper samples were cut manually into pieces of approximately 1 mm^2. Xylan (Xyl) was obtained as a powder. The procedure of hydrolysis is explained below.

The practical test was performed with biorefinery derived samples. Except sample “I”, which appeared as a powder, all samples were liquid, partly viscous. 200 mg of “I” were filled up to 50 ml with water. It was stirred with a magnetic stirrer at room temperature for 30 minutes and 800 rpm. Finally all samples were centrifuged (2.700 rpm, 15 minutes) and the supernatant was used for analysis. Some of the samples were diluted up to 1:400 with water due to very high loads of carbohydrates. To dilute the samples into the working range, an iterative approach was chosen.

2.2.2 Mobile Phase and modification of commercial plates

Preparation of the Mobile Phase

The used mobile phases were trinary mixtures. Solvent A was composed of acetonitrile, water, pentanol in a ratio of 4:1:1 (v/v/v). Solvent B contained acetonitrile, water, butanol in the same ratio. The required volume of each solvent was measured separately with a pipet or a graduated cylinder and mixed in a flask.

Modification of the Stationary Phase

Silica gel 60 was used as stationary phase (Merck Millipore, HPTLC AMD F_{254}, extra thin). The layers were labelled with a soft pencil for the identification of different impregnations and batches. To prevent any contamination, the plates were handled with disposable gloves. Prior to chromatography, each plate was checked for damages under 254 nm light.

Plate washing was performed according to Maxwell [32]: Two tanks were filled up with a solution of methanol and water in a ratio of 6:1 (v/v). While the second tank, labelled number 2, was filled up with a fresh solution, the first tank, labelled number 1, was filled up with a reused solution from tank 2. The content of each tank was used for 25 plates, before it was replaced. The plates were dipped into tank 1 for 5 minutes. The plates were removed, the backside was wiped with a tissue and the plates were air dried in a horizontal position for 15 min. The air-dried plates were then immersed in tank 2 for 1 minute. After removing and 15 minutes of air drying, the plates were heated in an oven to 105°C for 20 minutes.

Plates were impregnated with a sodium dihydrogen phosphate / disodium hydrogen phosphate buffer (pH 6.8, 0.2M). To prepare the buffer, 39.01 g of NaH_2PO_4 respectively 35.5 g of HNa_2PO_4 were dissolved each in 500 ml of water. Then a volume of 92.52 ml of sodium dihydrogen phosphate was mixed with 107.5 ml of disodium hydrogen phosphate and filled up with water to 500 ml. The pH was checked and corrected if necessary. The plates were dipped into the phosphate buffer for 3 minutes. After 20 minutes of air-drying in an upright position, the plates were put on the TLC heating plate for 10 minutes at 120 °C. After prewashing and impregnating, the plates were stored in a desiccator over mole sieve under exclusion of light.

2.2.3 HPTLC methods

This paragraph provides an overview on the methods developed and optimized. Figure 2 is giving an overview of the different chromatographic steps, which will be explained in detail below.

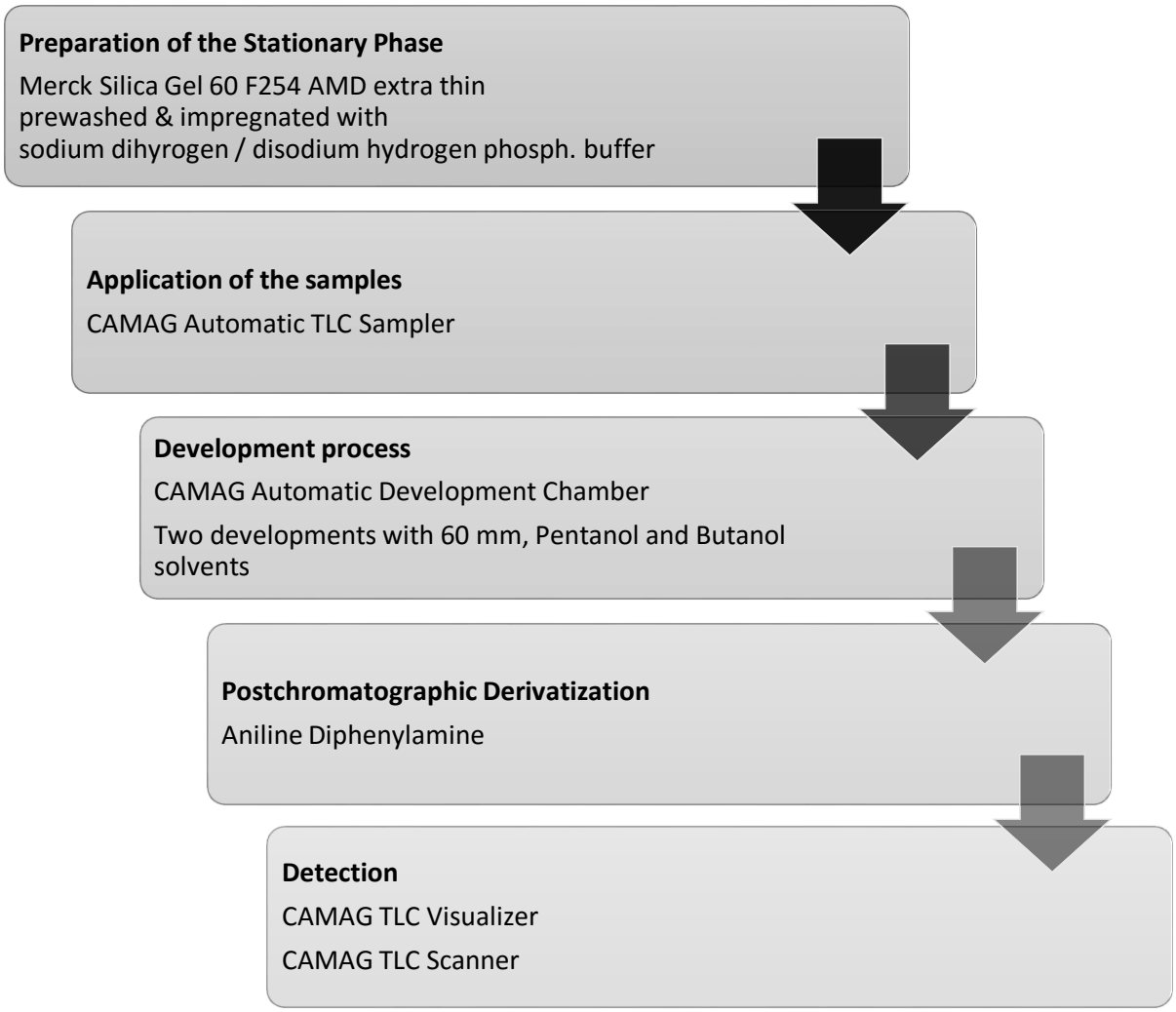

Figure 2: Method Overview

Sample application was performed with an Automatic TLC Sampler 4 (ATS 4, CAMAG). The bands were 8 mm long and had a distance of 12 mm from the bottom of the plate. The filling quality was set to Quantification and, hence, there were two rinsing cycles. The filling speed was set to 8 µl / s. The dosage as well as the retraction volume was set to 200 nl with a dosage speed of 50 nl / s. Nozzle heating was activated (40 °C). All samples were applied with a volume of 1 µl, hence, all samples and standards were diluted in the requested concentration before.

For the developing process, an Automatic Developing Chamber 2 was used (ADC2, CAMAG). The standard method was performed with two developments, both with a solvent front of 60 mm. There was no saturation used. There was no activation applied, hence, the relative humidity depends on the surrounding air and reached in our case approximately 30 %.

Before the development was started, 10 ml of the solvent were filled into the solvent funnel of the ADC2. After the process started, the solvent was flowing from the funnel into the TTC (Twin Trough Chamber). The chromatographic plate, which was placed in the holdfast, was fully automated immersed in the solvent, while the solvent front was measured and stopped at 60 mm by removing the plate. After drying the plate, the second development was started.

Development 1 was performed with a solution of acetonitrile, water, pentanol in a ratio of 4:1:1 (v/v/v) with a following drying time of 15 minutes. After cleaning the glass chamber, the second development was performed without pre-drying and a solution of acetonitrile, water, butanol in the same ratio. After a drying time of 15 minutes the developments were accomplished.

2.2.4 Derivatization methods

For the derivatization, two reagents were tested, a *N*-(1-Naphthyl)ethlyenediamine reagent and aniline diphenylamine phosphoric acid.

The *N*-(1-Naphthyl)ethlyenediamine reagent was a 6.5 mM solution. First, 6 ml of sulfuric acid were filled up with methanol to 200 ml. Then, 337 mg of *N*-(1-Naphthyl)ethlyenediamine were dissolved in this solution.

To prepare the aniline diphenylamine reagent, 5 ml of aniline were filled up to 250 ml with acetone and 5 g of diphenylamine were filled up to 250 ml with acetone and stirred. Then, 70 ml of each solution were mixed, before 10 ml of ortho-phosphoric acid was added. The derivatization reagent was stored in dark at a temperature of 4 °C.

After the development process, the plate was immersed in the solution of the derivatization reagent for 5 seconds. It was important to immerse the plate in an even way, a contact with the glass chamber was avoided. The layer then was air-dried for 15 minutes and the TLC heating plate (Ceran 500, LHG) was meanwhile heated up to 120 °C.

2.2.5 Data evaluation

First, the TLC Visualizer (CAMAG) was used for taking pictures of the plates. The pictures were taken with white light, UV 254 nm and UV 366 nm. Then, the TLC Scanner 3 (CAMAG) was used. The scanner was set to absorption. For the standard method, the lamp deuterium & tungsten was used with a wavelength of 354 nm.

The chromatograms were visually inspected and the R_f-values marked. The values for height and area for the known standard peaks were taken from winCATS after scanning. With these values, the calibration function was drawn. As the R_f-values of the standards were known, the height and area were obtained for the investigated substances in the samples. By using the calibration equation, the recovery value was calculated considering the dilution factor.

The following parameters were investigated:

Accuracy and precision

By applying the pure samples, the pure standard solution and a mixture of both, the method was tested for accurate results. A defined amount of the sugar mix was added to the sample solution, hence, the approximate result was already known. The accuracy was calculated as shown in the following formula:

$$Accuracy\ [\%] = \frac{expected\ amount\ of\ sugar}{detected\ amount\ of\ sugar} * 100$$

The expected amount of sugar is the sum of sugar (e.g. glucose) found in the sample plus the amount of the same sugar found in the standard solution. The detected amount of sugar means the amount of sugar found in the mixed solution of a defined volume of sugar and standard solution. Hence, a percentage of 100 means that the expected amount of sugar equals the detected amount. The more the accuracy matches the 100 % level, the more accurate the method is.

Repeatability

To proof the repeatability, the same samples were applied on three chromatographic plates at the same day. All parameters were set equally, the same stock solutions were used. After detecting the signals for height and area, the calibration curve was drawn and the average amount of sugar was calculated for each sample. These results were compared by the absolute and relative standard deviations.

Limit of Detection and Limit of Quantification

The calculation of the limit of detection (LOD) and limit of quantification (LOQ) are based on the signal-to-noise ratio (S/N), where the signals of low concentrated analytes are compared with those of blank samples. This is giving the lowest amount of analyte that can be detected.

The standard solution containing 200 ng / µl of each sugar was applied 14 times and the blank sample containing only water was applied four times. To gain the Signal-to-Noise ratio, all the small peaks except the peaks of the sugars were marked manually. With these peaks, the standard deviation of each track was calculated, which is equal to the signal-to-noise ratio. Finally, the limit of detection is three times S/N and the limit of quantification ten times S/N.

$$LOD = 3 * S/N$$

$$LOQ = 10 * S/N$$

LOD… Limit of Detection
LOQ… Limit of Quantification
S/N… Signal to Noise ratio

2.2.6 Total hydrolysis of pulp and paper samples

All pulp and paper samples were cut manually into pieces of approximately 1 mm^2, only xylane (Xyl) was obtained as a powder. 10 mg of each substance was weighed in a 10 ml test tube with a screw cap. A teflon coated magnetic stir bar (6x3 mm) was

added. The vials were put on ice and 1.5 ml sulfuric acid (72 % w/w) were slowly added. The samples were stirred at room temperature for two hours using heating/stirring modules (200 rpm, Pierce Reacti Therm III). Then, 2 ml water (HPLC grade) was added and the samples were heated to 80 °C for 1 hour while being stirred again. Finally, the tubes were centrifuged at 2.000 rpm to collect all solid material at the bottom.

After the acid hydrolysis, the samples have to be neutralized to enable a proper separation on HPTLC. An aliquot of 150 µl was mixed with 310 µl of NaOH (4.0M) and a tiny amount of solid sodium hydrogen carbonate was added. Each 100 µl of this sample solutions were mixed with 400 µl water (1:5) before they were applied to the HPTLC plate.

3 Results and Discussion

The results are presented in four chapters. First, 3.1 gives an overview of the development of a new method developed with the aim to separate and quantify wood derived sugars. In 3.2, the validation of the method is described in detail. Testing of the new approach based on HPTLC with samples from industrial processes is described in 3.3. In chapter 3.4 the acid hydrolysis of pulp is optimized with respect to meet the requirements of HPTLC as a detection system for the sugars generated.

3.1 HPTLC method development for lignocellulosic carbohydrates

The following chapters describe the development of a new method for the separation and quantification of the wood derived sugars cellobiose, glucose, galactose, mannose, arabinose and xylose on high-performance thin-layer chromatography. As a first step, the influence of the stationary phase was investigated, different solvent combinations were tested and the impregnation was optimized. The development procedure was improved and the derivatization reagent with the best results was chosen.

3.1.1 Stationary Phases

The stationary phase or chromatographic layer is the core part of the chromatographic accessories. In every chromatographic system, no matter if it is a simple paper chromatography or a high-tech procedure such as HPTLC, the stationary phase is needed to separate the investigated substances. To optimize the results, an appropriate layer must be chosen and possibly impregnated according to the chemical properties of the samples.

Selection of the Stationary Phase

According to Rashmin and co-workers, commercially available pre-coated HPTLC plates with Silica gel 60 F_{254} on either glass or aluminium backing are mainly chosen, as silica gel can be easily used for all kinds of compounds [33]. Major influence

parameters for the practicableness of a chromatographic plate are the pore distribution, the quality of the plate's back panel and the silanol groups, furthermore the size of the particles and their distribution [34].

In this study, two different layers were tested. A silica gel 60 F_{254} AMD extra thin (hereinafter called AMD plate) from Merck Millipore with a size of 20 x 10 cm and a layer thickness of 100 µm was used. It first was compared to other HPTLC silica gel plates with thicker layers. The AMD plate was chosen, as the optical evaluation of the classical plates was unrewarding, these ones were not regarded any longer. The second plate was a LiChrospher® HPTLC Silica gel 60 RP-18 $WF_{254}s$ plate (Merck Millipore) with a size of 20 x 10 cm and a layer thickness of approximately 180 µm.

To compare the results of the AMD and the LiChrospher plate, they were prewashed and impregnated with a phosphate buffer (pH=6.8) in an identical way, the same sample and standard solutions were applied and the chromatography was performed with the same reagents. These tests were performed in an early stage of the method development, as neither the impregnation nor the chromatographic processes were optimized yet.

Figure 3 is showing an AMD plate, while a LiChrospher plate can be seen in figure 4, both after the post-chromatographic derivatization with aniline diphenylamine. Besides the six sugars, the standard solution contained furfural, hydroxymethylfurfural (HMF), rhamnose, fructose and glucuronic acid. The bands on the AMD plate showed wavy structures, while the bands on the LiChrospher plate seemed to be more stable. Nevertheless, the AMD plate for this method was the better choice, as it gave a better separation for the six sugars with the described setting. The optical evaluation revealed, that the bands on the LiChrospher plate were lying closer together. It is not possible to distinguish the sugars galactose, glucose, mannose and arabinose from each other by optical means. This circumstance is also represented in a range of R_f-values of only 0.31 units ($0.15 < R_f < 0.46$) while the AMD shows a range of 0.38 ($0.2 < R_f < 0.58$) between the first and the last band. Even though the LiChrospher plate seems to be a quite promising layer, it needs more efforts to achieve a better range of R_f-values as there was no setting found to solve the described problem.

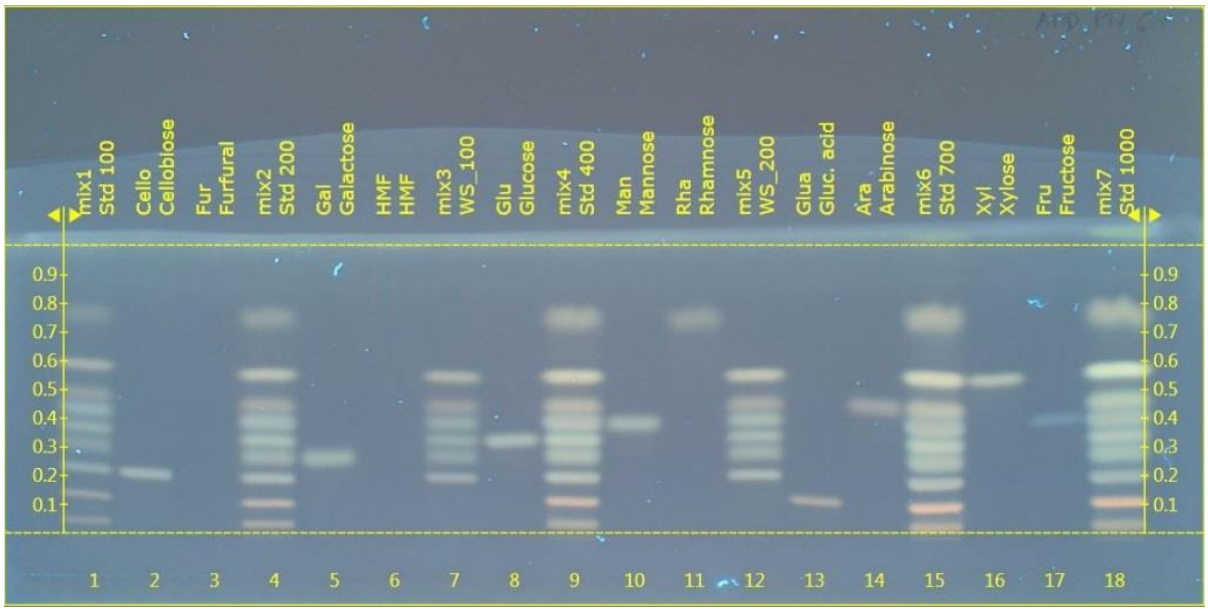

Figure 3: HPTLC with Silica gel 60 F254 AMD extra thin, prewashed and impregnated with a phosphate buffer (pH=6.8), two developments and post-chromatographic derivatization with aniline diphenylamine

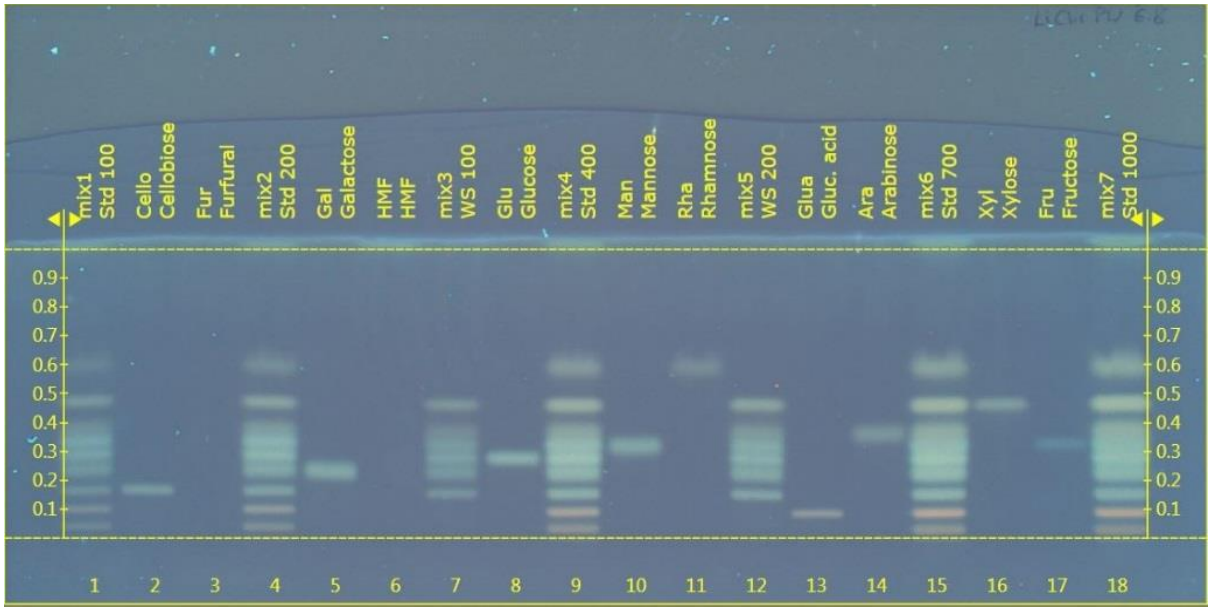

Figure 4: HPTLC with LiChrospher® HPTLC Silica gel 60 RP-18 $WF_{254}s$, prewashed and impregnated with a phosphate buffer (pH=6.8), two developments and post-chromatographic derivatization with aniline diphenylamine

Impregnation

The modification of the stationary phase is an important process for the chromatography of carbohydrates. Without impregnation, even the best chromatographic method is useless, as not even one sugar may be identified. This is illustrated in figure 5, which shows an AMD plate after derivatization, with a stationary phase that was not impregnated at all.

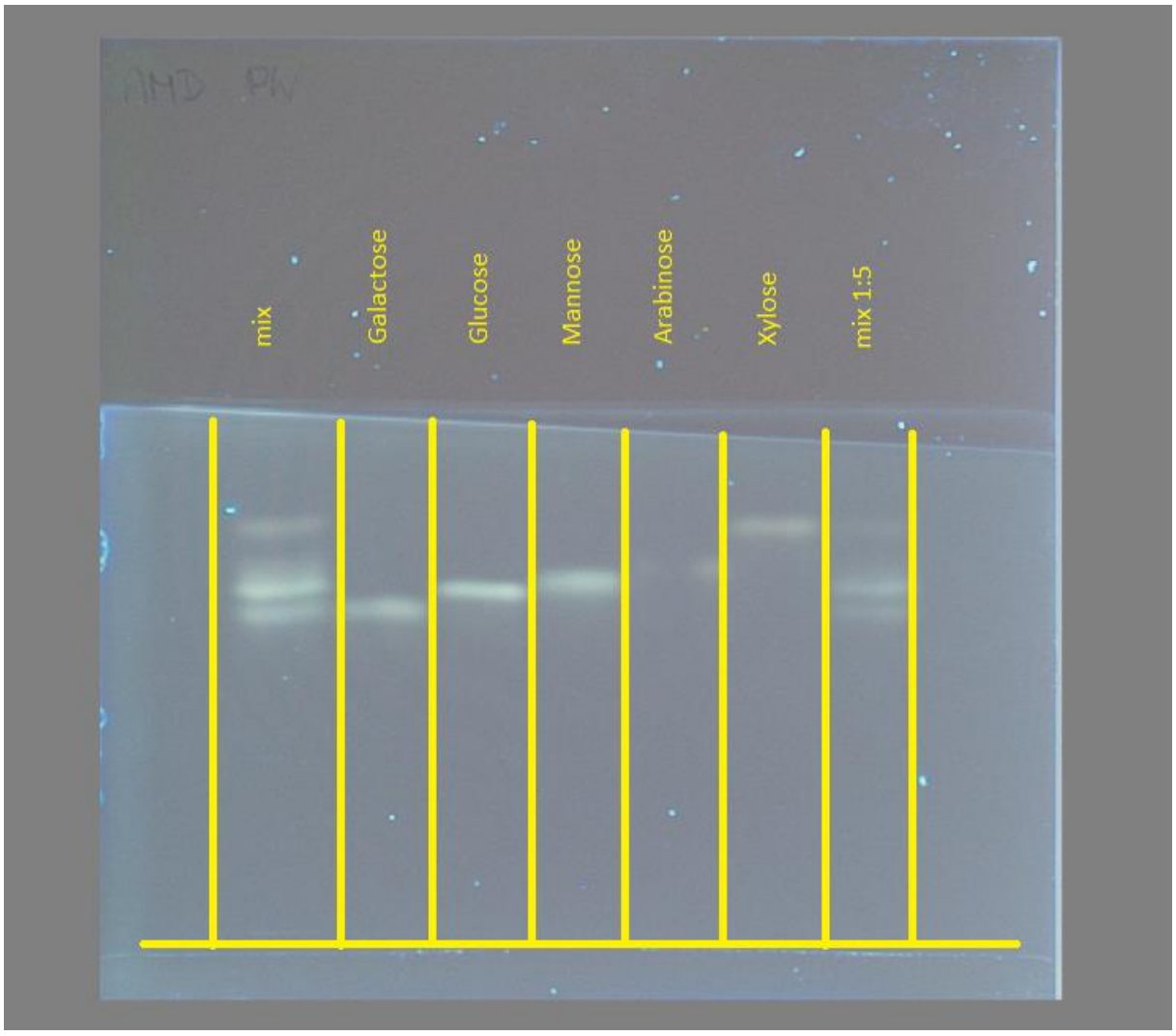

Figure 5: HPTLC with AMD, prewashed, no impregnation, two developments and post-chromatographic derivatization with aniline diphenylamine

According to Wilson, impregnating the stationary phase has got a major influence on the selectivity and the performance of the chromatographic process. The detectability can be enhanced as well, e.g. by supporting the development of fluorescent derivatives. Wilson was reporting, that a separation of complex mixtures of

carbohydrates on plates that were not impregnated is not satisfying. Good results for separating monosaccharides are achieved by using the salts of sodium, such as phosphates with a concentration of 0.05 – 0.1 mol L^{-1} [35].

Other workers, such as Batisse [29], Hansen [36] or Doner [37] were using phosphate buffer to impregnate their chromatographic layers as well and achieved good results in separating carbohydrates. Doner showed, that the use of 0.1 M monosodium dihydrogen phosphate for the impregnation significantly reduced the tailing of the spots compared to non-impregnated layers. By impregnating the plates with the double concentration, the tailing even completely disappeared [37].

Before the impregnation process, all plates were prewashed in a solution of methanol, water in a ratio of 6:1 (v/v). By cleaning the layers, contaminants are removed, which has beneficial effects on the reproducibility and the robustness of the results [33]. After prewashing, the plates were impregnated with a sodium dihydrogen phosphate / disodium hydrogen phosphate buffer with different pH.

Figure 6 shows an AMD plate impregnated with a buffer of a pH=6.2. The AMD plate in figure 7 was impregnated with a pH of 6.8. Both plates were dipped in the buffer for 3 minutes and then heated up for 20 minutes at 105 °C after air-drying. The plates before the chromatography showed no optical differences under white nor UV-light. After the impregnation, the tracks on the plate impregnated with the pH=6.8 buffer were more clear to detect and gave less diffusion. The standard mix on track one contained 200 ng/µl of each sugar, while the standard mix on track seven was diluted 1:5 and therefore contained only 40 ng/µl. It is obvious, that the sensitivity of the stationary phase is better when a buffer with a pH of 6.8 is used compared to 6.2.

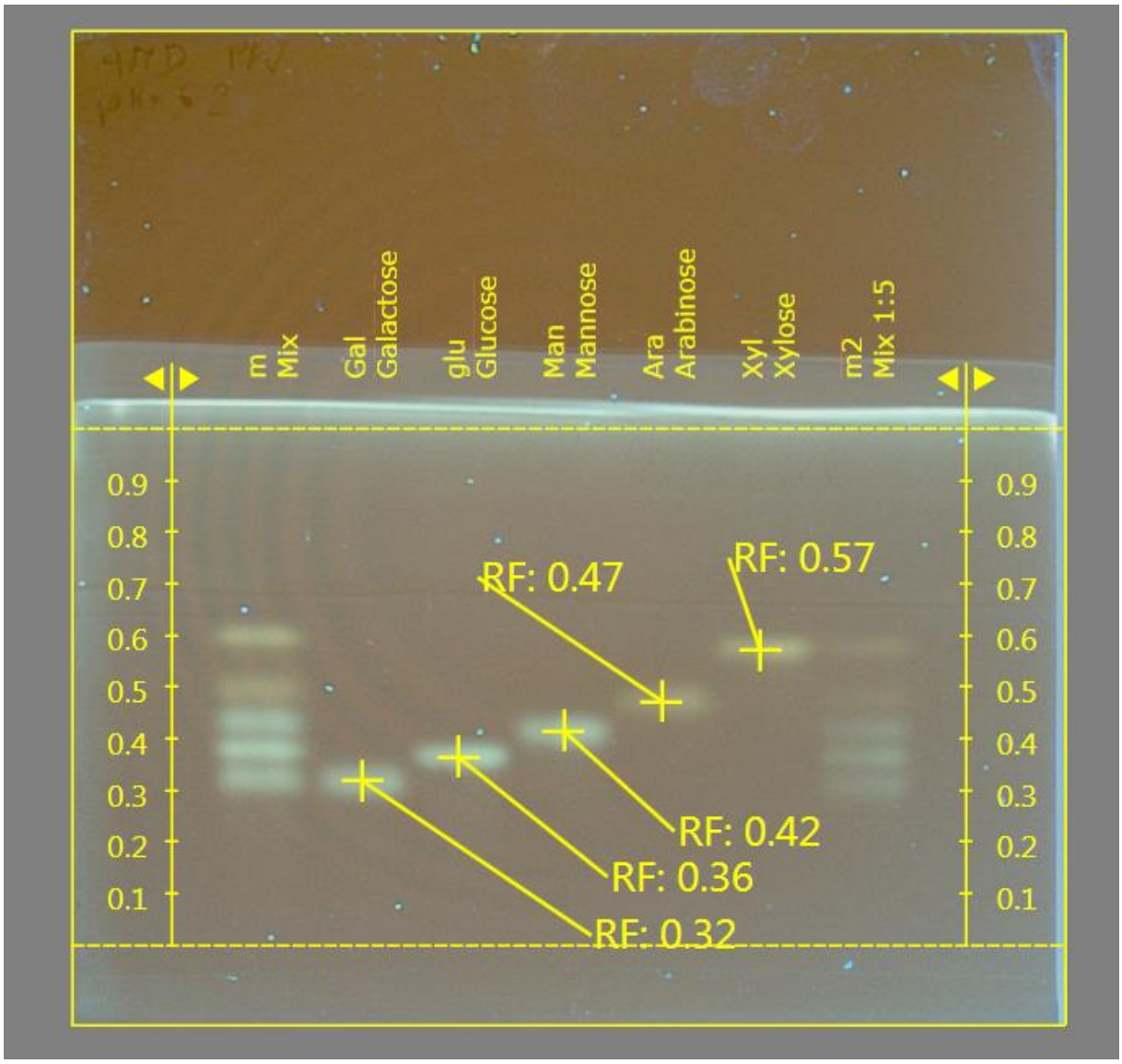

Figure 6: HPTLC with AMD, prewashed, impregnated with a phosphate buffer pH=6.2, two developments with acetonitrile, water, pentanol

The range of the R_f-values is not a parameter for the separation capability of a method, but in this case, as the bands of the investigated sugars are so close, a bigger range is definitely advantageous. The AMD plate impregnated with the buffer of the pH=6.8 was showing slightly better results in this way. In figure 6 (pH=6.2), galactose is showing an R_f-value of 0.32 and xylose 0.57. This is a difference of 0.25 units. The impregnation with pH=6.8 resulted in an R_f difference of 0.27, as galactose was showing 0.26 and xylose 0.53, as referred to figure 7.

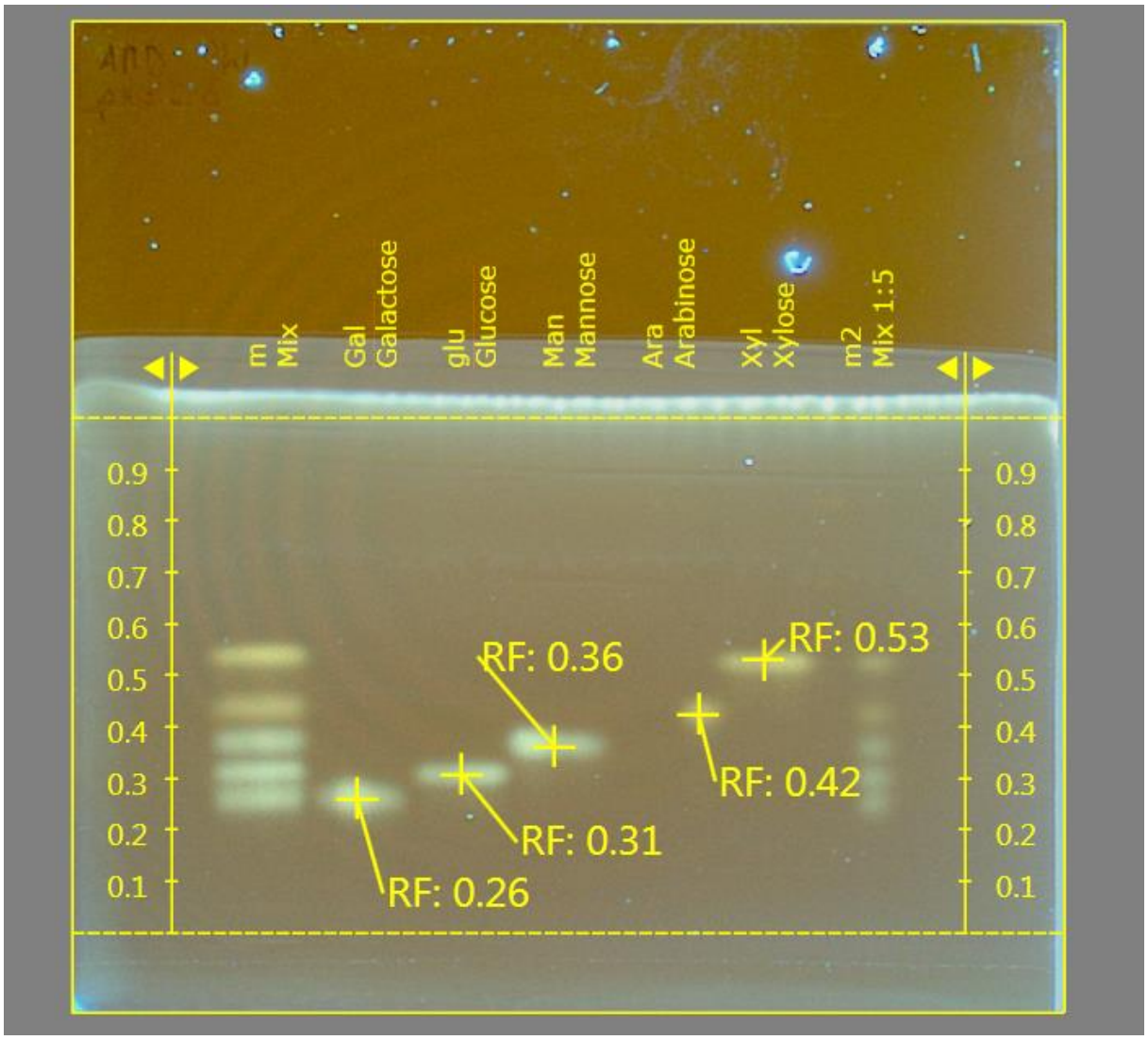

Figure 7: HPTLC with AMD, prewashed, impregnated with a phosphate buffer pH=6.8, two developments with acetonitrile, water, pentanol

As impregnation is one of the core processes during the HPTLC of carbohydrates, it is essential to follow the protocol strictly. Any small change in this procedure has an effect on the results of the quantification and, hence, on the comparability of the results of different operators.

It was shown that it is important to dry the plates after dipping into the impregnation reagent in an upright position. For some reason, the chromatograms in the first stage of the method development were showing a wave-effect, as illustrated in figure 8. This behaviour was attributed to the defectiveness of other chromatographic steps, which were not optimized yet. Optimization in other procedures, e.g. using different relative

humidity during the development process, did not show any improvement to this problem. Finally, the fault was found in the procedure of the impregnation step.

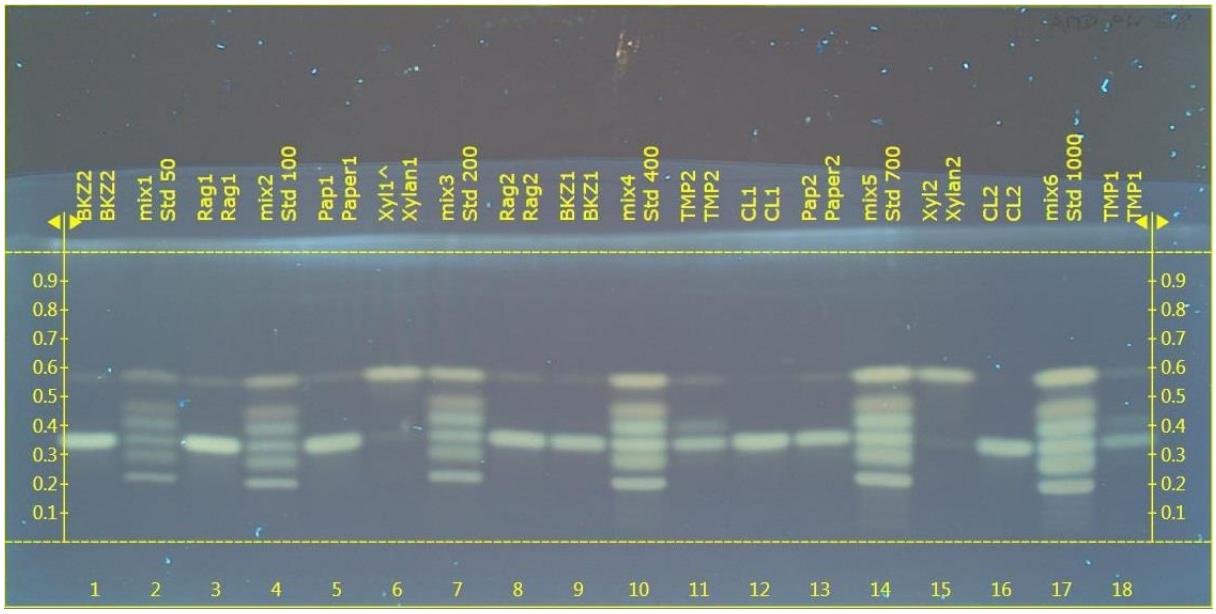

Figure 8: HPTLC showing wave-effect due to drying in a horizontal position after impregnation [AMD plate, prewashed and impregnated, two developments, both 60 mm: a) acetonitrile, water, pentanol b) acetonitrile, water, butanol; derivatization with aniline diphenylamine]

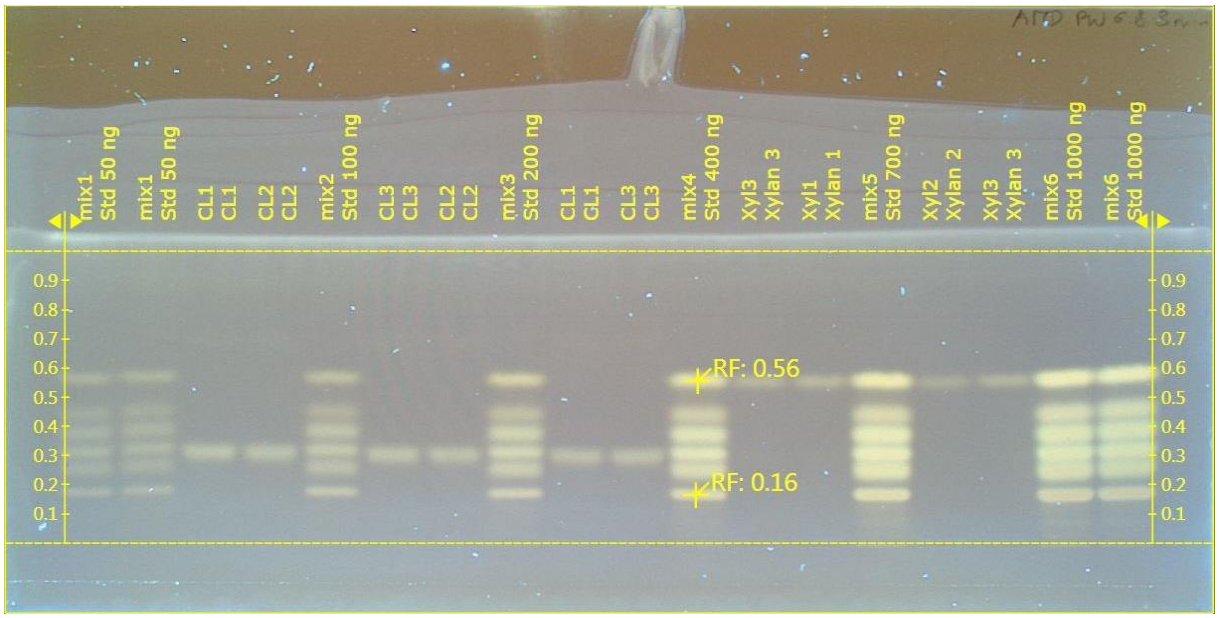

Figure 9: HPTLC showing no wave-effect after drying in an upright position after impregnation [AMD plate, prewashed and impregnated, two developments, both 60 mm: a) acetonitrile, water, pentanol b) acetonitrile, water, butanol; derivatization with aniline diphenylamine]

Some plates were dried in an upright position. These ones did not show this wave-effect, as can be seen in figure 9. When the plate is dried in the horizontal position the remaining of the buffer on the wet plate leads to an uneven drying, while the vertical position allows a drain off of the buffer and, hence, a consistent impregnated layer. This little change in the procedure was leading to an improvement in the stability of all upcoming chromatograms.

3.1.2 Application process

With devices such as the Automatic TLC Sampler 4 (ATS 4), this step of the HPTLC is fully automated. Samples and standards are prepared in autosampler vials and placed in the ATS 4. According to Spangenberg [38], the samples should be applied as lines instead of spots. Due to an uneven mass distribution in spots, they would lead to half-moon-shaped bands. Spangenberg is reporting, that the bands when applied should be as thin as possible to take full advantage of the number of theoretical plates. Other important factors are the application position and the volume of the samples [38].

By using the spray-on technique, the ATS 4 allows application volumes for bands of 0.5 µl up to more than 50 µl. Through contact transfer, it would be possible to apply spots with a volume of 0.1 to 5 µl. For the operator it is quite straight forward to apply different volumes of the same stock solution to get different amounts of substances per sample for the calibration.

Unfortunately, the tests were showing some difficulties when different volumes were applied. The bands often looked somehow cut off, as can be seen in figure 7. This problem occurred for application volumes for 1 µl as well as for less or more than 1 µl, but only when different volumes were applied. By applying the same volume for every track, this problem could be avoided. Hence, it was necessary to prepare standard solutions with different concentrations to get the desired amount of substance for the calibration. An application volume of 1 µl gave good results for wood derived sugars. Higher volumes often showed a diffusion of the bands, lower volumes had difficulties with the needle that was used.

The software visionCATS allows a precise setting for the position of the bands applied on the stationary phase. Possible settings are the length of the bands, the distance between the bands and the position of the bands in the X and Y direction. Different application positions from 10.0 to 15.0 mm in the Y direction were tested, a position of 12.0 mm from the bottom was chosen by optical evaluation.

3.1.3 Development process

The development is the core process of the chromatography, where the substances are finally separated. Hence, there is a high potential for optimization, even though the Automatic Developing Chamber (ADC 2) allows a fully automated development, for which a lot of parameters can be set. By choosing the optimal solvents, the amount of developments, the solvent front or the humidity, the operator has a strong influence on the success of the chromatography.

According to [39], solvents for the chromatography of sugars are usually containing two or three components. Solvents should contain water, otherwise the spots are getting blurred. Usually, the percentage of water ranges between 10 and 20 % with an optimum of 15 %. The choice of the solvent reagent was based on the results of [29], where acetonitrile, 1-pentanol, water in a ratio of 60:20:20 (v/v/v) was used to separate sugars. For this method acetonitrile, water, pentanol (hereinafter called solvent A) as well as acetonitrile, water, butanol (hereinafter called solvent B) both in a ratio of 4:1:1 (v/v/v) were used for the development of the chromatograms.

Solvent A results in a higher resolution of the chromatogram. The bands in figure 10 are much better to identify and to distinguish from each other, while the bands in figure 11 developed with solvent B are showing more diffusion.

On the other hand, solvent B leads to a better separation as can be seen by comparing the R_f-values in table 2. The distance galactose - glucose, glucose - mannose, mannose - arabinose and arabinose - xylose is approximately 0.02 units bigger when the development was performed twice with the butanol solvent compared to two developments with the pentanol solvent. The overall distance between galactose and

xylose is 0.26 units for the pentanol solvent and 0.34 units for the butanol solvent after two developments.

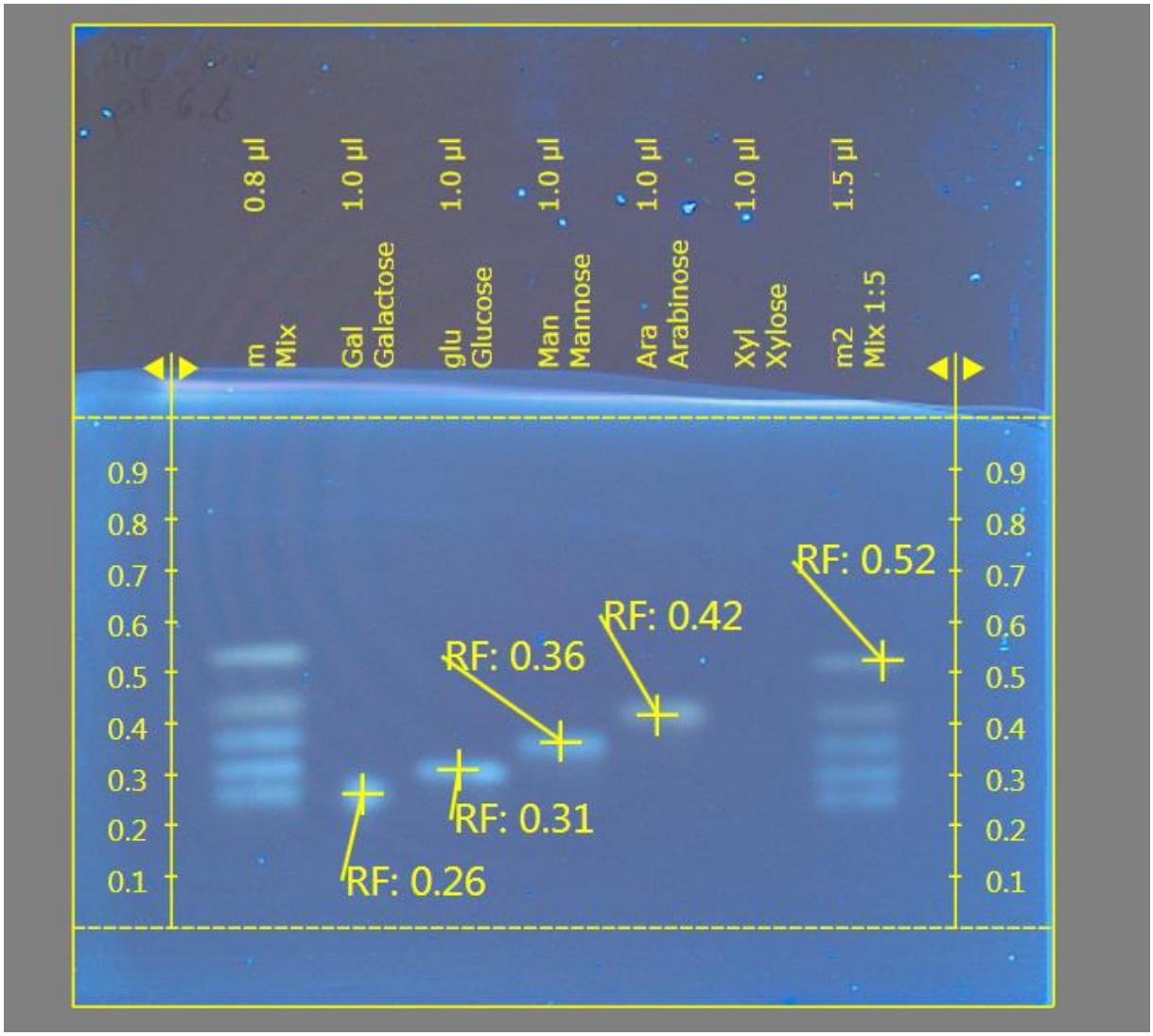

Figure 10: HPTLC after derivatization, two developments with acetonitrile, water, pentanol (4:1:1) twice; solvent front: 50 mm

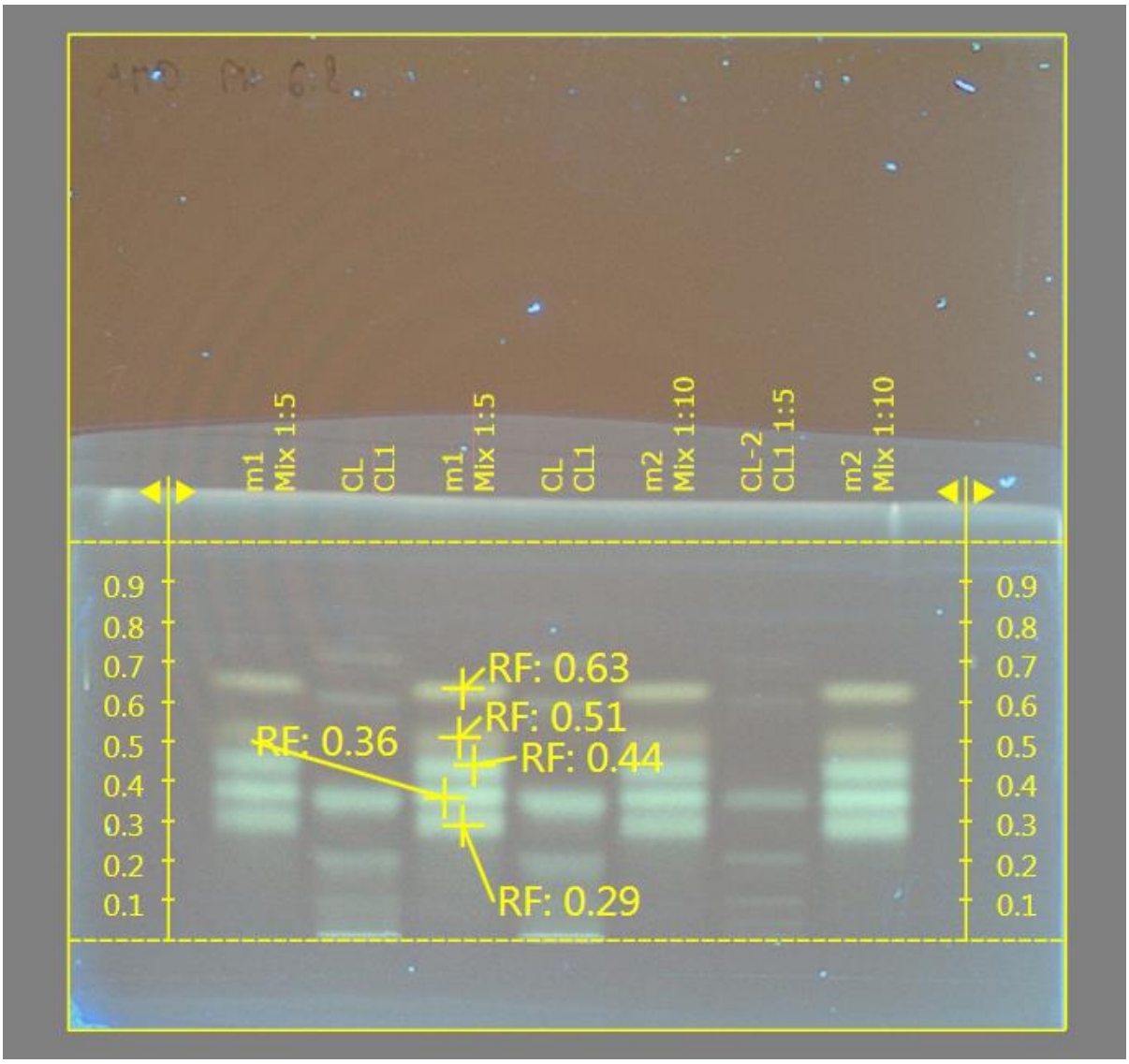

Figure 11: HPTLC after derivatization, two developments with acetonitrile, water, butanol (4:1:1); solvent front: 50 mm

Table 2: R_f-values after two developments with solvent A taken from fig. 10 and solvent B taken from fig. 11

Substance	**R_f-values** Solvent A			**R_f-values** Solvent B		
Galactose	0.26			0.29		
		Δ 0.05			Δ 0.07	
Glucose	0.31			0.36		
		Δ 0.05	Δ **0.26**		Δ 0.08	Δ **0.34**
Mannose	0.36			0.44		
		Δ 0.06			Δ 0.07	
Arabinose	0.42			0.51		
		Δ 0.10			Δ 0.12	
Xylose	0.52			0.63		

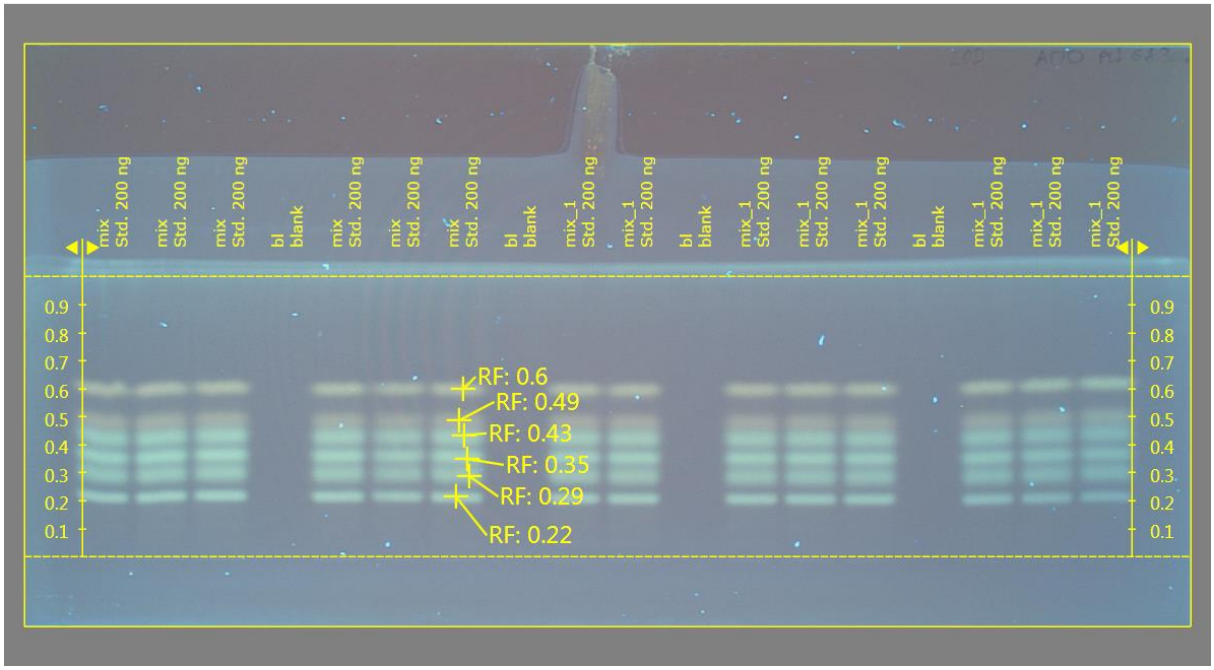

Figure 12: HPTLC after one development with acetonitrile, water, pentanol and a second development with acetonitrile, water, butanol; picture is showing R_f-values of the following substances: Cellobiose, Galactose, Glucose, Mannose, Arabinose, Xylose

To combine both, the higher resolution of solvent A and the better separation properties of solvent B, the two reagents were used sequenced. The order of the two solvents was tested as well. Finally, the best results were shown, when solvent A was applied for the first development and solvent B for the second. Figure 12 is showing a chromatogram in a high resolution with a satisfying separation.

Table 3: R_f-values after two developments, first with solvent A, then with solvent B

Substance	**R_f-values**		
Galactose	0.29		
		Δ 0.06	
Glucose	0.35		
		Δ 0.08	
Mannose	0.43		Δ **0.31**
		Δ 0.06	
Arabinose	0.49		
		Δ 0.11	
Xylose	0.60		

The R_f-values for this chromatogram can be seen in Table 3. The migration distance galactose - glucose, glucose - mannose, mannose - arabinose and arabinose - xylose are similar to a double development with solvent B only. The overall migration distance galactose - xylose is satisfying and still better compared to a double development with solvent A only.

Humidity control

During the first tests an activation step was performed for 15 minutes with molecular sieve to reduce the relative humidity to 0.1 %. Mistakenly, the low relative humidity was thought to cause the "wave-effect". Using a saturated lithium chloride solution for the activation resulted in a relative humidity of 13 - 15 %. The increased relative humidity did not show any improvement to this problem. Finally, this step was deactivated at all. Hence, the chromatographic plate was exposed to changing relative humidities depending on the conditions in the laboratory. The tests e.g. in the end of March showed a relative humidity of 40 % during the development step while the relative humidity in the end of June was rising up to 60 %. However, the results obtained without any humidity control did not show any impacts on the quality of the analysis.

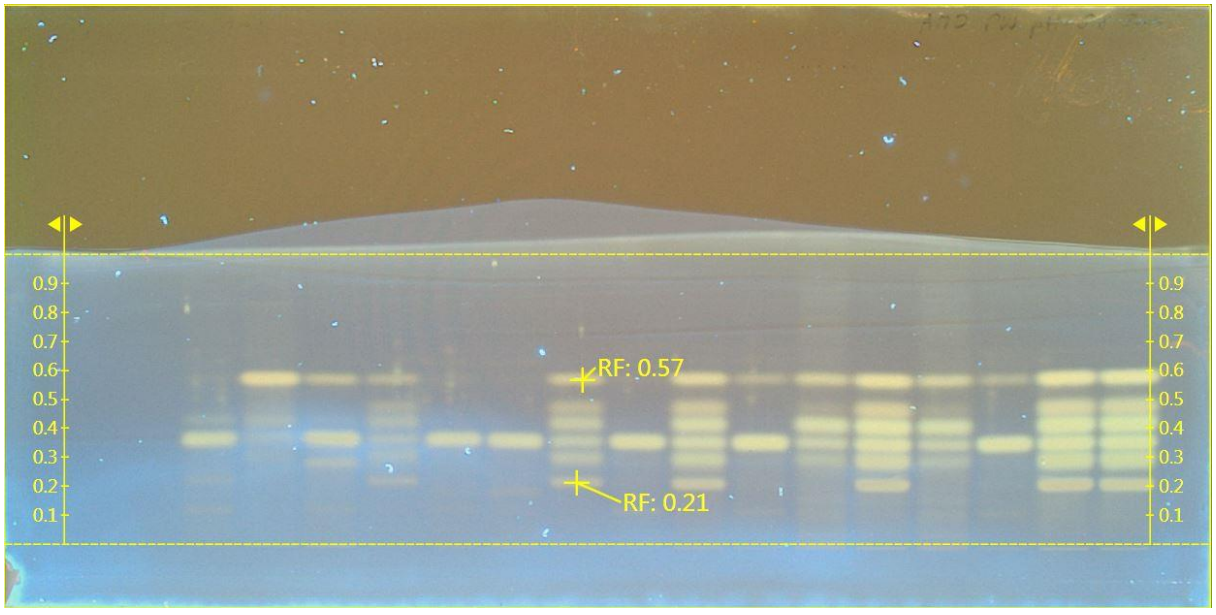

Figure 13: HPTLC of different concentrations of the standard solution and different samples, developed with an activation of 15 minutes with a saturated lithium chloride solution and a relative humidity of approx. 14 %. Bands 1 and 2 are applied with standard solution of 50 ng/µl

The chromatograms that were developed without any activation (but with changing relative humidity) were much more sensitive to low concentrations of the sugars. When the humidity control was applied, it was not possible to detect a concentration of 50 ng/µl on the plate, as can be seen in figure 13. Even in the evaluation of the scanning pictures there were no peaks to spot at all. On the contrary, by deactivating the humidity control the plates were showing higher sensitivity. A concentration as little as 50 ng/µl could be analysed qualitatively and quantitatively, as shown in figure 14.

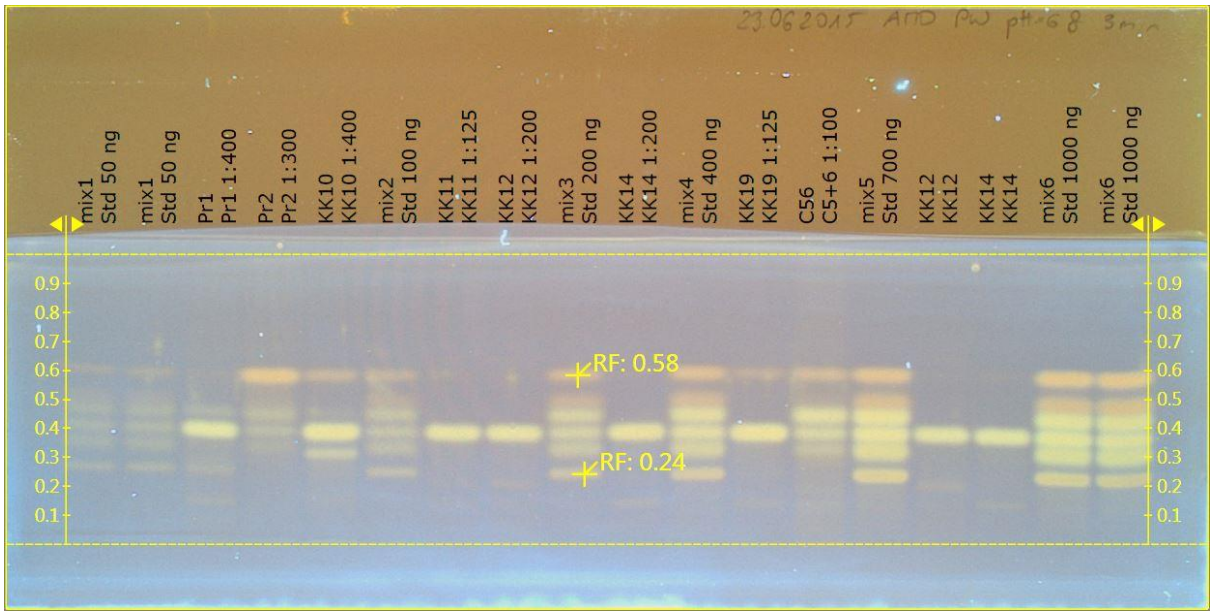

Figure 14: HPTLC of different concentrations of the standard solution and different samples, developed without activation and a relative humidity of approx. 50 %. Bands 1 and 2 are applied with standard solution of 50 ng/µl

3.1.4 Derivatization

Some substances are coloured or exhibit fluorescence. These are easy to detect on a chromatographic plate. Substances without chromatographic groups can be visualized by a chemical reaction introducing suitable chromophores. Hence, the derivatization process is transubstantiating them into a detectable form, so that an evaluation of the chromatogram is possible. The derivatization is either possible in universal reactions or through reactions targeting specific functional groups of the substances. However, the post-chromatographic derivatization is not only making the substances detectable,

but also increasing the sensitivity and the selectivity [40]. In the past, the procedure of detection was quite challenging. In the works of [41], first a solution of sodium periodate and then a solution of benzidine had to be sprayed on the plate. The layer then was placed in a chamber with an ammonia steam, before a solution of silver nitrate was sprayed on the plate. Today, this process is much easier by simply dipping the plate after the development into the derivatization reagent.

Choosing the right reagent for the derivatization is a challenging task. Today, the post-chromatographic derivatization is the most common one. The aim is to make the substances easier to detect and therefore to evaluate, to optimize the selectivity and to increase the sensitivity [40]. To meet all these requirements, it is necessary to find the optimal derivatization reagent. During the method development, two reagents were tested: the *N*-(1-Naphthyl)ethlyenediamine reagent and the aniline-diphenylamine-phosphoric acid reagent

The first one, *N*-(1-Naphhtyl)ethlyenediamine, was based on the investigations of Bounias [42], where this reagent allowed an improvement of the quantitative analysis and Han and his co-worker [30], who used the reagent for the detection of various aldoses and alditols. In their case it was very sensitive for sugars with a limit of detection of 50 ng. However, in this work the reagent was showing unstable results with differing colours, hence, the *N*-(1-Naphthyl)ethlyenediamine reagent was not giving satisfying results.

Aniline diphyenylamine phosphoric acid is a known derivatization reagent for sugars (mono- and disaccharides, oligosaccharides and starch hydrolysates), thickening agents and glycosides. During the reaction, the sugar is heated with strong acids. Furfural and furfural derivatives are formed, reactions with amines results in Schiff's bases, which are coloured. However, the aniline reagent has some disadvantages as well. Aniline is a toxic substance and the reagent is perishable. It shouldn't be stored longer than 14 days. The mixture turns dark yellow over time, which is an indication that the reagents should not be used any longer [40].

The procedure of preparing aniline diphenylamine was based on the investigations of Jork and his co-workers [40]. After the development process, in which the plate was already dried for 15 minutes, the layer was air-dried for 15 more minutes to make sure

that all the mobile phase was removed. There are two options to apply the derivatization reagent: spray-on and immersion. According to [33], immersion is the better option because of its improved reproducibility. In these tests, immersion was used only.

The chromatographic plate was immersed in the reagent solution for 5 seconds. It is important to dip the plate in an even way, a contact with the glass chamber should be avoided. The layer then was air-dried for 15 minutes and the TLC heating plate was meanwhile heated up to 120 °C. The plate was not heated for 10 – 15 minutes as in the method of Jork [40], but only for 3 minutes. This period of time is sufficient to obtain derivatives for the evaluation and it makes sure that no further chemical reactions occurs.

3.1.5 Detection and Evaluation

For the evaluation of the chromatogram, two systems were used. The qualitative analysis was performed with the CAMAG TLC Visualizer, which allows a visual inspection under white and ultraviolet light. The quantitative analysis was performed with the CAMAG TLC Scanner, which uses a spectral range from 190 to 900 nm and allows the identification of substances by using absorption and fluorescence. The detection with fluorescence was not possible in this case, as there was a problem with the software.

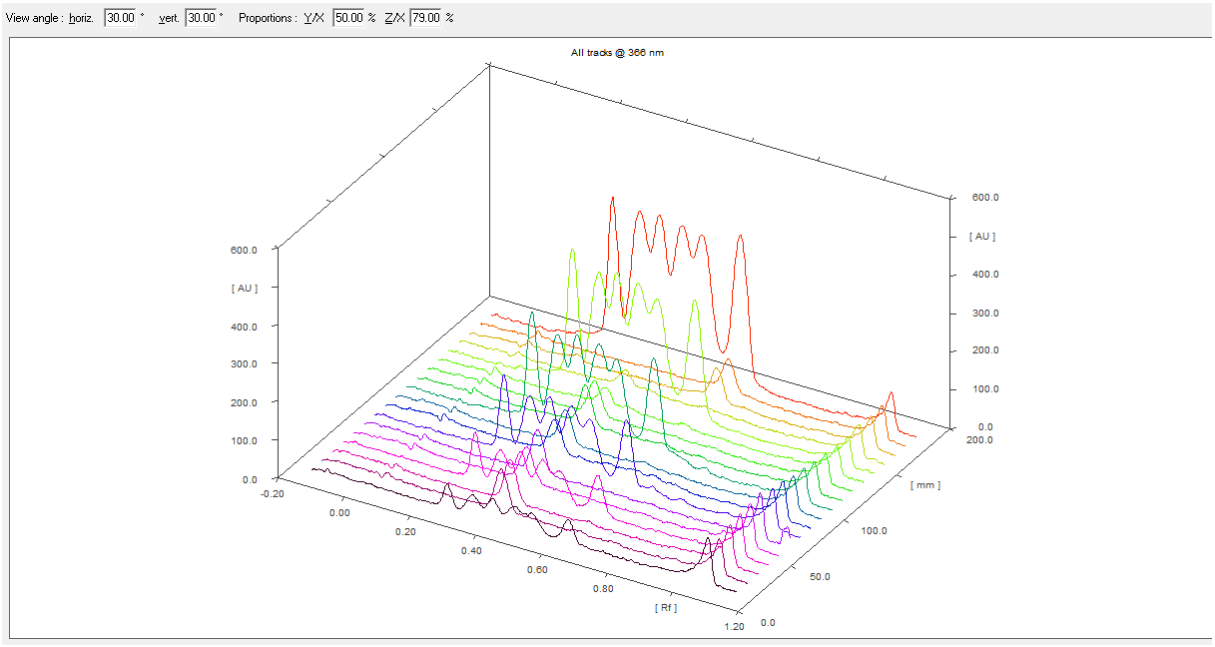

Figure 15: Picture of all 18 tracks after scanning using the software winCATS

First, the CAMAG TLC Visualizer was used for taking pictures of the TLC plate. It is possible to take pictures with white light, UV 254 nm and UV 366 nm. The pictures allowed to measure the R_f-values and to evaluate the chromatograms by optical means. Finally, the CAMAG TLC Scanner 3 was used for the evaluation. It is possible to use absorption at different wavelengths. For the standard method, a wavelength of 354 nm was used. Figure 15 is giving an example of the obtained peaks after scanning.

Track 14, ID:

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.05 Rf	2.5 AU	0.07 Rf	15.7 AU	0.76 %	0.08 Rf	10.1 AU	152.8 AU	0.30 %	unknown *
2	0.08 Rf	10.3 AU	0.13 Rf	25.6 AU	1.24 %	0.15 Rf	19.8 AU	589.4 AU	1.15 %	unknown *
3	0.18 Rf	30.4 AU	0.23 Rf	360.9 AU	17.50 %	0.26 Rf	80.8 AU	7291.1 AU	14.21 %	unknown *
4	0.27 Rf	84.1 AU	0.31 Rf	319.0 AU	15.46 %	0.34 Rf	34.0 AU	8705.6 AU	16.97 %	unknown *
5	0.34 Rf	235.9 AU	0.37 Rf	332.7 AU	16.13 %	0.40 Rf	04.8 AU	7774.1 AU	15.16 %	unknown *
6	0.40 Rf	205.9 AU	0.43 Rf	321.2 AU	15.57 %	0.46 Rf	51.3 AU	8888.9 AU	17.33 %	unknown *
7	0.47 Rf	252.6 AU	0.49 Rf	293.4 AU	14.23 %	0.55 Rf	59.2 AU	8015.2 AU	15.63 %	unknown *
8	0.55 Rf	59.3 AU	0.60 Rf	320.0 AU	15.51 %	0.66 Rf	14.7 AU	8259.3 AU	16.10 %	unknown *
9	1.00 Rf	17.3 AU	1.07 Rf	74.0 AU	3.59 %	1.10 Rf	0.2 AU	1619.5 AU	3.16 %	unknown *

Figure 16: Evaluation of one track using winCATS; each peak is described with the R_f-value (start, max and end position), the height (start, max and end height) and the area

For the evaluation of the qualitative and quantitative data, the software visionCATS and winCATS were used. Basically, visionCATS allows a fully automated evaluation by directly using the data for the integration of the peaks and the calibration of the standard substances. The software provides the operator directly with the

concentration of the substances and therefore it is highly reproducible. Unfortunately, during these tests the software showed some major errors in the evaluation process, hence the software winCATS was used.

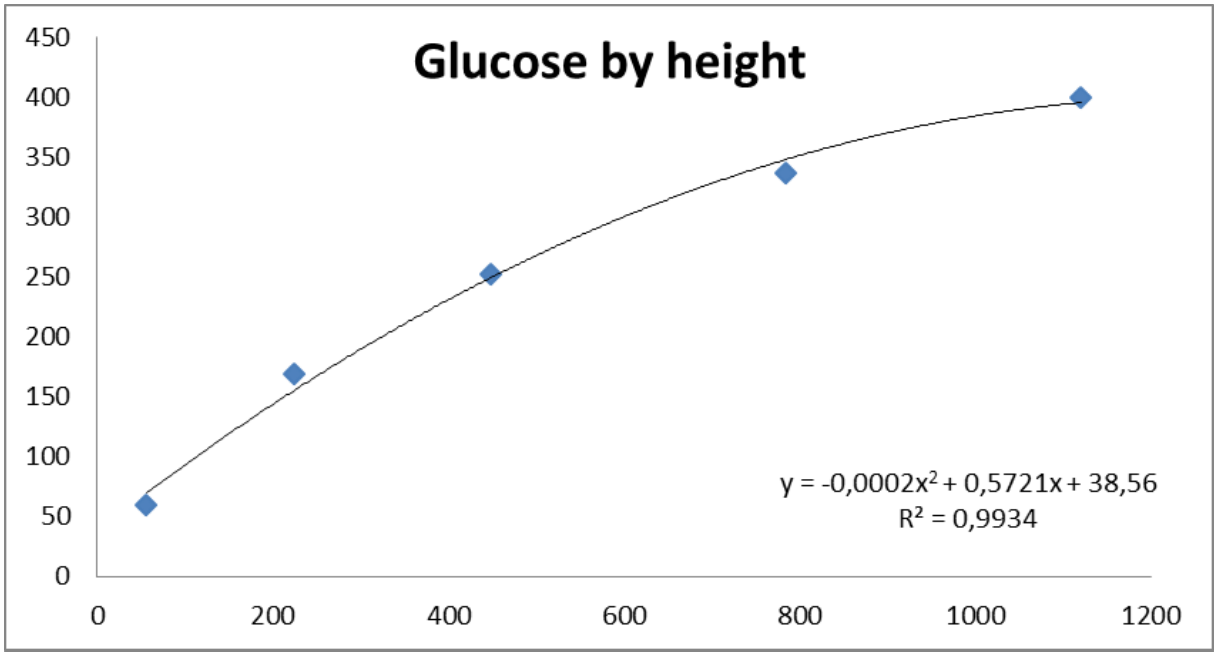

Figure 17: Example of a calibration curve for glucose calibrated by height

After scanning the plate, winCATS provides the R_f-values, the height and the area of all peaks found, as can be seen in figure 16. With these values, the calibration function was drawn. Figure 17 is giving an example of a calibration function for glucose. As the R_f-values of the standards were known, the height and area were obtained for the investigated substances in the samples. By using the calibration equation, the recovery value was calculated considering the dilution factor.

3.2 Validation of the HPTLC-Method for Carbohydrates

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) was defining standards to validate a new method. According to these requirements, the results of the quantification are tested for specificity, accuracy, precision, and limit of detection, limit of quantification, linearity, range and robustness [43].

3.2.1 Specifity/Selectivity

Specifity means the distinction of a substance from other substances that might be present, such as impurities. A method is selective when an analyte is separated from the matrix of the sample in a way that the analyte can be detected. To make sure that a peak is not containing any other substance, the peak must be compared to a reference sample. A method is selective, when the same result can be obtained even under different parameters during chromatography [38].

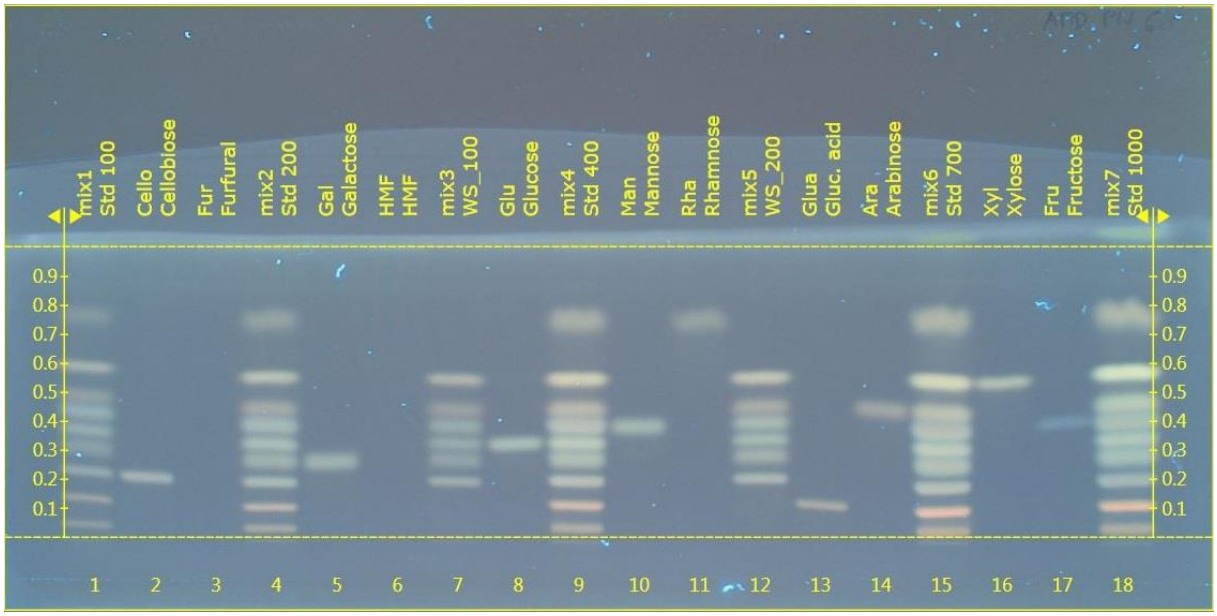

Figure 18: HPTLC with standards containing cellobiose, galactose, glucose, mannose, arabinose, xylose, furfural, HMF, rhamnose, gluconic acid and fructose

The present method allows a separation of cellobiose, galactose, glucose, mannose, arabinose and xylose. This was shown by using different layers, different solvents and derivatization reagents. As can be seen from figure 18, it was possible to separate sugar acids such as gluconic acid or rhamnose. Fructose was comigrating with mannose and could not be separated. This method was highly selective under various parameters and in presence of unknown substances.

3.2.2 Accuracy

The accuracy describes how close the expected value is from the value measured [43]. According to [33], there are several ways to obtain the accuracy. One option is to use a reference method to compare the result with. In this case, the reference method is an established one with known uncertainty. Another method is using different concentrations of the sample. The value obtained during the test is compared to the reference value of the material.

The accuracy test was performed for glucose and xylose. By applying the pure samples, the pure standard solution and a mixture of both, the method was tested for accurate results. A defined amount of the sugar mix was added to the sample solution. Figure 19 is showing the chromatogram of the accuracy test. The accuracy is calculated as shown in the following equation:

$$Accuracy\ [\%] = \frac{expected\ amount\ of\ sugar}{detected\ amount\ of\ sugar} * 100$$

The expected amount of sugar is the sum of one sugar (e.g. glucose) found in the sample plus the sugar found in the standard solution. Together, this is the amount of sugar that can be expected in a mixture of both, the sample and the standard solution.

The detected amount of sugar means the spotted sum of sugar detected in the mixed solution of a defined volume of sugar and standard solution. Hence, a percentage of 100 means that the whole expected amount of sugar is found in the detected amount of sugar.

Table 4 gives the results of the accuracy test. Glucose and xylose were both calibrated by height and area. The results for the calibration by height were better than by area. Glucose is showing an accuracy of 103.4 % for height, but only 87 % for area. Xylose is showing an accuracy of 93.5 % for height and 89.6 % for area.

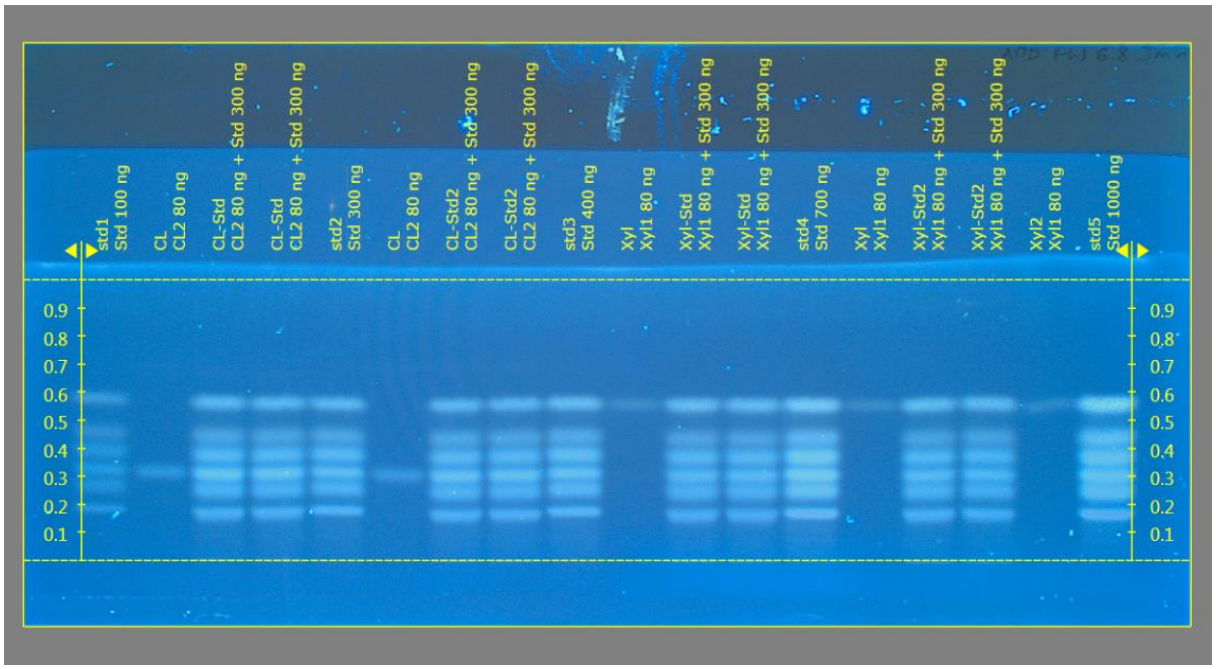

Figure 19: HPTLC with samples, standards and a mixture of both for the accuracy test; std1-std5…pure standard samples in different concentrations; CL…pure Cotton Linters sample; Xyl… pure Xylane sample; CL-Std / Xyl-Std…mixture of sample and standard solution

Table 4: Results of the accuracy test for glucose and xylose, both calibrated by height and area

Substance	Expected [ng]	Detected [ng]	Accuracy [%]
Glucose (height)	460	445	103.37
Glucose (area)	442	508	87.01
Xylose (height)	386	413	93.46
Xylose (area)	386	431	89.56

3.2.3 Precision

An analytical method is called precise when there is a high degree of conformity among the results of a numerous tests, where the same samples were applied under equal conditions. To obtain the precision of a method, its repeatability must be investigated [43].

The repeatability is the short-term precision, also known as intra-assay or within-day precision [33]. It is obtained in a short interval of time, sometimes even in one day. All the equipment used is the same, the same solutions and the same batch of chromatographic plates are used. All the laboratory work is performed by one analyst. This means, that the risk of an error caused by the equipment or surrounding parameters, which can't be influenced, is low. The results of the repeatability test are providing information about the precision of the preparation, application and evaluation of samples.

To proof the repeatability, the same samples were applied on three chromatographic plates at the same day. All parameters were equal, the same stock solutions were used. After detecting the signals for height and area, the calibration curve was drawn and the amount of sugar was calculated for glucose and xylose. The results are shown in tables 5 and 6 for glucose and in tables 7 and 8 for xylose.

Concerning the repeatability, the standard deviation should be considered. When calibrated by area, the results are more precise. When calibrated by height, the standard deviation for glucose is about 10.1 % and 9.4 % for xylose, the calibration by area is showing a standard deviation of 6.5 % for glucose and 5.0 % for xylose.

The intermediate precision, which is a long-term test with different operators and, if possible, with different equipment, could not be investigated within this work. The reproducibility is testing the agreement of the results obtained by different laboratories. The reproducibility could not be determined within this experiment neither.

Table 5: Recovery, absolute standard deviation (Abs. Std. deviation) and relative standard deviation (Rel. Std. deviation) of glucose (Glu) calibrated by height

HEIGHT	Recovery [g Glu/g sample]	Abs. Std. deviation [g/g]	Rel. Std. deviation [%]
Glu (Sample 1)	0.898	0.081	8.999
Glu (Sample 2)	0.892	0.119	13.323
Glu (Sample 3)	0.896	0.072	8.047
Glu (Total)	**0.895**	**0.091**	**10.117**

Table 6: Recovery, absolute standard deviation (Abs. Std. deviation) and relative standard deviation (Rel. Std. deviation) of glucose (Glu) calibrated by area

AREA	Recovery [g Glu/g sample]	Abs. Std. deviation [g/g]	Rel. Std. deviation [%]
Glu (Sample 1)	1.096	0.064	5.821
Glu (Sample 2)	1.119	0.085	7.567
Glu (Sample 3)	1.058	0.063	5.913
Glu (Total)	**1.091**	**0.070**	**6.448**

Table 7: Recovery, absolute standard deviation (Abs. Std. deviation) and relative standard deviation (Rel. Std. deviation) of xylose (Xyl) calibrated by height

HEIGHT	Recovery [g Xyl/g sample]	Abs. Std. deviation [g/g]	Rel. Std. deviation [%]
Xyl (Sample 1)	0.909	0.080	8.845
Xyl (Sample 2)	0.815	0.087	10.712
Xyl (Sample 3)	0.919	0.061	6.687
Xyl (Total)	**0.881**	**0.083**	**9.425**

Table 8: Recovery, absolute standard deviation (Abs. Std. deviation) and relative standard deviation (Rel. Std. deviation) of xylose (Xyl) calibrated by area

AREA	Recovery [g Xyl/g sample]	Abs. Std. deviation [g/g]	Rel. Std. deviation [%]
Xyl (Sample 1)	0.961	0.038	3.995
Xyl (Sample 2)	0.894	0.031	3.465
Xyl (Sample 3)	0.944	0.053	5.621
Xyl (Total)	**0.933**	**0.047**	**5.036**

3.2.4 Calibration by height or area

Tables 6 to 9 are showing the results for the quantification of glucose and xylose. The calibration by height gives a recovery value for glucose of approximately 90 % for xylose of 88 %, while the calibration by are results in 109 % for glucose and 93 % for xylose.

Furthermore, the relative standard deviation gives much more precise results for the calibration by area. While the standard deviation for the results of glucose is about 10.1

% and 9.4 % for xylose, the calibration by area is showing a standard deviation of only 6.5 % and 5.0 %.

Even though the precision in this test was better when calibrated by area, it must be considered that the reproducibility (the precision between laboratories or operators) not necessarily is showing the same ratio. The software permits the operator to define the borders of the peaks. An experienced operator will do the definition always in an equal way, e.g. cutting off tails. This leads to a very high precision for one operator, but the repeatability between different operators will be affected negatively.

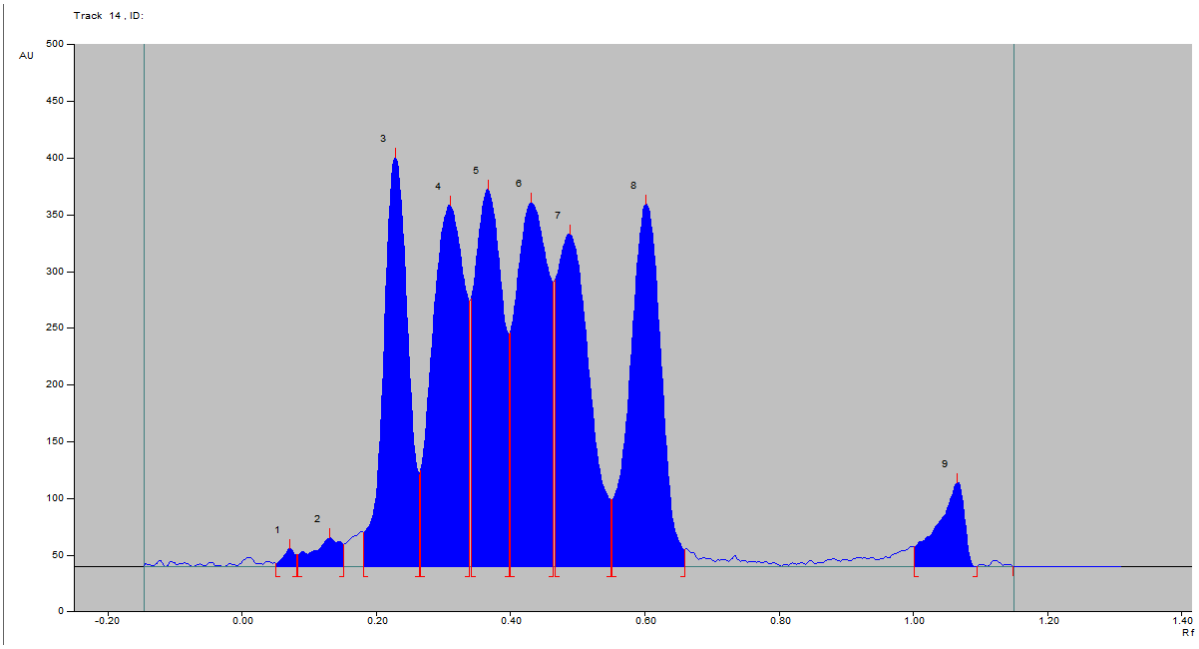

Figure 20: Scan of a HPTLC, peaks are representing sugars

Figure 20 is showing a typical picture of the peaks of a standard sample after scanning using the software winCATS. The peaks are tagged automatically but can be influenced by the operator. It is necessary for the operator to check all the peaks for each track before the quantification first of all to assure the selectivity when it comes to unidentified samples and secondly, in case of a calibration by area, the operator must make sure that the marked peaks have accurate borders. If the area of a peak is including a tail, then the borders must be adopted. This procedure can lead to problems when results of different operators or laboratories would like to be compared.

Regarding the repeatability it is highly recommended to use the calibration by height, as the signals by height can't be affected by the operator. Therefore, the results are better to compare, even though the recovery values may suffer slightly.

3.2.5 Limit of Detection and Limit of Quantification

The limit of detection (LOD), also known as the detection limit, is the lowest amount of a substance that can be detected in a sample. Usually, this amount can't be quantified precisely, but only detected. The limit of quantification (LOQ), also known as the quantitation limit, is the lowest amount of a substance in a sample that can be quantified precisely with good results of accuracy. These two parameters are important in comparing methods. The lower the LOD and LOQ are, the more sensitive a method is.

The calculation of the LOD and LOQ are based on the signal-to-noise ratio, where signals of samples with known concentrations are compared to water samples (or blank samples). This is giving the lowest amount of a substance that can be detected or quantified. The Signal to Noise ratio (S/N ratio) is obtained by the standard deviation of all peaks, as seen in figure 21. According to the equation, the LOD is 3 times of the S/N ratio and the LOQ is 10 times the S/N ratio.

$$LOD = 3 * S/N$$

$$LOQ = 10 * S/N$$

LOD… Limit of Detection
LOQ… Limit of Quantification
S/N… Signal to Noise ratio

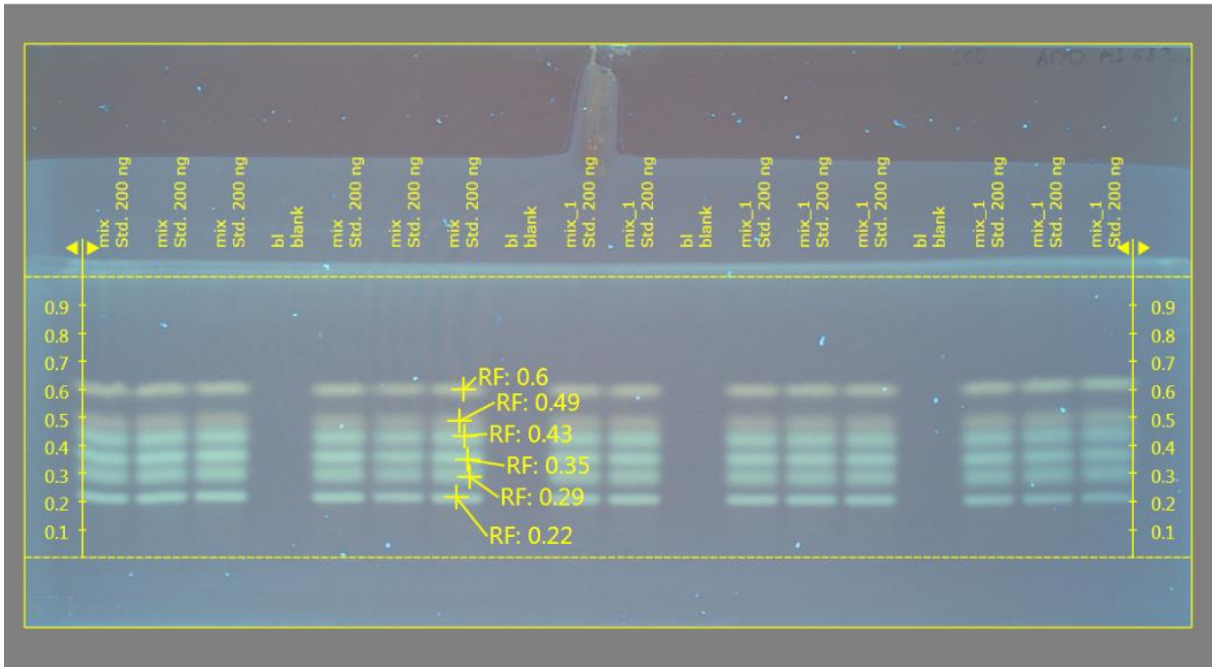

Figure 21: HPTLC of the LOD/LOQ test: 14 samples of a standard solution containing 200 ng / µl of each sugar 4 blank samples (water) were applied

Figure 21 gives the chromatogram of the LOD/LOQ test. The standard solution containing 200 ng / µl of each sugar was applied 14 times, the blank sample containing only water was applied four times. Track one had to be excluded. To gain the Signal-to-Noise ratio, all the small peaks except the peaks of the sugars were marked manually as seen in figure 21, which is showing a blank sample. With these peaks, the standard deviation of each track was calculated. The overall standard deviation is giving the S/N ratio, which was 2.9 in these tests. This means, that the smallest amount of substance that can be detected (LOD) is 9 ng and the smallest amount of substance that can be quantified (LOQ) is 29 ng.

These results in the very low range of nanograms can be attributed to the use of aniline diphenylamine in the derivatization process. The use of *N*-(1-Naphthyl)-ethylenediamine gives a limit of detection of 50 ng, as reported by Han and Robyt [3].

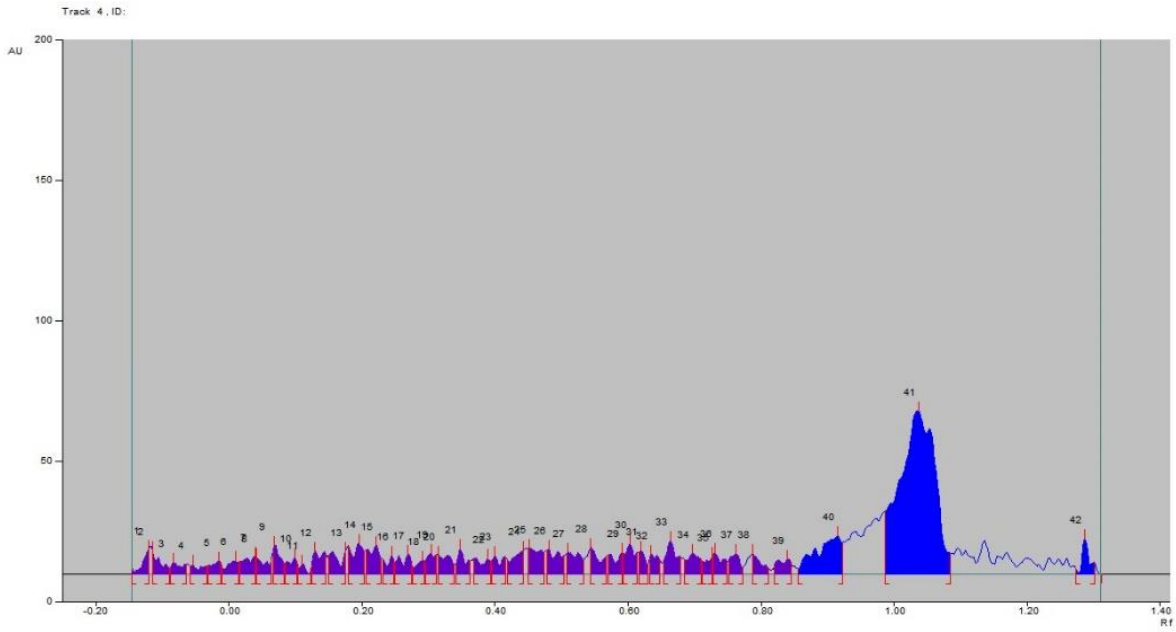

Figure 22: Scan of a blank sample; violet marked peaks were used for the S/N ratio, the blue peaks represent the solvent front

3.2.6 Linearity

General speaking, the aim of a method is to obtain a linear relationship between the results of a test and the concentration of a sample. Optical signals not necessarily give a linear relationship of the results. However, for the quantification, a linear relationship between concentration and signal would be needed. Equations such as the Kubelka and Munk equation are giving approximate linearity over a certain range of concentration [44]. The functions obtained by calibration in HPTLC are mostly non-linear. In some cases it is still possible to find a quasi-linear calibration in a well-defined range. If this is not possible, a non-linear calibration should be chosen, such as a polynomial [33]. During this work, only non-polynomial functions were used with precise and accurate results.

3.2.7 Robustness

The robustness of a method means the ability to persist little variations of the parameters giving a reference point about the reliability. Being robust means, that the result of an analysis remains unaffected by small unintended deviations. This measure

does not include intended variations with the aim to optimize a method [5]. According to [33], the important parameters are temperature and humidity, small variations in applying (volume, shape and size of the spots), scanning and evaluating the samples, the composition of the reagents and their pH, differences in the production of the plate batches or the conditions of drying. To test the robustness to its full extent was not possible within this work. However, in a practical test with samples from industrial processes as described below, the robustness was tested to some extent.

3.3 Biorefinery samples: A practical test

The new method was not only tested and validated with pure sugar samples, but also with samples from industrial processes containing impurities with the aim to proof the usefulness of the HPTLC-method for sugars in a practical test.

The samples used for the test were from paper mills. The exact composition was unknown, but it was imaginable which sugars the samples may contain. Before the test it was not clear if the sugars could be detected to a full extent, as it could have been possible that some other unknown substances would overlay some of the sugars and therefore distort the result.

3.3.1 Quantification and Repeatability

The first problem was to find a proper dilution for the samples to enable the quantification within the range of the calibration. Undiluted, some of the samples were viscous and dark brown to black. A dilution of 1:50 resulted in an overloaded band for some samples, which means, that the quantification was not possible due to diffusion. Table 9 is showing at which dilution it was possible to quantify the sugars. The table is also showing the sugars contained in each sample.

Cellobiose was found only in “A”, galactose in “B” and “H”, mannose in “A”, “B”, “C” and “H”. Xylose and glucose were found in every sample, while arabinose was not found at all. If a sugar was not found in one sample, it doesn’t mean that it is not existing. It could be possible, that e.g. arabinose could be detectable at a dilution of 1:20, which was not possible to perform because of the high load of glucose leading

to diffused and overloaded bands. The samples were showing high loads of glucose, this is why the samples had to be diluted up to 1:400. At higher concentrations, the height of the peak was outside the calibration range, which means, that a polynomial calibration was inapplicable.

Table 9: Biorefinery derived samples and their dilution to allow a proper quantification; X… polynomial quantification is possible

		Cellobiose	Glucose	Galactose	Mannose	Arabinose	Xylose
A	1:50	X					X
	1:100	X			X		X
	1:200	X			X		X
	1:400	X	X		X		
B	1:50		X	X	X		
	1:125		X	X	X		
	1:300		X	X	X		X
C	1:50				X		
	1:100				X		X
	1:200				X		X
	1:400		X		X		X
D	1:50						X
	1:100		X				X
	1:125		X				
E	1:50						X
	1:100						X
	1:125						X
	1:200		X				
F	1:50						X
	1:100						X
	1:125						X
	1:200		X				
G	1:50						X
	1:100						X
	1:125		X				X
H	1:50		X	X			X
	1:100		X	X	X		X

Table 10 is showing the results for the quantification of glucose. The samples were diluted according to table 9. The samples were applied on 3 different chromatographic plates and quantified after the detection. The results for glucose are stable, only “G” is showing a relative standard deviation above 10 %. The loads are different with the highest concentration of glucose in sample “C” with more than 300 mg / ml. Especially sample “H” is showing good results with a relative standard deviation less than 2 %.

Table 10: Quantification of glucose in biorefinery samples; Dilution according to Table 9

Glucose			
Samples	Average [mg/ml]	Std. Dev. abs. [mg/ml]	Std. Dev. rel. [%]
A	224.60	17.83	7.94
B	20.08	1.59	7.91
C	317.98	22.48	7.07
D	68.43	2.92	4.27
E	116.59	7.21	6.19
F	135.64	6.47	4.77
G	108.62	13.06	12.02
H	27.99	0.50	1.77

The results for xylose can be found in table 11. Xylose yields more variable results than glucose with higher relative standard deviations. Anyway, the concentration of xylose for “D” and “E” is too low for a proper quantification, hence, the standard deviation is quite high. These results should not be taken into account. All other samples gave a relative standard deviation of 10 % or less. Again, it is “H” with the best results with a variation of only 0.20 %.

Table 11: Quantification of xylose in biorefinery samples; Dilution according to Table 9

Xylose			
Samples	Average [mg/ml]	Std. Dev. abs. [mg/ml]	Std. Dev. rel. [%]
A	7.38	0.74	10.05
B	225.62	18.60	8.24
C	71.05	1.38	1.94
D	2.18	0.57	25.94
E	1.36	0.52	38.67
F	9.27	0.74	8.04
G	13.23	0.73	5.54
H	28.55	0.06	0.20

A quantification of mannose was possible for “A”, “B”, “C” and “H” (compare Table 12). Only C is showing a very high variation of 38 % even though the concentration seems to be high enough. The other samples gave good results with higher loads of mannose. Galactose was found only in “B” and “H”. The concentration was quite low, hence, the variation is high according to table 13. Table 14 is showing the results for cellobiose, which was found in “A” only. The concentration is low. Still, galactose and cellobiose are proofing the high sensitivity of the method to detect even low amounts of sugars.

Table 12: Quantification of mannose in biorefinery samples; Dilution according to Table 9

Mannose			
Samples	Average [mg/ml]	Std. Dev. abs. [mg/ml]	Std. Dev. rel. [%]
A	68.80	7.76	11.28
B	32.29	1.36	4.21
C	9.41	3.58	38.05
D			
E			
F			
G			
H	46.25	2.63	5.69

Table 13: Quantification of galactose in biorefinery samples; Dilution according to Table 9

Galactose			
Samples	Average [mg/ml]	Std. Dev. abs. [mg/ml]	Std. Dev. rel. [%]
A			
B	1.67	7.64	21.83
C			
D			
E			
F			
G			
H	0.62	3.83	16.33

Table 14: Quantification of cellobiose in biorefinery samples; Dilution according to Table 9

Cellobiose			
Samples	Average [mg/ml]	Std. Dev. abs. [mg/ml]	Std. Dev. rel. [%]
A	0.57	3.26	17.55
B			
C			
D			
E			
F			
G			
H			

3.3.2 Specifity/Selectivity

As the biorefinery samples contain matrix impurities, it was not clear, whether the selectivity would allow a proper separation of the sugars and the other substances of the samples. Figure 23 is showing a chromatogram, where the samples were not diluted to the final extent. It can be seen, that “A” and “B” are showing a long shadow from the start of the chromatographic development until the solvent front. It is obvious, that the substance, which is causing this shadow, is overlaying the sugars and, hence,

incorrectly increasing the amount of sugar found in the substance. By using a higher dilution, the shadow disappeared and the negative effect was decreasing to a minimum.

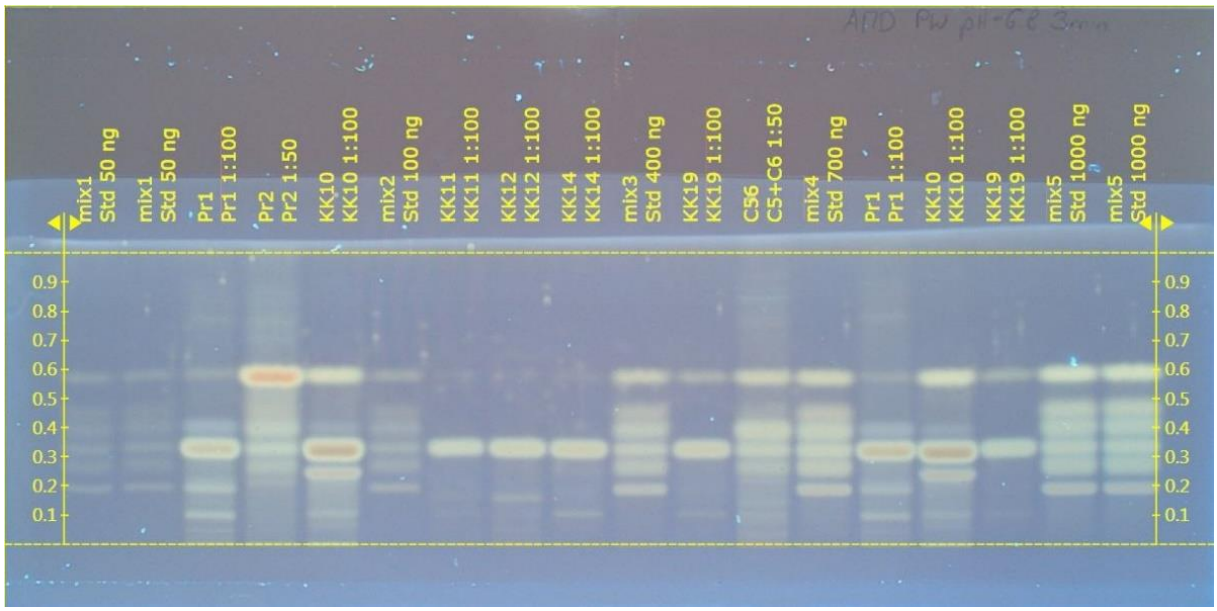

Figure 23: HPTLC of biorefinery derived samples with too high loads

Another effect that can be seen in figure 23 is the high load of glucose in “A” and “C” with a dilution of 1:100, and of xylose in “B” with a dilution of 1:50. The peaks of these bands first of all were outside the calibration range and second, were overlaying the neighbouring sugar.

3.3.3 Accuracy

The accuracy test was performed with “A” and “H”. By applying the pure samples, the pure standard solution and a mixture of both, the method is tested for accurate results. A defined amount of the sugar mix is added to the sample solution, hence, the approximate result is known.

Table 15 and table 16 are showing the results of the accuracy test for the samples “A” and “H”. Sample “A” is a sample with a low matrix containing only glucose and mannose. The result of the accuracy for glucose was 94 % and for mannose 104 %.

“H” is a sample with a matrix containing galactose, glucose, mannose and xylose. The results reached an accuracy of 93 to 97 %. The results are highly accurate, the quantification with this method is reliable.

Table 15: Results of the accuracy test for the sample “A” diluted 1:400 and calibrated by height

Substance	Expected [ng]	Detected [ng]	Accuracy [%]
Glucose	654	693	94.37
Mannose	300	288	104.17

Table 16: Results of the accuracy test fort the sample “H” diluted 1:100 and calibrated by height

Substance	Expected [ng]	Detected [ng]	Accuracy [%]
Galactose	291	295	94.37
Glucose	403	416	96.88
Mannose	602	624	96.47
Xylose	398	428	92.99

3.3.4 Robustness

To proof how robust the method in this practical test is, the results of the quantification for different dilutions are considered. As it was not clear in the beginning of the practical test, which dilution would be needed for each sample, a lot of tests have been performed giving finally the chance to compare results of different dilution. There have been 4 successful tests with these samples. Table 17 is showing the dilution of each sample in the particular test.

Table 17: Dilution of each sample as considered for robustness test

Dilution				
	Test # 1	Test # 2	Test #3	Test #4
A	1:50	1:100	1:400	1:400
B	1:50	1:50	1:300	
C	1:50	1:100	1:400	
D	1:50	1:100	1:125	
E	1:50	1:100	1:200	
F	1:50	1:100	1:200	
G	1:50	1:100	1:125	
H	1:50	1:50	1:100	1:100

Table 18 is showing the results of the quantification for glucose. The results for “H” are stable. The average concentration of glucose is 28.96 mg/ml with a relative standard deviation of 5.18 %. The sample “B” was diluted 1:50 in the first two tests, but 1:300 in the third one, which was necessary for the quantification of xylose. This higher dilution might be the reason for the high standard deviation of 14.44 %. The quantification of glucose for “A” is showing a high content of more than 230 mg / ml with a low standard deviation of about 5 %. The same is true for “C”. The samples “D”, “E”, “F” and “G” are containing between 70 and 135 mg / ml of glucose. The standard deviation for these samples is quite low, “G” is quite high with 12 %.

Table 18: Robustness test, results of glucose in mg/ml

GLUCOSE															
Samples	Test # 1			Test # 2			Test #3			Test #4			Average	Std. Dev. abs.	Std. Dev. rel.
	1.a	1.b	1.c	2.a	2.b	2.c	3.a	3.b	3.c	4.a	4.b	4.c	[mg/ml]	[mg/ml]	[%]
A							240.83	227.47	205.51	236.72	232.79	238.96	230.38	13.08	5.68
B	24.77	27.92	24.40	27.57	28.17	24.30	19.88	21.76	18.60				24.15	3.49	14.44
C							332.30	329.57	292.08				317.98	22.48	7.07
D				70.01	81.50	66.97	71.50	68.11	65.68				70.63	5.72	8.10
E							123.39	117.36	109.03				116.59	7.21	6.19
F							142.14	135.56	129.21				135.64	6.47	4.77
G							122.58	106.57	96.70				108.62	13.06	12.02
H	29.61	28.89	26.89	28.51	31.56	28.06	28.48	27.49	28.01	28.12	30.52	31.39	28.96	1.50	5.18

Table 19 is showing the results for xylose. The standard deviations are higher compared to glucose. Especially “F” has a variation of almost 60 %, but the concentration of 5.63 mg / ml is very low, also for “D” and “E”. Obviously, these amounts are too low for a proper quantification. Again, “H” is showing the best results with a relative standard deviation of only 3.89 %.

Table 19: Robustness test, results of xylose in mg/ml

XYLOSE															
Samples	Test # 1			Test # 2			Test #3			Test #4			Average	Std. Dev. abs.	Std. Dev. rel.
	1.a	1.b	1.c	2.a	2.b	2.c	3.a	3.b	3.c	4.a	4.b	4.c	[mg/ml]	[mg/ml]	[%]
A	6.97	8.03	7.82	10.46	11.33	8.64	7.20	6.75	8.20				8.38	1.57	18.69
B							208.04	245.09	223.74				225.62	18.60	8.24
C				83.81	84.75	93.15	72.48	69.74	70.92				79.14	9.48	11.98
D	2.60	1.85	2.12	2.27	2.93	1.32							2.18	0.57	25.94
E	2.14	1.33	1.02	1.65	1.39	0.61							1.36	0.52	38.67
F	3.22	3.03	2.85	8.61	3.26	1.94	8.52	10.01	9.27				5.63	3.34	59.25
G	13.31	12.49	12.20	17.80	16.58	16.52	12.43	13.38	13.88				14.29	2.11	14.75
H	27.59	29.10	29.18	30.24	28.99	30.32	28.61	28.53	28.50	28.46	31.05	31.27	29.32	1.14	3.89

Table 20 is showing the results for mannose. In the samples “D” to “G” there was no mannose found at all. Sample “A” is showing the highest content of this sugar with more than 70 mg / ml and a standard deviation of 9 %. “B” contains 32 mg / ml showing the lowest relative standard deviation of only 4 %. The content of mannose in “C” is too low for a proper quantification, as a result the deviation reaches almost 40 %. Again, “H” has very stable results with a content of mannose of almost 50 mg / ml.

Table 20: Robustness test, results of mannose in mg/ml

MANNOSE															
Samples	Test # 1			Test # 2			Test #3			Test #4			Average	Std. Dev. abs.	Std. Dev. rel.
	1.a	1.b	1.c	2.a	2.b	2.c	3.a	3.b	3.c	4.a	4.b	4.c	[mg/ml]	[mg/ml]	[%]
A							77.11	67.56	61.74	77.57	75.97	76.77	72.79	6.59	9.05
B							32.11	33.73	31.02				32.29	1.36	4.21
C							12.72	5.61	9.90				9.41	3.58	38.05
D															
E															
F															
G															
H							47.86	43.22	47.69	46.79	51.86	52.17	48.26	3.36	6.96

The results for galactose can be found in table 21. The sugar was detected only in “B” and “H”. In both samples the content is about 20 mg / ml and a relative standard deviation of 7 %.

Table 21: Robustness test, results of galactose in mg/ml

GALACTOSE															
Samples	Test # 1			Test # 2			Test #3			Test #4			Average	Std. Dev. abs.	Std. Dev. rel.
	1.a	1.b	1.c	2.a	2.b	2.c	3.a	3.b	3.c	4.a	4.b	4.c	[mg/ml]	[mg/ml]	[%]
A															
B							19.92	23.01	22.55				21.83	1.67	7.64
C															
D															
E															
F															
G															
H							16.17	17.02	15.80	16.53	17.91	18.78	17.04	1.12	6.60

3.4 Acid Hydrolysis: Procedure and Optimization

So far, it was shown that the developed HPTLC method is able to analyse mixtures of carbohydrates even in the presence of impurities. For biorefinery processes, it is also important to monitor the overall content of different carbohydrates (hemicellulose, cellulose) of the lignocellulosic biomass e.g. by total hydrolysis to monomeric components. As HPTLC is able to separate monomeric and oligomeric compounds as well as degradation products at the same time, it is a suitable approach to monitor the hydrolysis. Different hydrolysis protocols are available from literature and where compared with respect to completeness and possible side reactions.

First, the hydrolysis was performed according to TAPPI T 249 cm-00, which is the standard method of hydrolysis with a subsequent detection of monomers with gas chromatography. For this method, $Ba(OH)_2$ is used for the neutralisation of the samples. The following HPTLC was unsatisfying, as can be seen in figure 24. The bands showed tailing and a wave-effect. The R_f-values were not stable and fluctuated even within one plate. This effect was put down to the high load of salts in the samples due to neutralisation. The TAPPI standard T249 cm-00 might be suitable subsequent

analysis of the monomers by gas chromatography, but it is not suited for TLC chromatography.

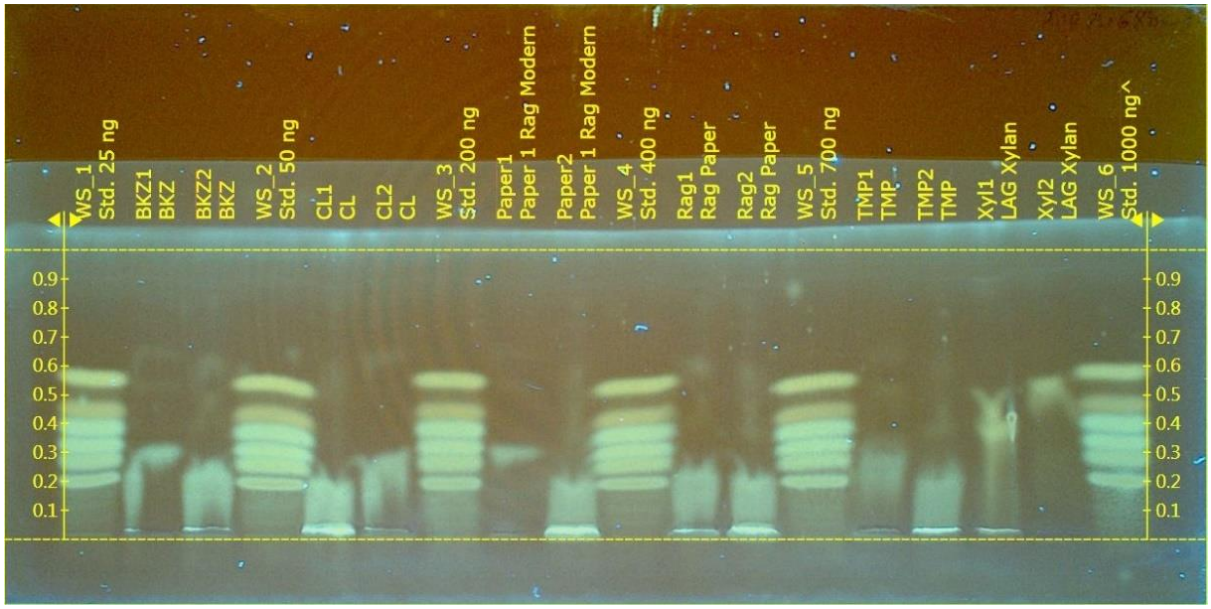

Figure 24: HPTL of pulp and paper samples hydrolysed according to TAPPI T-249 cm-00 after derivatization

A more appropriate method of hydrolysis was found in the investigations of Bose and co-workers [45], who were using a 72% sulfuric acid in a two-step hydrolysis. In the first step, the tubes were put in a water bath of 25°C for 2 hours. The suspension was stirred every 15 minutes. The second step of hydrolysis was performed in the water bath at 80 °C for 1 hour without stirring.

After the acid hydrolysis, the samples have to be neutralized to enable a proper separation on HPTLC. In batch A, 300 µl of the hydrolysates' supernatant was extracted and transferred into a vial. Then, 750 µl of NaOH (4M) and subsequently 450 µl of a saturated solution of $NaHCO_3$ were added. After the neutralization, the pH should be 7. Each 100 µl of this sample solutions were mixed with 400 µl water (1:5) before they were applied to the HPTLC plate.

The samples obtained by Bose's method did not show any negative effect during the HPTLC. However, it was found that the pulp and paper material was not fully converted into its monomeric compounds, as cellobiose was detected during HPTLC. Obviously, the hydrolysis was incomplete. Bose's hydrolysis was modified with one crucial

modification: during the second step of hydrolysis, mechanical energy was added by stirring. This little modification allowed a complete degradation of the pulp material. As shown in figure 25, no oligomeric compounds were detected in the qualitative analysis.

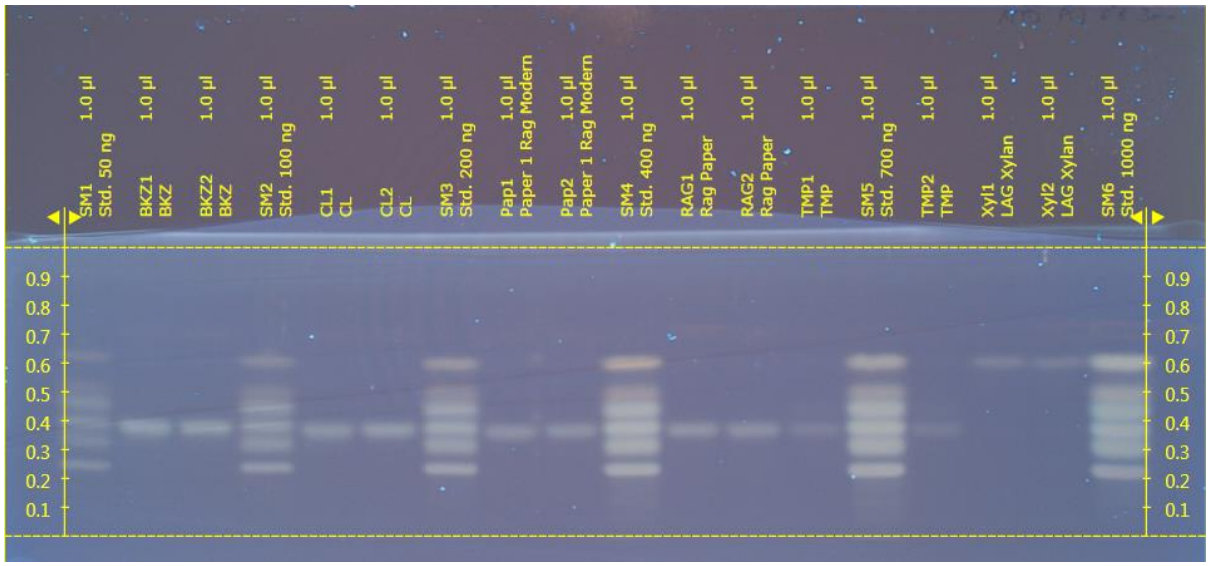

Figure 25: HPTLC of pulp and paper samples hydrolysed according to a modified method of hydrolysis based on investigations of Bose [45] after derivatization

The results of the quantification for batch A can be found in table 22 and 23. It is obvious, that the calibration by area gives more stable results, as the standard deviation is much better than by height. The samples 1 and 2 of each sample were prepared exactly the same way, but in different vials. The results for the two samples of sulphite dissolving pulp (BKZ) and paper rag modern (Paper) are very similar. The standard deviation is less than 9 %. The quantification of glucose for thermomechanical pulp (TMP) was not satisfying, the standard deviation is too high, as the amount of substance found in the sample was too low. Cotton linters (CL) gave standard deviations less than 7 %, but obviously some mistake happened in the preparation of the sample material, as the difference between CL1 and CL2 is significant.

Table 22: Results of the quantification of batch A for glucose, calibrated by height

Samples	Average [g/g]	Std. Dev. Abs. [g/g]	Std. Dev rel. [%]
BKZ1	0.93	0.07	7.78
BKZ2	0.95	0.05	5.49
CL1	0.65	0.04	6.27
CL2	1.07	0.02	1.83
Paper1	0.68	0.04	6.42
Paper2	0.65	0.06	8.58
Rag1	0.79	0.09	11.09
Rag2	0.68	0.07	10.39
TMP1	0.10	0.07	63.54
TMP2	0.12	0.04	35.12

Table 23: Results of the quantification of batch A for glucose, calibrated by area

Samples	Average [g/g]	Std. Dev. Abs. [g/g]	Std. Dev rel. [%]
BKZ1	1.03	0.08	7.98
BKZ2	1.03	0.02	2.02
CL1	0.83	0.03	4.08
CL2	1.19	0.03	2.65
Paper1	0.85	0.00	0.45
Paper2	0.83	0.06	6.74
Rag1	0.88	0.06	6.43
Rag2	0.85	0.04	5.21
TMP1	0.26	0.04	16.41
TMP2	0.24	0.01	3.78

The difference between CL1 and CL2 was very likely caused by an erroneous neutralization process, as it was quite difficult to adjust the pH with the saturated solution of $NaHCO_3$. Therefore, batch B was prepared slightly different. An aliquot of 150 µl was mixed with 310 µl of 4 M NaOH and a tiny amount of solid sodium hydrogen carbonate was added. Each 100 µl of this sample solutions were mixed with 400 µl water (1:5) before they were applied to the HPTLC plate.

Batch B was performed with cotton linters and xylane. The calibration by height gives about 0.9 g of glucose per 1 g of cotton linters and about 0.88 g of xylose per 1 g of xylane, i.e. a recovery value of approximately 90 % for glucose and 88 % for xylose is obtained. The calibration by area shows different results. The calculated amount of sugar per 1 g of the sample material is 1.09 g of glucose for cotton linters and 0.93 g of xylose for xylane. The recovery value in this case is 109 % for glucose and 93 % for xylose. The results include the conversion factor polymer to monomer.

4 Conclusion

Lignocellulosic biorefinery concepts require fast and accurate analytical methods for mixtures of carbohydrates derived from industrial processes. Matrix free mixtures of monosaccharides can be readily analysed by conventional chromatographic methods such as gas chromatography (GC) or high-performance liquid-chromatography (HPLC). However, samples with a more complex matrix, a high load of degradation products and inorganic impurities may cause problems with GC or HPLC. In this work high-performance thin-layer chromatography (HPTLC) was tested as an alternative system for the analysis of carbohydrates in more complex mixtures of hydrolysed lignocelluloses.

It was attempted to develop an optimized method of HPTLC with respect to the challenges mentioned above. Based on previous studies, each step was investigated and improved. Different commercial plates were tested resulting in a decision for the layer silica gel 60 F_{254} AMD extra thin. The impregnation of the stationary phase is essential for the separation of sugars on silica gel. It could be shown that the pH of the reagent has a major influence on the sensitivity of the layer. The position of the plate during the process of drying is very important. Only after drying in an upright position, the optical evaluation of the chromatographic plate after the development was satisfactory. The application process is a fully automated step in HPTLC, still some optimization was achieved. For a successful development, the same volume of each sample had to be applied. Somehow, different application volumes on the same plate were resulting in irregular bands.

The development is the core process of planar chromatography, where the substances of a sample are separated. Choosing the appropriate solvents in a right order was one of the major challenges. By using the solvent acetonitrile, water, pentanol in a first step and the solvent acetonitrile, water, butanol in a second step, best results were achieved. Different derivatization reagents were tested. Aniline diphenylamine showed the best results. Finally, the chromatographic plates were evaluated using modern equipment and software. All investigated sugars were separated successfully allowing both, a qualitative and quantitative analysis.

In a next step, the method was validated according to the ICH guideline for validating an analytical method. The guideline includes categories such as selectivity, accuracy, precision, limit of detection and limit of quantification. The tests of selectivity showed a complete separation of glucose, galactose, mannose, arabinose, xylose and cellobiose even under varying conditions and in presence of unknown substances.

The accuracy was tested for glucose and xylose with results of close to 100 %. The parameters repeatability, long-term comparison and reproducibility between laboratories fall into the category precision. Only the repeatability could be tested showing a standard deviation of about 10 % for the calibration by height, but only around 6 % for the calibration by area. The discrepancy between these results was discussed. Regarding the repeatability it is highly recommended to use the calibration by height, as the results are better to compare between laboratories, even though the recovery values may suffer slightly.

Previous studies reported a limit of detection in the range of 50 ng for the use of *N*-(1-Naphthyl)ethylenediamine as a derivatization reagent. In this work, the limit of detection with 9 ng and limit of quantification with 27 ng were located in the very low range of nanograms. These results can be attributed to the use of aniline diphenylamine in the derivatization process.

To show the method's practicability, it was tested with samples derived from industrial processes, which had impurities and an unknown overall composition. After finding the correct dilution for each sample, the substances were quantified with a standard deviation of less than 10 %. The results were accurate with accuracy levels between 93 and 104 %. The results of the robustness test confirmed the high reliability of the method.

To find a good procedure for the complete hydrolysis of lignocellulosic samples such as pulp or paper was another challenge of this work. Current standard methods for the acid hydrolysis of lignocellulosic samples were causing problems when applied for HPTLC due to the high content of salt. Other reported methods were not fully converting pulp or paper into its monomeric compounds. By modifying the procedure,

a complete degradation was achieved, as no oligomeric compounds were detected in the following qualitative analysis.

The method presented in this work provides a useful tool of analysing monosaccharides in a rapid, precise and accurate way. It was carefully developed and validated, before it was tested with impure samples in a practical test. This work showed that high-performance thin-layer chromatography does not only complement other chromatographic methods, but is a reliable and fully functional analytical stand-alone method of detecting carbohydrates both in a qualitative and quantitative way.

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ADC……	Automatic Developing Chamber
AMD ……..	Automated multiple development
ATS ……	Automatic TLC Sampler
GC……...	Gas Chromatography
GLC…….	Gas-Liquid Chromatography
HPLC……..	High-Performance Liquid Chromatography
HPTLC……	High-Performance Thin-Layer Chromatography
LOD……..	Limit of Detection (or detection limit)
LOQ……..	Limit of Quantification (or quantification limit)
MS……	Mass Spectrometry
PC ……	Paper Chromatography
S/N……	Signal-to-Noise ratio
TL ……	Thin-Layer
TLC……...	Thin-Layer Chromatography
TTC……...	Twin-through chamber
UV-VIS……	Ultra-Violet visible