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ADVANCED ANALYTICAL STRATEGIES FOR IN DEPTH CHARACTERISATION OF WHEAT BRAN FRACTIONS

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Abstract

Wheat bran comprises an underutilized resource. As a byproduct of white flour production, it is used primarily as animal fodder. This work was conducted as part of a project that deals with the biorefinery of wheat bran investigating advanced analytical strategies for in depth characterization of wheat bran fractions. The approach taken was to establish three sets of tools: structural analysis of polysaccharides, visualization techniques and rapid screening methods.

Methylation analysis followed by methanolysis revealed the linkage of sugars constituting arabinoxylan and was applied to characterize the selectivity of arabinosidases rendered from *Lactobacillus brevis*.

For visualization, antibody-based fluorescent labeling of arabinoxylan coupled with confocal laser scanning microscopy was established. This allowed for the spatial resolution of arabinoxylan within a wheat bran sample after selected pretreatments.

Finally, a rapid screening method based on near- and mid-range infrared spectroscopy using multivariate calibration was developed for nutritional parameters of wheat bran intended to facilitate income control. These were: water, protein, ash, starch, insoluble as well as soluble dietary fiber and lipids.

The established techniques were applied in a concerted effort for the thorough elucidation and assessment of changes in wheat bran and fractions thereof.

Additionally, the synthesis of a fluorescent label to measure oxidative damage perpetrated on polysaccharides during pretreatments was attempted. Various synthetic routes were tried, but none could be brought to fruition. They are detailed to serve as a basis for future investigations.

Kurzfassung

Weizenkleie stellt eine unzureichend genutzte Ressource dar. Als Nebenprodukt der Weißmehl-Produktion wird es aktuell vor allem als Tierfutter verwendet. Diese Arbeit wurde im Rahmen eines Projekts durchgeführt, das sich mit der Bioraffinerie von Weizenkleie beschäftigt, und untersuchte fortschrittliche, analytische Strategien zur tiefgehenden Charakterisierung von Weizenkleie Fraktionen. Unsere Herangehensweise war es, drei Arten von Werkzeugen zu etablieren: Strukturanalyse von Polysacchariden, Visualisierungstechniken und schnelle Screening-Methoden.

Methylierungsanalyse gefolgt von Methanolyse zeigte die Bindung von Zuckern auf, aus denen Arabinoxylan besteht, und wurde angewendet, um die Selektivität von Arabinosidasen aus *Lactobacillus brevis* zu charakterisieren.

Zur Visualisierung wurde Antikörper-basierte Fluoreszenzmarkierung von Arabinoxylan in Verbindung mit konfokaler Laser Scanning Mikroskopie etabliert. Diese ermöglichte die räumliche Auflösung von Arabinoxylan in einer Weizenkleieprobe nach verschiedenen Vorbehandlungen.

Schließlich wurde ein schnelles Screening-Verfahren auf Basis von Nah- und Mittelwelleninfrarotspektroskopie mit multivariater Kalibrierung für Ernährungsparameter von Weizenkleie etabliert, um Eingangskontrollen zu erleichtern. Folgende Parameter wurden kalibriert: Wasser, Eiweiß, Asche, Stärke, unlösliche sowie lösliche Ballaststoffe und Lipide.

Die etablierten Techniken wurden gemeinsam zur gründlichen Aufklärung und Bewertung von Veränderungen in Weizenkleie und Weizenkleiefraktionen angewendet.

Darüber hinaus wurde die Synthese eines Fluoreszenzmarkers unternommen, der den oxidativen Schaden an Polysacchariden durch Vorbehandlungen messbar machen sollte. Verschiedene Synthesewege wurden ausprobiert, jedoch konnte keiner realisiert werden. Diese werden diskutiert, um als Grundlage für weitere Forschung zu dienen.

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1 Introduction

1.1 A brief introduction to biorefinery

While the first documented recovery and use of oil goes back some two and a half millennia, it wasn't until 1745 that the first attempt at distilled fractionation, albeit very simplistic, was described for lamp oil from petroleum. Yet only the rising energy demands during the industrial revolution in the middle of the 19th century propelled the development to bigger scales and widespread use. The 1940s, almost a century later, saw the emergence of a new generation of oil refineries based on multiple classes of products. Olefins and aromatics obtained through cracking of the high-boiling fraction of crude oil were developed into suitable building blocks for plastics, solvents and adhesives among others. The rise of the petrochemical industry helmed most of the technological advances we consider household commodities today. (Matar & Hatch, 2001)

In the 1970s, first scientific concerns were voiced about the lavish release of carbon dioxide from non-renewable resources leading to global warming. It is not without irony that the first plant-based multi-product refinery, built in the same decade, was not conceived as a substitute for oil refineries but as a factory for the production of high-fructose corn syrup.

The onset of the so-termed 'green revolution' in the 1990s rang the bell for renewable energy and thus led to the development of biorefineries based on first-generation biomass, i.e. food crops, primarily corn and soy. Centerpiece products are currently biodiesel, ethanol and polylactic acid. While these biorefineries do rely on renewable resources, their competition with the food market poses ethical questions. Therefore, the utilization of second-generation biomass, i.e. forest and agricultural residues, food and wood waste as well as energy crops, is being pursued (Lynd, Wyman, Laser, Johnson, & Landucci, 2005).

In theory, the carbon-based synthesis in nature would be easily sufficient to cover the world energy demands. E.g., cellulose production alone is estimated at 10^{11}-10^{12} tons per year (Klemm, 1998). Even though second-generation biorefineries are technically feasible, none have been successfully realized on an industrial scale as of yet, because their profitability is not deemed by ecological benefits, but by the ability to compete with prize and quality of oil-based fuels and petrochemicals, in which regard the oil industry draws from a significant head start. As of 2015, about 80% of global energy needs, more than 90% of organic chemicals and more than 95% of fuels are covered by and produced from oil (Rodrigue, Comtois, & Slack, 2013).

Global warming is tightly bound to the release of carbon dioxide into the atmosphere, to which fossil fuels are the major contributor. From the beginning of the Industrial Revolution to

2014, the concentration of carbon dioxide has gone from 280 ppm to 401 ppm (Meinshausen et al., 2009). We are beyond the point where global warming could still be prevented, but even a 2 °C rise in temperature, which is currently being considered the optimistic scenario, would require a stabilization of the current carbon dioxide levels, i.e. global, carbon-neutral operations henceforth (Meinshausen et al., 2009). Yet, we are presently running at a net increase of atmospheric carbon dioxide of 18 billion tons per year and the emerging economies and growing populations of both China and India are expected to raise worldwide energy demands by over 30% within the next 25 years (Agency, 2013). There is pressing need for the switch to a sustainable resource.

The terminology implies analogous approaches in oil refineries and biorefineries, yet they are inherently different. Oil mainly poses a mixture of desirable end products that can be separated through distillation and tailored through cracking of long-chain hydrocarbons into shorter ones. In comparison, second-generation biomass faces some intrinsic properties that demand intensive and diverse treatments, which nullify the low cost and abundance of the source material.

Biomass usually boasts a recalcitrant matrix, the constituents of which are both non-volatile and thermolabile, which complicates fractionation. Additionally, fractionation alone is rarely sufficient to obtain a valuable product. In the case of polylactic acid (PLA), starch needs to be isolated from a complex matrix such as corn and tapioca, monomerized, fermented and finally polymerized (Sin, Rahmat, & Rahman, 2012). Production so far is only cost-effective from first-generation biomass with high starch content. Cellulose, which is abundant in second-generation biomass, is inherently harder to degrade than starch, since the former is employed by nature as a structural building block whereas the ladder is used as energy storage and hence needs to be accessible by design.

The most successful practice of a biorefinery to date comprises the kraft pulp mill. It achieves selective isolation of cellulosic fibers from wood while recycling most of the chemicals used. With the logistics established and most of the infrastructure already in place, it is regarded as one of the most promising approaches to upgrade a kraft pulp mill by integrating a wood biorefinery into its process that makes use of hemicelluloses, lignin and salts (Van Heiningen, 2006).

As an alternative approach, thermal conversion of biomass into char, oil and gas is currently being investigated intensely. The main processes are combustion, gasification and pyrolysis.

Combustion, i.e. burning, only produces energy from the conversion of biomass to carbon dioxide and water in the presence of air. This is the simplest and oldest conversion for biomass, however it is limited by a small lower heating value, low energy density and a lack of fuel-like products (Demirbas, 2004).

Gasification of biomass employs a controlled amount of oxygen at temperatures above 700 °C. In addition to the exothermal energy of the reaction, about 80% of the biomass is converted to syngas, a mixture of carbon monoxide, carbon dioxide and hydrogen, which can be used as a fuel (Higman & Burgt, 2008).

Pyrolysis exposes biomass to 400-550 °C under inert atmosphere for 1-2 s. Particles need to contain less than 10% moisture and be smaller than 2 mm in diameter for the heat to be able to penetrate fully. The main product is pyrolysis oil followed by gasses, char and water, which quite detrimentally mixes with the pyrolysis oil. Pyrolysis oil is a complex mixture of more than 400 compounds. Its low pH and high oxygen content lead to a low shelf life and poor thermostability as well as corrosive properties, which put a lot of strain on the processing facilities. For better storability and possible use as fuel, pyrolysis oil needs to be upgraded, e.g. through catalytic hydrodeoxygenation (Venderbosch & Prins, 2011).

These technologies, while having been feasible for decades, have never been applied successfully on biomass to commercial profit. Heterogeneous source material and high water content are among the major concerns (Bridgwater, 2012).

1.2 Biorefinery of wheat bran

This work was done as part of a Christian Doppler Laboratory for “Innovative Bran Biorefinery”, which is a project to investigate and apply approaches to utilize wheat bran within the concept of a biorefinery. By definition, wheat bran would have to be regarded as first-generation biomass. But as a byproduct in the production of white flour it only plays an insignificant role in human nutrition and is almost exclusively used as animal fodder. Therefore it is generally understood to be second-generation biomass.

Wheat bran is composed of three distinct layers. From the outside moving inwards they are the pericarp containing epidermis, hypodermis as well as cross and tube cells, the seed coat made up of testa and nucellar layer and the aleurone layer comprising aleurone cells. The protective function of the outer layers is on the one hand achieved through a barrier of dead, elongated cells stacked at right angles with thick cell walls and on the other hand through embedding of inhibiting enzymes to ward off pathogens. The living tissue of the aleurone layer harbors metabolic enzymes that initiate the germination process as well as the

bulk of antioxidants and still a few protective enzymes as a last fence before the endosperm (Jerkovic et al., 2010).

In the milling process, about 20% of the wheat kernel is separated as bran. The ratio heavily depends on the settings and efficiency of the mill, since it is desirable to minimize residual endosperm in the bran while keeping the flour free from fibrous particles. This, in combination with seasonal and genetic variety, makes wheat bran prone to large fluctuations in composition. Roughly, wheat bran contains 15% protein, 15% water, 5% lignin, 5% minerals, 3% fat and 57% carbohydrates. The carbohydrate fraction is made up of 52% arabinoxylan, 25% starch, 18% cellulose and 5% β-glucan (Sun, Liu, Qu, & Li, 2008). While the amount of carbohydrates is comparable to whole cereals, wheat bran differs in that it is mainly composed of dietary fiber and only little starch. Nutritionally, this is its strongest asset but at the same time its current downfall. Despite a plethora of proven health benefits of dietary fiber (Anderson, 2004; Aune et al., 2012; Liu, 2002; Painter, 1982), it is the fibrous texture and bitter taste that thwarts consumer acceptance (Delcour & Poutanen, 2013; Saricoban, Yilmaz, & Karakaya, 2009). Within our project, Prückler et al. (2014) have compiled a detailed account of possible functionalization strategies to overcome the detriments of wheat bran in food, but none of them is currently marketable. In animal feed, the lack of starch is critical to high growth rates and therefore wheat bran can only be employed to a limited extent (Taylor-Pickard & Spring, 2007). This dilemma, however, opens the door for a biorefinery approach of utilization, which can be two-fold:

One, fractionating wheat bran into its constituents and utilizing them as such. Especially protein and arabinoxylan could be of interest in this scenario. At 15%, wheat bran has a protein content that is relatively high for cereals and superior in Amino Acid Score (76 for wheat bran compared to 54 for whole wheat (source: www.nutritiondata.self.com)). Thus it would comprise a suitable fortification for animal feed or a vegan alternative to dairy protein supplements in human nutrition. Arabinoxylan is the most abundant compound in wheat bran with about 30%. Since the western diet is largely deficient in dietary fiber, it could be used as a food additive or supplement (Clemens et al., 2012). Analogous formulations already exist for arabinoxylan from rice bran and ensuing health benefits have been demonstrated (Kamiya et al., 2014).

Two, conversion of wheat bran constituents into fine chemicals. Here, the emphasis is on the use of carbohydrates. Polysaccharides, especially glucose-based ones, could be monomerized and fermented to plastic starting materials such as lactic acid (Tirpanalan et al., 2015). However, to rely on starch as a constant resource in wheat bran is a daring assumption, since its presence is regarded as a loss from white flour production and will

likely decrease as milling technology advances. As a pentose-based polysaccharide, arabinoxylan could be converted to furfural or xylitol, which are high-value products (Sarmala et al., 2000; Yemis & Mazza, 2011). Additional valorization strategies have been described in a review, which was published in the journal “LWT – Food Science and Technology” (Apprich et al., 2014). For the full text see chapter 6.1.

Since wheat bran is quite adamantine a matrix, every fractionation necessitates a pretreatment to make its structures more accessible. These pretreatments can be mechanical, chemical or enzymatic. Within the Christian Doppler Laboratory for “Innovative Bran Biorefinery”, comprehensive data for hydrothermal, organosolv and enzymatic treatment was gathered (Reisinger, Tirpanalan, Huber, Kneifel, & Novalin, 2014; Reisinger et al., 2013). A pretreatment is usually followed up with a hydrolysis step that can be based on acid, lye or enzymes and should result in monomerization of carbohydrates or protein.

This work mainly focused on arabinoxylan, as it poses the biggest and, we propose, potentially most valuable fraction in wheat bran. Whether the aim is to isolate arabinoxylan or to remove it to access other compounds, it is invariably necessary to deal with it. High temperatures, strong mechanical forces and aggressive chemicals during treatments bear a strong impact on arabinoxylan. In order to be able to follow these changes, one must first delve into its native structure.

1.3 On the nature of arabinoxylan

Arabinoxylan comprises a structural polysaccharide and therefore can be found exclusively in the cell wall of plants. It is the most ubiquitous hemicellulose in the Poaceae family (true grasses), to which all cereals belong (Albersheim, 2011). Arabinoxylans differ slightly depending on the type of plant, but they all share a common foundation: a linear β-D-1,4-linked xylopyranose backbone with arabinofuranosyl side chains α-L-1,2- and/or α-1,3-linked to it in a random pattern. Galactosyl, rhamnosyl, glucuronic acid and 4-*O*-methyl glucuronic acid units as well as phenolic acids, predominantly ferulic acid, have been identified as possible alternative side chain constituents. Furthermore, these side chains can form a covalent bond to lignin over the phenolic acids (Albersheim, 2011).

Consequently, the nature and frequency of a side chain greatly determine the physicochemical properties of arabinoxylans such as pH, viscosity, solubility and recalcitrance, and plants deploy them accordingly: in wheat bran, the testa and nucellar epidermis show an arabinose to xylose ratio of only 0.13 compared to 1.12 for the outer pericarp, where high rigidity is required (Parker, Ng, & Waldron, 2005). Moreover, cross-linked phenolic acids, which are associated with improved cell-to-cell adhesion (Parr,

Waldron, Ng, & Parker, 1996), are found almost exclusively in the pericarp (Parker et al., 2005).

With about 2% of galactose and less than 1% of glucuronic acid, wheat bran arabinoxylan contains only minuscule amounts of sugars other than arabinose and xylose. About 38% of xylose units are unsubstituted, 23% are mono- and 39% are di-substituted. Arabinose side chains are chiefly composed of a single arabinose unit. Only 25% of arabinose units are involved in oligomeric side chains (Edwards, Chaplin, Blackwood, & Dettmar, 2003). Figure 1 shows a typical section of the structure of arabinoxylan.

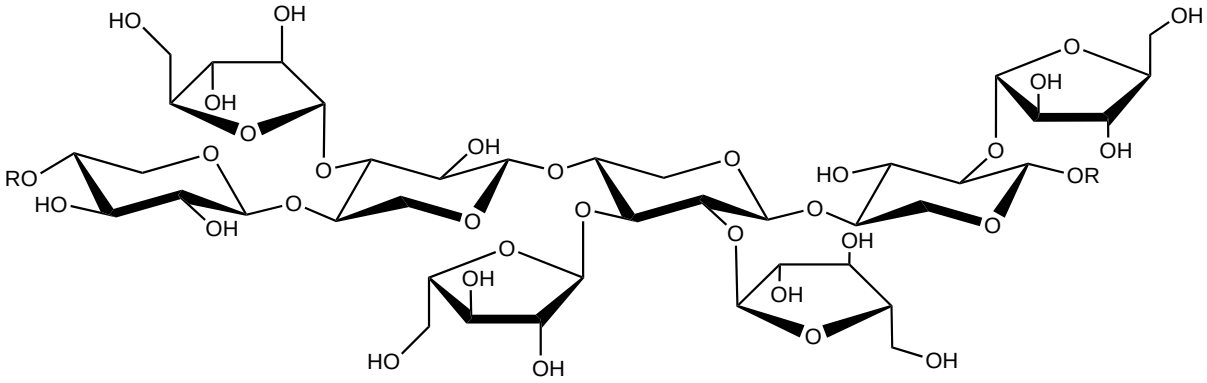

Figure 1. Structural elements of arabinoxylan

This intricate structure and hence the overall properties of arabinoxylan are undoubtedly affected by almost any treatment employed within the frame of a biorefinery. Taking further into consideration that arabinoxylan represents the largest fraction in wheat bran, it is integral not only to be able to follow structural changes, but also to be able to monitor its path in the course of processing as meticulously as possible.

2 Aim of the thesis

As part of a project that deals with the biorefinery of wheat bran, the aim of this thesis was to establish analytical strategies in order to be able to elucidate and characterize changes in wheat bran constituents effected by treatments applied in the biorefinery approach. Since the most suitable progression of treatments for wheat bran was only to be elucidated in the course of the project, we set out to deliver a broad overview over analytical methods and then institute tools that provide key information yet are applicable to a wide variety of processes. Within our purview were: structural analysis, visualization techniques and rapid screening methods.

3 Results and Discussion

3.1 Literature review

The topic of this work was initially broached with extensive literature research, which resulted in the publication of two review papers.

3.1.1 Valorization strategies for wheat bran

As already mentioned earlier, several potential valorization strategies for wheat bran were investigated and compiled in a review published in the journal “LWT – Food Science and Technology” (Apprich et al., 2014). For the full text see chapter 6.1.

Briefly, products from either the carbohydrate or non-carbohydrate fraction were considered. As feasible extracts from the carbohydrate fraction were identified arabinoxylan, starch and β-glucan. They could be used as functional food ingredient and dietary fiber supplement. Possible derivatives thereof via fermentation or acid and thermal treatment were lactic and succinic acid as acidulants or biopolymer starting material, acetone, ethanol and butanol as biofuels and solvents, and furfural and hydroxymethylfurfural as fine chemical building blocks. From the non-carbohydrate fraction was discussed the extraction of protein, wheat bran oil and ferulic acid as food additives and anti-oxidant supplement. Vanillin as a flavoring agent and amino acids as food and feed additives were envisioned as follow-up products thereof.

3.1.2 Modern analytical techniques for wheat bran

A plethora of official, standardized methods for the analysis of dietary fiber have been issued over the years by the Association of Official Analytical Communities (AOAC). However, these methods are merely focused on bulk quantification mostly by means of gravimetric analysis and fail to elucidate or even distinguish dietary fibers. The topic of standard analyses, as well as modern analytical techniques that provide more in-depth information about wheat bran constituents in general and dietary fiber in particular, have been discussed extensively as part of this work in a review article published in the journal of “Trends in Food Science and Technology” (Hell, Kneifel, Rosenau, & Böhmdorfer, 2014). For more details see the full text in chapter 6.2.

3.2 Analytic approach

In order to be able to draw from a broad spectrum of comprehensive data in the assessment of a process, the analytic approach taken in this work was to establish three domains: structural analysis of polysaccharides, visualization techniques and rapid screening methods.

3.2.1 Structural analysis of polysaccharides

Methanolysis allows for the determination of the quantitative composition of polysaccharides and fibers. 2 M hydrochloric acid in methanol is used to cleave glycosidic bonds and methyl glucosides are subsequently silylated and analyzed by GC-MS with sorbitol as an internal standard. Methanolysis thus works analogously to sulfuric acid hydrolysis, which is the more common approach in polysaccharide analysis. However, methanolysis is the milder of the two approaches and infers less degradation on sugars, especially pentoses, which makes it the method of choice for the analysis of arabinoxylan. Also, sample workup is facilitated by the use of easy to evaporate solvents as compared to water in acid hydrolysis, which requires a freeze-drying step (Sundberg, Sundberg, Lillandt, & Holmbom, 1996).

However, methanolysis alone cannot preserve any structural information, since after cleavage, monosaccharides of one type of sugar are identical regardless of their position within the polysaccharide. Methylation can help overcome that obstacle. The intact polysaccharide is permethylated with methyl iodide in alkaline dimethyl sulfoxide prior to methanolysis. Since only free hydroxyl groups are methylated, the pattern of methoxy groups on monosaccharides after methanolysis reveals their linkage within the polysaccharide chain. However, it has to be taken into account that complexity of analysis also scales up with the ability to discriminate. Whereas methanolysis generates four peaks per type of sugar (α-, β- as well as pyranoside and furanoside form respectively), methylation followed by methanolysis generates up to four peaks per type of linkage, e.g. typically four peaks for xylose from arabinoxylan in methanolysis become 20 peaks after methylation and methanolysis.

Despite the specificity of the MS detector, peak identification is only possible by retention time, because the MS cannot discern diastereomers of sugars. Unfortunately, commercially available standards do not cover all types of linkage. Therefore, retention times had to be mapped by measuring standard substances such as xylose, arabinose, xylan and arabinoxylan, a selection that does not suffice to cover all types of linkages individually. However, Laine et al. (2002) have provided a comprehensive summary of retention times for

complete linkage types of xylose and arabinose relative to the internal standard sorbitol. By reproducing the setup used in this work, it was possible to assign every peak. Since the response factors provided by Laine et al. did not give sensible results, they had to be determined manually. The methyl glycosides corresponding to C1,2,3,4/5-linked xylose and arabinose were obtained by methanolysis of the respective monosaccharides. Terminal (C1-linked) xylose and arabinose were produced by methylation of monosaccharides. C1,4-linked xylose was obtained from methylating xylotriose. C1,2,4- and C1,3,4-linked xylose, however, is not commercially available in defined amounts. By assuming the two response factors to be nearly identical due to structural similarities, the response factors could be calculated inversely from the arabinose/xylose ratio of pure arabinoxylan obtained from methanolysis. The response factors showed good reproducibility, as did the overall methylation of arabinoxylan. Since the content of terminal xylose is determined, the average degree of polymerization can also be estimated with this technique.

This method was used to characterize enzyme specificity of arabinosidases from *Lactobacillus brevis* DSM 20054 in the hydrolysis of arabinoxylooligosaccharides, which poses an integral step towards complete enzymatic hydrolysis of wheat bran. The results have been published in the journal of “Applied and Environmental Microbiology” (Michlmayr et al., 2013). For a detailed account see the full text in chapter 6.3.

3.2.2 Visualization

Methylation analysis, like most analytic methods, is an invasive technique, i.e. samples are extracted, depolymerized or analyzed in very selective ways. So as to get a more complete picture, we found it necessary to also be able to evaluate changes in a non-invasive way. Therefore, antibody-based fluorescent labeling of arabinoxylan coupled with confocal laser scanning microscopy was established for wheat bran. This technique is comparable to a two-dimensional enzyme-linked immunosorbent assay (ELISA) assay performed on the cross-section of a sample resulting in a fluorescent map, i.e. an image, of the analyte. This made possible the localization of arabinoxylan and other key constituents in cell material and let us follow their course through digestion and extraction processes on a spatial level. Acid, lye and hydrogen peroxide treatment, ball-milling, extrusion, fermentation and treatment with esterase, xylanase and a combination thereof were examined.

The microscopic observations were backed up by methanolysis and enzymatic peeling, which provided a cross-sectional sugar profile of samples. The results have been published in the journal “Carbohydrate Polymers” (Hell et al., 2015). For the full text see chapter 6.4.

3.2.3 Rapid screening

Within the project, a set of standard analyses was established in order to characterize incoming samples and check them for compositional variation. These standard analyses are very laborious and need to be performed frequently on account of seasonal and production-derived deviation. They are indispensable to be able to gauge the starting material for further processing yet the return of valuable information is rather small compared to the effort required to collect it. This is a classic case of wet-chemical analyses waiting to be replaced by a screening method.

Spectroscopic techniques are usually used as screening methods. Since the method has to be sensitive towards all analytes to be encompassed, it needs to be able to collect a wide range of information in a single spectrum. Extracting meaningful information from the raw data usually requires computer-based deconvolution and multivariate analysis in order to reveal the contribution of specific compounds. Large sets of samples are necessary to train the computer algorithm and establish sound correlation, which is usually the limiting factor for laboratory application.

Near-infrared (NIR) and mid-infrared (MIR) spectroscopy are prevalent examples of such screening techniques. Since both have been used on numerous accounts for relevant parameters in cereal matrices but never for wheat bran in particular, we decided to perform a comparison of the two methods using a range of fermented and untreated samples. Selected parameters were the contents of water, protein, ash, starch, soluble as well as insoluble dietary fiber and lipids. NIR came out as the method of choice for wheat bran and proved to be superior to MIR in both precision and robustness. The overall accuracy achieved was sufficient for our screening purposes. The results were published in the journal “Food Control” (Hell et al., 2016). A full account can be found in chapter 6.5.

3.2.4 Determination of oxidative damage in water-soluble polysaccharides

Basically all pretreatments performed on wheat bran perpetrate a certain amount of damage to the contained polysaccharides. One approach to assess these unwanted changes is to quantify carbohydrate degradation products such as furfural and hydroxymethyl furfural as markers of damage. Since these are usually end-stage products, they are favorably generated from monosaccharides and short-chain polysaccharides, implying that the constitution of the starting material bears great influence on the final measure. Furthermore, quite severe conditions are required for these degradation products to be formed at all (Reisinger et al., 2013). In order to rule out the influence of the starting material

and to be able to also register lighter forms of degradation, it is preferable to account for damage at an earlier stage.

Our approach was to synthesize a fluorescent label that would bind to the site of oxidative damage in polysaccharides, i.e. carboxylic groups, and then separate them from other wheat bran constituents through size-exclusion chromatography according to chain length with fluorescence detection. Hence, we would get a reading of the severity of a process based on both depolymerization and oxidative damage. These trials are detailed in chapter 7.

4 Conclusion

In summary, a versatile toolbox for the characterization of wheat bran fractions was established and employed accordingly. Going beyond what is currently available as standardized methods has proven valuable not only for collecting more detailed information, but also for pointing towards new strategies of optimization.

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Wheat bran-based biorefinery 2: Valorization of products

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ABSTRACT

Wheat bran is multi-layered and consists of different cell types with different chemical compositions. The relatively high content of protein as well as the small amount of lignin is the key differentiator to other lignocellulose containing biomasses. Following the classical route of a biorefinery, bran may be disintegrated to a high extent and separated into fractions of high purity in order to build up new chemicals which serve as precursors for higher polymerized compounds. Secondly, bran contains substances that are *per se* valuables, but need to be further separated and purified.

There are basically two directions of valorization, aiming at carbohydrate and non-carbohydrate based products. Both groups are of great heterogeneity, thus candidates are usually grouped according to their functionality.

Due to this theoretical approach the production of glucose and fermentation to lactic acid, succinic acid or ethanol, the extraction of proteins and production of essential amino acids including γ-aminobutyric acid (GABA), the extraction of ferulic acid (FA) and its conversion to vanillin, as well as the extraction of arabinoxylans (AX) seem to be feasible and of economic relevance, which will be highlighted within this review.

1. Introduction

A great part of the worldwide produced industrial products and energy carriers depends on fossil fuel refinery. However, fossil resources like oil and gas become more and more scarce, so the global economy needs to switch to a new system based on renewable raw materials. In this technological system, biorefineries will take the key position through sustainable processing of biomass into a spectrum of marketable products and energy ("European Biorefinery Joint Strategic Research Roadmap Star-COLIBRI," 2011; Kamm & Kamm, 2007). In addition, it has to be taken into consideration that in the long term, energy production can also be facilitated by the utilization of regenerative energy sources like wind, sun or water, whereas the economy of substances fundamentally depends on compounds, which are currently found in fossil resources and therefore have to be replaced by biomass. Because of the high diversity of possible feedstocks, generally four biorefinery concepts have been suggested: the lignocellulosic feedstock-, the whole-crop-, the green biorefinery and in addition to that, the two-platform concept, where the production of syngas is also included (Kamm & Kamm, 2007).

As an abundantly produced low-cost by-product of the milling industry, wheat bran was traditionally intended mainly for animal feed and for human food, but considering its composition and availability, it could also perfectly serve as a feedstock in a possible biorefinery (Cherubini, 2010; Javed et al., 2012). Whilst the enzymatic hydrolysis of the starch fraction is easily done, the more recalcitrant compounds like cellulose and hemicelluloses must be processed with some kind of pre-treatment and therefore constitute a significant underutilized source of sugars (Palmarola-Adrados, Choteborská, Galbe, & Zacchi, 2005). Overall, the easily accessible starch fraction and also the relatively high protein concentration add an extra value to wheat bran as a resource compared to other lignocellulosic feedstocks like wood or straw (Javed et al., 2012; Yu, Lou, & Wu, 2008).

2. General composition of wheat bran

In general, wheat bran comprises the outer tissues of the wheat kernel and includes botanically distinct tissues of the pericarp (fruit coat), the testa (seed coat), the hyaline layer and

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the aleurone layer which is part of the endosperm (Evers & Millar, 2002). Bran is histologically and chemically heterogeneous, and treatments to isolate valuables largely depend on the targeted tissue.

Wheat bran is a by-product of the roller milling of wheat grain to produce white flour and contains some of the underlying starchy endosperm. Apart from dietary fiber, wheat bran also contains protein, starch (as 'contamination' from the starchy endosperm), moisture and ash. Other minor components including phenolic acids, flavonoids, lignans and phytic acid have been independently implicated in the protection against cancer, although it is unknown, which of these compounds have the highest bioactive potential (Harris, Chavan, & Ferguson, 2005). Table 1 lists the general composition of wheat bran as found in published data.

The utilization of wheat bran is of interest, as there are different possibilities to follow different strategies to obtain valuables. Firstly, following the classical route of a biorefinery, bran can be disintegrated to a high extent and separated into fractions of high purity in order to build up new chemicals which serve as precursors for higher polymerized compounds. Secondly, bran as a histologically and chemically heterogeneous material contains substances that are valuables *per se*, but need to be separated and purified.

Different candidate compounds have already been isolated from wheat bran. These candidates can be categorized into four major groups. The first group deals with soluble and insoluble dietary fiber, e.g., arabinoxylan (Du et al., 2009; Hollmann & Lindhauer, 2005; Schooneveld-Bergmans, Beldman, & Voragen, 1999) and β-glucan (Li, Cui, & Kakuda, 2006). The second group comprises the group of sugars and their derivatives, e.g., starch (Xie, Cui, Li, & Tsao, 2008), glucose (Chotěborská et al., 2004; Elliott et al., 2002) and succinic acid (Bechthold, Bretz, Kabasci, Kopitzky, & Springer, 2008; Dorado et al., 2009). The secondary plant metabolites, of which ferulic acid (Buranov & Mazza, 2009; Di Gioia, Sciubba, Ruzzi, Setti, & Fava, 2009) is the most important one, can be allocated to another important category. In addition, proteins (Di Lena, Vivanti, & Quaglia, 1997; Roberts, Simmonds, Wootton, & Wrigley, 1985) can be used as platform for the production of specific amino acids (Nogata & Nagamine, 2009).

Finally, it should not be forgotten that bran can be regarded as an important source for minerals and salts, a fact, which hitherto has not been considered. For example, the growing demand for fertilizers like phosphate can be seen as a driving force for research in this field.

Table 1
General composition of wheat bran.

Compound	Amount [%]	References
Water	12.1	(Roberts et al., 1985)
Protein	13.2–18.4	(Di Lena et al., 1997; Dornez et al., 2006)
Fat	3.5–3.9	(Babiker et al., 2009; Hemery et al., 2007)
Phytosterols	0.16–0.17	(Fardet, 2010; Hemery et al., 2007)
α-Linolenic acid	0.16	(Fardet, 2010)
Total carbohydrates	56.8	(Beaugrand, Crônier, Debeire, & Chabbert, 2004)
Starch	13.8–24.9	(Dornez et al., 2006; Hemery et al., 2007)
Cellulose	11.0	(Hemery et al., 2007)
Total arabinoxylans	10.9–26.0	(Dornez et al., 2006; Gebruers et al., 2008; Hemery et al., 2007)
Total β-glucan	2.1–2.5	(Li et al., 2006)
Phenolic acids	1.1	(Fardet, 2010)
Ferulic acid	0.02–1.5	(Anson et al., 2012; Hemery et al., 2007, 2010)
Phytic acid	4.2–5.4	(Fardet, 2010; Hemery et al., 2007)
Ash	3.4–8.1	(Babiker et al., 2009; Dornez et al., 2006; Fardet, 2010; Hemery et al., 2007)

3. Valuables in wheat bran

3.1. Valuable derivates of the carbohydrate fraction

Chapter 3.1 summarizes the *status quo* regarding extraction and/or production of valuable compounds from the carbohydrate fraction of wheat bran. An overview is given in Table 2.

The carbohydrate fraction of wheat bran comprises starch, hemicelluloses as well as cellulose. While starch can be easily hydrolyzed *via* enzymatic treatment, hemicelluloses and cellulose need harsher pre-treatment steps, including heat treatment, acid and enzymatic hydrolysis. At the moment only few papers are available dealing with the application of pre-treatment processes specifically on wheat bran. Reisinger et al. (2013) recently published some information about the pre-treatment of wheat bran, including hydrothermal and enzymatic pre-treatment.

3.1.1. Wheat bran starch

Starch is one of the major compounds present in wheat bran, accounting for 14–25% depending on the degree of milling (Dornez, Gebruers, Wiame, Delcour, & Courtin, 2006; Hemery, Rouau, Lullien-Pellerin, Barron, & Abecassis, 2007). Generally, wheat starch consists of two different types of granules (A- and B-type granules), which differ in diameter as well as in chemical and functional properties such as amylose and lipid content, pasting properties and baking qualities (Xie et al., 2008). A-type granules have a diameter >10 μm and make up about 70% of commercial wheat starch. B-type granules are smaller with diameters <10 μm and account for about 30%. According to Xie et al. (2008), the granules size distribution of starch isolated from wheat bran is different to commercial wheat starch, resulting in different chemical, physicochemical and functional properties, which are described in Prückler et al., (2013). Xie et al. (2008) described a procedure for the extraction of starch from wheat bran (compare Table 2) including wet-milling followed by extraction using 70% ethanol, water, 0.5 M NaOH and a water/toluene mixture (4:1). The recovery rate was 90%; the protein contamination was below 0.15% compared to 0.35% in commercial wheat starch.

Additionally, starch extracted from wheat bran can be hydrolyzed to glucose and thus be used as sugar feedstock for fermentation processes producing lactic acid, succinic acid, ethanol and/or butanol. These issues will be described in the following sections. For this purpose, the slurry of wheat bran is incubated at a predetermined temperature, filtered *via* vacuum filtration, and the filtrate is subjected to liquefaction *via* addition of α-amylase, followed by saccharification using glucoamylase at elevated temperature. Finally, the solution is filtered again and further used as fermentation broth (Elliott et al., 2002).

3.1.2. Lactic acid (LA) and poly lactic acid (PLA)

LA is a versatile chemical that is widely used in the food, cosmetic, pharmaceutical and chemical industry, mainly as acidulant, preservative and flavor compound. Additionally, growing interest has been driven to LA as the monomer of PLA, which is a biodegradable polymer derived from renewable resources. Worldwide over 250 kt of LA are produced per year (Jem, van der Pol, & de Vos, 2010) and it is estimated to reach 330 kt by the year of 2015 (PlasticsToday Staff, 2011). Only 10% of LA is produced synthetically *via* lactonitrile hydrolysis, while 90% is produced *via* bacterial fermentation of starch, including biomass from renewable resources (Hofvendahl & Hahn-Hägerdal, 2000).

The fermentative production of LA is usually initiated with the hydrolysis of the substrate to C6- and C5-sugars *via* enzymatic treatment (liquefaction and saccharification; compare 3.1.1),

Table 2
Target substances in the carbohydrate fraction of wheat bran.

Target substance	Yield (max.)	Process	Challenges	Application	Reference
Wheat bran starch	90%	Wet-milling and extraction using EtOH (70%) and H_2O-Toluene (4/1)	Reduction of protein contamination.	Ingredient in functional food industry	(Xie et al., 2008)
LA	98%	Continuous fermentation of glucose (40 g/L) using *Lactobacillus coryniformis* subsp. *torquens* DSM 20004	Selection of homofermentative LAB strain, optical purity of L-LA, decrease in by-product formation.	Acidulant, preservative, flavor, monomer for PLA-production	(González-Vara et al., 1996)
Succinic acid	93%	Batch fermentation of glucose (50 g/L) with addition of several nutrients using *Anaerobiospirillum succiniciproducens*	Reduction of production costs; selection of suitable fermentation strains (concerning requirements of nitrogen source, mixed acid fermentation, by-product formation).	Acidulant, flavoring agent or as preservative	(Song & Lee, 2006)
ABE (Acetone, Butanol, Ethanol)	32%	Acid/heat treatment, H_2O extraction, fermentation using *C. beijerinckii* 55025	Reduction in by-product formation (furfural and 5-HMF), increase of product yields (genetically engineered MOs).	Biofuel, additive to gasoline	Liu, Ying, Li, Ma, & Xu, 2010
Furfural/HMF	<60%	Acid-catalyzed hydrolysis and dehydration of pentoses and hexoses	Typical yields are below 60%, even if theoretically a yield of 100% is possible.	Substitutes for petroleum-based building blocks in the production of fine chemicals, fuels and polymers	Zeitsch, 2000
Arabinoxylan	76.9%	Washing with ethanol and water, extraction with saturated $Ba(OH)_2$	Enhancement of extraction yield (including enzymatic treatment).	Dietary fiber supplement	(Gebruers et al., 2008; Sun et al., 2011)
β-Glucan	n.s.	Ethanol precipitation, enzymatic treatment (α-amylase), saponification, ammonium sulfate precipitation	Optimization of extraction yields.	Ingredient in functional food industry	(Li et al., 2006)

followed by fermentation to LA using appropriate strains of LA bacteria (LAB) or even moulds such as *Rhizopus oryzae* or yeasts like *Saccharomyces cerevisiae* (Sauer, Porro, Mattanovich, & Branduardi, 2008). The process continues with the separation of bacteria cells and solids from the broth, and finally purification of LA. In some studies hydrolysis and fermentation was performed simultaneously by introducing batch amylolytic enzymes or amylase producing bacteria (Hofvendahl, Akerberg, Zacchi, & Hahn-Hägerdal, 1999; Hofvendahl & Hahn-Hägerdal, 2000; Petrov, Urshev, & Petrova, 2008). Depending on the fermentation medium and the selected LAB strain (homo- or heterofermenters), different by-products may be formed during fermentation. While the homofermentative LAB convert 1 mol of glucose almost exclusively to 2 mol of LA, the heterofermentatives convert 1 mol of glucose into 1 mol of LA, 1 mol of CO_2 and 1 mol of ethanol or acetate (Wee, Kim, & Ryu, 2006). In case of pentose utilization by LAB, 1 mol of acetate is produced together with 1 mol of LA. Heterofermentation of sugars to produce LA is unfavorable, since it decreases the yield of LA and thus enhances the costs for purification processes.

Regarding the bran-based biorefinery, LA production seems to be a valuable approach, since it presents the possibility of simultaneous saccharification and fermentation, thereby eliminating costly pre-treatment processes. Additionally, the protein fraction as a source for nitrogen as well as the provision of vitamins by bran could meet the nutrient requirements of LAB for the fermentation process. Rojan, Nampoothiri, and Pandey (2006) reported that protease treated wheat bran medium decreased the costs for supplementation by e.g. yeast extract or peptone by tenfold and increased the recovery of LA.

LA exists as two optical isomers, namely L(+)-LA and D(−)-LA. L(+)-LA has an advantage over D(−)-LA, since elevated levels of D(−)-LA are recognized as harmful to humans, resulting in decalcification and acidosis (Wee et al., 2006). While the fermentative production of LA enables to produce optically pure LA, the synthetic production leads to the formation of racemic LA, which is not preferred in industrial applications due to the amorphous structure of the polymerized product (Gupta & Kumar, 2007; Wee et al., 2006). The degree of crystallinity, hence the mechanical properties of the polymer, can be controlled by arranging the ratio of D- and L-enantiomers (Lunt, 1998; Nampoothiri, Nair, & John, 2010). Most common commercial polymers of LA have 99.5% optical purity with L-LA (Farrington, Davies, & Blackburn, 2005; Henton, Gruber, Lunt, & Randall, 2005). Nonetheless, optical purity is not the only parameter determining the mechanical properties of PLA; also the molecular weight of PLA has a significant impact (Gupta & Kumar, 2007). While the optical purity mainly depends on the fermentation process, the molecular weight depends on the polymerization technique applied. Possible polymerization techniques that enable the production of high molecular weight PLA are ring-opening polymerization, azeotropic condensation polymerization as well as solid state polymerization. In contrast, direct poly-condensation polymerization yields low molecular weight PLA, unless chain coupling agents are used (Farrington et al., 2005; Gupta & Kumar, 2007; Henton et al., 2005; Kingsland, 2010, p. 13).

3.1.3. Succinic acid

Succinic acid is used as acidulant, flavoring agent or as preservative in the food industry, and in the pharmaceutical industry mainly to control the acidity. Moreover, it serves as building block being the precursor for many industrially valuable chemicals such as, 1,4 butanediol, tetrahydrofuran, γ-butryolactone, adipic

acid as well as for biodegradable polymers such as polybutrate succinate (PBS).

The U.S. Department of Energy has assigned succinic acid as one of the top twelve sugar-derived building-block chemicals which can be produced from biomass (Aden, Bozell, Holladay, White, & Manheim, 2004). Approximately 16 kt of succinic acid is produced annually (Sauer et al., 2008), while the market potential, including the applications as building block, was estimated with 270 kt (Kurzrock & Weuster-Botz, 2010; Willke & Vorlop, 2004). It turned out that glucose derived succinic acid can clearly compete with its petroleum derived equivalent (Song & Lee, 2006), however, the high processing costs especially arising from the downstream process due to by-product formation still have to be optimized.

There are several natural succinic acid producing bacteria, which are generally isolated from rumen. Among them, the most prominent ones are *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens* and *Mannheimia succiniciproducens*. Additionally, there is a great effort to develop recombinant *Escherichia coli* to be used in biocatalysis. *A. succiniciproducens* is a strict anaerobic bacterium that produces succinic acid and acetic acid as the major metabolites together with minor amounts of ethanol and LA (Bechthold et al., 2008). One of the major problems other than by-product formation is its requirement of complex nitrogen sources (Lee, Lee, Lee, Chang, & Chang, 2000). *A. succinogenes* is also a CO_2 fixing species, requiring a nitrogen source and has the same problem of producing by-products such as formic, propionic, acetic, lactic and pyruvic acid (Bechthold, Bretz, Kabasci, Kopitzky, & Springer, 2008). It can utilize a wide range of carbon sources and is moderately osmophilic, thus, has the ability of tolerating high concentrations of glucose in the fermentation batch (Song & Lee, 2006). *M. succiniciproducens* produces succinic acid as the major product, in addition to acetic acid and formic acid. It is able to utilize xylose efficiently, and the conversion to succinic acid from 1 mol glucose could be as high as 1.7 mol. However, mixed acid formation is still a problem that should be solved for a cost effective production (Song & Lee, 2006). Recombinant *E. coli*, which enables the aerobic fermentation, has been studied widely and remarkable performances were reported compared to wild strains. Yet, the productivity of succinic acid thereby obtained is lower than that of natural succinic acid producers and the by-product formation has not been solved completely (Bechthold et al., 2008; McKinlay, Vieille, & Zeikus, 2007; Song & Lee, 2006).

3.1.4. Acetone/Butanol/Ethanol (ABE)

Bioethanol has been used as biofuel since the beginning of the 1970s. Due to its high content in starch and non-starch polysaccharides (mainly consisting of hemicelluloses and cellulose) wheat bran can be used for the production of bioethanol and thus forms a promising alternative for the use of petroleum. Ethanol production from biomass always comprises the same main steps: hydrolysis of starch, hemicellulose and cellulose to monomeric, reducing sugars, fermentation, product recovery and distillation (Galbe & Zacchi, 2002). While starch can be enzymatically hydrolyzed *via* amylases to glucose, hemicelluloses and celluloses are not completely degradable *via* enzymatic treatment. Therefore, additional chemical and/or physical processes are required. Hydrolysis of hemicellulose and cellulose comprises combinations of heat treatment, acid and enzymatic hydrolysis. However, these processes are high in costs and low in yields (Sun & Cheng, 2002).

Palmarola-Adrados et al. (2005) as well as Chotěborská et al. (2004) evaluated different methods for the hydrolysis of starch and non-starch polysaccharides in wheat bran in order to produce a sugar feedstock suitable for fermentation to bioethanol. Firstly, the starch content was separated using liquefaction *via* α-amylases and saccharification *via* amyloglucosidase. Then, the starch-free bran (SFB) was hydrolyzed in different ways, including heat treatment, acidic and/or enzymatic hydrolysis and combinations thereof. They concluded that the recovery of sugars from hemicellulose, cellulose and glucan requires different pre-treatment conditions, and that acid addition (1% H_2SO_4 (w/v)) during pre-treatment and heating (130 °C) is essential for proper enzymatic hydrolysis. The overall sugar yield of the SFB was about 80% of the theoretical, mainly consisting of xylose, glucose and arabinose accounting for 22.8, 16.7 and 13.5 g/100 g SFB, respectively. Glucose can directly be fermented to ethanol using *S. cerevisiae*, while currently no pentose-fermenting microorganisms are used in commercial ethanol production. A problem during sugar hydrolysis is the formation of by-products such as furfural and 5-hydroxymethylfurfural (HMF), which are inhibitors during the fermentation step, even if the concentrations formed during pre-treatment and enzymatic hydrolysis are under the limit of yeast inhibition.

Wheat bran hydrolysate can also be used as fermentation media for the combined production of acetone, butanol and ethanol (ABE) using mesophilic strains of *Clostridium* spp. (Liu, Ying, Li, Ma, & Xu, 2010). In general, butanol is superior to ethanol, since it has increased hydrophobic properties, higher energy density, and can be mixed with gasoline at any ratio. However, butanol production *via* fermentation is limited due to high substrate costs, the low product yield and high recovery costs. In their experiments Liu et al. (2010) used wheat bran hydrolysate as fermentation medium. Wheat bran was hydrolyzed by the addition of 0.75% sulfuric acid (w/v) at 121 °C. The hydrolysate was neutralized and detoxified by $Ca(OH)_2$ addition and filtrated. Then, the clear supernatant was inoculated with the mutant strain *Clostridium beijerinckii* 55025, and fermentation was performed for 72 h at 37 °C. The wheat bran hydrolysate contained a total amount of reducing sugar of 53.1 g/l, consisting mainly of glucose, xylose and arabinose with 21.3, 17.4 and 10.6 g/l, respectively. After 72 h the total concentration of ABE was 11.8 g/l with 2.2, 8.8 and 0.8 g/l of acetone, butanol and ethanol, respectively. During the fermentation time of 72 h, 20.1, 12.9 and 5.3 g/l of glucose, xylose and arabinose were utilized, respectively. This shows that 94% of glucose was utilized during the fermentation, while only 74 and 50% of xylose and arabinose were metabolized, respectively. The yield of ABE was 0.32 g/g total sugars with a productivity of 16 g/l/h. The productivity was nearly equivalent to the productivity in a glucose broth. Butanol accumulation in the medium led to a deceleration of the fermentation process after 24 h, since butanol is a potent inhibitor of bacterial growth. Thus, microorganisms with a high tolerance to butanol have to be selected for butanol fermentations or butanol has to be removed from the fermentation broth continuously.

3.1.5. Furfural and 5-Hydroxymethylfurfural (5-HMF)

On the one hand, the furan derivatives furfural and 5-HMF are naturally occurring and hazardous toxins, which are produced during pre-treatment processes of biomass (compare 3.1.4). On the other hand, due to the wide range of intermediate and end-products, they are important chemicals which possess the potential to substitute petroleum-based building blocks in the production of fine chemicals, fuels and polymers (Chheda, Román-Leshkov, & Dumesic, 2007). Actually, only limited scientific information is available concerning the syntheses of furfural or 5-HMF from wheat bran. In principle, these compounds can be produced from renewable biomass-derived carbohydrates *via* acid-catalyzed dehydration of pentoses (e.g., xylose → furfural) or hexoses (e.g., glucose → 5-HMF). Quaker Oats Company for example has produced furfural from oat hulls since 1921 (Zeitsch, 2000). Furfural is mainly produced from xylan as well as xylose with a molar yield of approximately 50%. The production volume is about 200 kt/year (Binder, Blank, Cefali, & Raines, 2010). In analogy, 5-HMF is

produced from hexoses such as fructose or glucose with a rather low production volume. Chheda et al. (2007) published the production of these two furan derivatives from polysaccharides such as sucrose, starch or xylan, eliminating the need to obtain monosaccharides prior to dehydration process. In a biphasic reactor system, they showed that it is feasible to process the corresponding polysaccharides to its monomers in the same reactor system with good selectivity and high conversion rates. The process is mainly influenced by the pH of the solution, the dimethylsulfoxide concentration, the type of acid, and the extraction solvent.

3.1.6. Arabinoxylan – xylan and xylose

As described by Prückler et al., (2013) arabinoxylan is a major structural component of cell walls of wheat bran, comprising 10.9–26.0% of total wheat bran based on dry matter (see Table 1). The large variation heavily depends on the variety of wheat analyzed. With wheat flour containing only 1.4–2.8% of arabinoxylan, the bran layer clearly poses the key source of arabinoxylan in wheat (Gebruers et al., 2008).

Structurally, arabinoxylan is based on a linear β-D-(1→4)-linked xylopyranose backbone, which can be substituted at C(O)-2, C(O)-3 or both in irregular patterns (Brillouet & Joseleau, 1987). The major substituent is single α-L-arabinofuranose linked to the xylan at C(O)-1 (Izydorczyk & Biliaderis, 1995). More complex branches such as oligo-α-L-arabinofuranose, terminal xylopyranose and β-D-glucuronic acid have been described, albeit in marginal amounts (Edwards, Chaplin, Blackwood, & Dettmar, 2003; Izydorczyk & Biliaderis, 1995; Pastell, Virkki, Harju, Tuomainen, & Tenkanen, 2009). The prevalence and characteristics of side chains are indicative of the wheat layer they originate from, with the ratio of arabinose to xylose and the occurrence of disubstituted xylose being exemplary parameters (Gruppen, Kormelink, & Voragen, 1993; Parker, Ng, & Waldron, 2005).

The dominant phenolic compound in wheat, ferulic acid, is found randomly esterified with single α-L-arabinofuranose residues in C-5, inferring its anti-oxidant properties to arabinoxylan (for further details see 3.2.1 Phenolic compounds – Ferulic acid). With 98% of total ferulic acid located in the bran layer, it is almost exclusive to this part of the kernel (Lempereur, Rouau, & Abecassis, 1997). It also plays a key role as a structural element by cross-linking single strands of arabinoxylan. In fact, 39% of ferulic acid is accounted for as dehydrodiferulic acid (Parker et al., 2005; Ralph, Quideau, Grabber, & Hatfield, 1994).

Only small amounts of arabinoxylan are water-extractable (2.8–3.9% of total arabinoxylan) (Gebruers et al., 2008). In order to achieve thorough extraction, more drastic means are required. Starting from destarched and deproteinized bran, the most common approach is alkaline extraction (Cui, Wood, Weisz, & Beer, 1999) using barium hydroxide due to its arabinoxylan-specific complexing behavior (Sun, Cui, Gu, & Zhang, 2011). The process can be assisted by the use of lignin-oxidizers such as hydrogen peroxide (Maes & Delcour, 2001) or by sonication (Hromádková, Kost'álová, & Ebringerová, 2008). The application of enzymatic extraction with xylanases has also been proposed (Swennen, Courtin, Lindemans, & Delcour, 2006).

Despite the EFSA health claim concerning the benefits of arabinoxylans from wheat germ (Prückler et al., 2013), application of arabinoxylan in the commercial market has been slow and scarce.

An interesting approach to produce fine chemicals from arabinoxylan is to hydrolyze arabinoxylan into its monomeric sugar constituents, namely arabinose and xylose. Xylose can be reduced to xylitol, which is a non-cariogenic sweetener that is metabolized insulin-independently and therefore suitable for diabetics (Kishore et al., 2012; Roberts et al., 2002). The hydrogenation of xylose is currently achieved chemically with Raney-Nickel as a catalyst, but extensive research is ongoing to develop a feasible, enzyme-based conversion as well as utilization of hemicelluloses from other resources besides hardwood and maize (Prakasham, Rao, & Hobbs, 2009; Saha, 2003).

3.1.7. Beta-glucan

Wheat-β-D-glucans are mainly located in the aleurone layer of the wheat kernel as described by Prückler et al., (2013).

Cereal-based β-D-glucans are water soluble, possess different beneficial health effects (therapeutic effects on cholesterol and blood glucose level or cardiovascular disease), and therefore are used as an ingredient in the functional food industry (Li et al., 2006; Prückler et al., 2013).

Although wheat bran is no typical source for β-D-glucan, Li et al. (2006) described an extraction and fractionation process,

Table 3
Target substances in the non-carbohydrate fraction of wheat bran.

Target substance	Yield (max.)	Process	Challenges	Application	Reference
Ferulic acid (FA)	100%	Pressurized alkaline extraction (NaOH) at 180 °C and 5.2 MPa	Alternative purification procedures (e.g. SPE), avoid use of organic solvents.	Anti-oxidant, raw material for vanillin production	(Buranov & Mazza, 2009)
Vanillin	70%	Release of FA using enzymes, isolation of free FA, fermentative conversion to vanillin	Avoid contaminations in ferulic acid hydrolysate, decrease in reduction of vanillin to vanillyl alcohol.	Flavor in food and beverages, fragrance	(Di Gioia et al., 2007)
Protein	72%	Extraction at pH 6.5, 16 h, room temperature using tap water	Avoid protein denaturation during extraction procedure, optimize extraction parameters e.g. pH, temperature, particle size.	Inexpensive source of protein	(Roberts et al., 1985)
Amino acids & GABA	n.s.	Autolysis *via* proteolytic enzymes enclosed in wheat seeds	Optimize genetically engineered MO strains regarding pentose utilization.	Food and feed additives	(Nogata & Nagamine, 2009)
Wheat bran oil	90%	Solvent extraction or CO_2-extraction	Minimize lipid peroxidation during extraction, avoid use of organic solvents	Food and feed additive	(Brandolini & Hidalgo, 2012)

which includes precipitation with ethanol at 70 °C, followed by enzymatic treatment using thermostable α-amylase at 90 °C. The residue was saponified, and purified wheat β-glucan was obtained by gradual ammonium sulfate precipitation. Since β-glucans and arabinoxylans have different solubility in ammonium sulfate solution, this precipitation method can be used for the fractionation of β-glucan (gradual ammonium sulfate precipitation from 20 to 55%).

3.2. Valuable derivates of the non-carbohydrate fraction

The following sub-chapters will give a survey of extraction or production conditions of valuable compounds from the non-carbohydrate fraction of wheat bran, as resumed in Table 3.

3.2.1. Phenolic compounds – ferulic acid

The main physiological function of the bran layers in nature is to protect the seed. Therefore, wheat bran is a rich source of bioactive components such as phenolic compounds.

Phenolic compounds in wheat bran mainly consist of phenols containing one aromatic ring represented by phenolic acids (e.g. ferulic acid, sinapic acid or *p*-coumaric acid) as well as alkylresorcinols or vitamin E (Anson, Hemery, Bast, & Haenen, 2012). Most of the phenolic acids are present in a bound form, which can be released by hydrolysation under either alkaline or acidic conditions. The alkaline hydrolysis is more efficient in releasing free phenolic acids, and fractions after alkaline treatment show higher antioxidant activities than acid hydrolysates (Kim, Tsao, Yang, & Cui, 2006).

As described by Prückler et al., (2013) alkylresorcinols are phenolic lipids which are basically located in the testa of the wheat grain, where they possess different biological effects.

The most frequent phenolic acid in wheat bran is ferulic acid with 20–1500 mg/100 g which is mostly linked to cell wall polysaccharides or to lignin *via* ester and ether bonds (lignin/phenolics–carbohydrate complex). Ferulic acid (3-(4-hydroxy-3-methoxyphenyl) propionic acid) is a bioactive compound in wheat bran since it possesses anti-oxidant, anti-microbial, anti-inflammatory, anti-thrombosis and anti-carcinogenic activities, and thus has a high potential for applications in the food, health and cosmetic industry (Ou & Kwok, 2004). Moreover, ferulic acid, extracted from other agricultural crop by-products (mainly sugar-beet pulp, rice bran oil and maize bran) can be used as raw material for the production of natural vanillin, which will be discussed below. Generally, the extraction process of ferulic acid starts with the release from the lignin/phenolics–carbohydrate complex either chemically using alkaline or acid hydrolysis at elevated temperatures or by enzymatic treatment with ferulic acid esterase, and is followed by a costly and precarious purification step. Commercially, ferulic acid is produced from γ-oryzanol in rice oil. Buranov and Mazza (2009) as well as Pourali, Asghari, and Yoshida (2010) describe the extraction of ferulic acid from wheat, corn or rice bran under subcritical water conditions. This method has the advantage that no toxic, flammable and expensive organic solvents have to be used, and the selectivity of extraction can be enhanced. They concluded that subcritical water extraction is a green alternative to conventional organic solvent extraction, also having the potential to be scaled up to industrial level.

A major problem of ferulic acid extraction from crop residues is the purification step, which comprises complicated adsorption and desorption processes with activated charcoal and resin chromatography. However, Buranov and Mazza (2009) describe a simple and alternative process, which is based on the solubility of ferulic acid in ethanol. The extract is purified by the addition of anhydrous ethanol to a concentration of 30%, followed by simple centrifugation.

3.2.2. Vanillin

Vanillin is one of the most important and universally used aromatic flavors, either applied as flavoring agent in foods and beverages (82%), as a fragrance in perfumes (5%) or for pharmaceuticals (13%) (Priefert, Rabenhorst, & Steinbüchel, 2001). Worldwide about 15–16 kt vanillin are produced per year with a predicted market growth of 2–3%.

Vanillin can be produced from natural sources such as *Vanilla planifolia* pods, but only at high costs and at insufficient amounts to meet the global demand. Thus, vanillin is mainly produced synthetically from guaiacol and lignin at lower costs, but also with lower quality. According to European Council Directive, 88/388/EEC (EC, 1988) and U.S. legislation (FDA, 2012) flavors can be considered as natural, if they are obtained by a biological process, i.e. enzymatically or microbiologically. Therefore, vanillin produced *via* biotechnical processes from substances such as ferulic acid can be considered as “natural” vanillin, and, in addition, is much cheaper (Di Gioia et al., 2009).

Priefert et al. (2001) summarized the major pathways of the biotechnical production of vanillin from ferulic acid *via* microbial degradation using fungi & yeast or bacteria, comprising (i) non-oxidative decarboxylation, (ii) side chain reduction, (iii) coenzyme-A-independent deacetylation, or (iv) coenzyme-A-dependent deacetylation.

The bioconversion of ferulic acid from plant-based by-products has been described by several authors. In principle the process includes the following steps: (i) release of ferulic acid form cell wall matrix using enzymes either *via* microbial fermentation (Thibault et al., 1998) or the addition of enzyme preparations (Di Gioia et al., 2007), (ii) isolation of free ferulic acid from interfering carbohydrates of the hydrolysate using activated carbon or resins, and finally (iii) vanillin conversion using different fungi or bacteria e.g. genetically engineered *Pycnoporus cinnabarinus* or *E. coli*, respectively. The isolation of free ferulic acid from carbohydrates in the wheat bran hydrolysate is necessary, since it increases the molar yield of vanillin up to 70%, and decreases the reduction of vanillin to vanillyl alcohol. Instead of expensive ion exchange resins, solid phase extraction (SPE) with commercial hydrophobic solvents can be applied, resulting in 95% recovery of ferulic acid. Further advantages of SPE are that ferulic acid is extracted under mild conditions, and that the resulting fermentation broth is free of any solvents (Di Gioia et al., 2009).

A large number of patents can be found concerning the extraction of ferulic acid from agricultural by-products, the biotechnological production of vanillin from ferulic acid and the extraction of vanillin after bioconversion. This reflects the high commercial interest in this field.

3.2.3. Protein

The world’s increasing population elevates the demand for sources of inexpensive dietary protein from plants and microorganisms, supplementing or even replacing expensive and limited sources of animal protein. Since wheat bran contains 13–18% protein, it can be regarded as a possible source for protein extraction. Wheat bran proteins have a chemical structure different to wheat flour proteins, which is reflected in the amino acid composition (Di Lena et al., 1997). The endosperm proteins are mainly formed by glutenins and gliadins, while the bran proteins primarily contain albumins and globulins which have a more equilibrated amino acid composition. Compared to endosperm proteins, the bran proteins contain higher amounts of lysine, arginine, alanine, asparagine and glycine, and less of glutamine, proline, phenylalanine and sulphur

containing amino acids. Thus, bran proteins possess a higher biological and nutritional value (Di Lena et al., 1997) and may therefore be used as food ingredients or in special feeds.

Wheat bran proteins can be extracted *via* acidic or alkaline conditions, whereby alkaline extraction shows higher yields (Roberts, Simmonds, Wootton, & Wrigley, 1985). However, the pH value is a restricting factor due to protein denaturation at severe conditions. The yield is mainly influenced by pH, time, temperature, extraction solvent and particle size. Roberts et al. (1985) concluded that optimum extraction conditions are at pH 6.5, for 16 h at ambient temperature simply using tap water and finer bran size. The addition of cellulase did not enhance the extraction yields.

Wheat bran protein may also be produced as a by-product of starch extraction. The filter cake after liquefaction and saccharification is high in protein and therefore can be used as feed additive to enhance the nutritional quality of feed (Elliott et al., 2002).

3.2.4. Amino acids and gamma-aminobutyric acid (GABA)

On an industrial level, amino acids are mainly produced *via* fermentation from molasses or starch, and are used as additives in food (65%), feed (30%) as well as in pharmaceutical and cosmetic products (Novalin-Canoy, 2011). The chemical composition of wheat as well as wheat bran is strongly influenced by intrinsic (e.g. breed) and extrinsic factors (e.g., soil, conditions of growth and storage), which is reflected in the amino acid composition. Thus, a great variability can be found in the literature (Babiker, Kijora, Abbas, & Danier, 2009; Di Lena et al., 1997).

Nogata and Nagamine (2009) described a simple and cheap process to produce free amino acids and GABA from whole grain flour and wheat bran using autolysis reactions with proteolytic enzymes such as thiol-proteases or carboxypeptidase that are enclosed in wheat seeds and bran. For this purpose, the milled fractions are suspended in sodium phosphate buffer and warmed at 40–45 °C for 1–4 h. The content of all α-amino acids and GABA increased, except for asparagine and glutamic acid. The reason for the lower harvest of the latter mentioned amino acids was considered to be due to glutamate decarboxylase activity and production of GABA. The pH optimum for GABA extraction was 5.5–6.0, while all other amino acids showed maximal productivity at pH 4.0–4.5. It was found that this simple and inexpensive approach of autolysis led to increased amounts of the branched chain amino acids (BCAA: valin, leucin, isoleucine), arginine, lysine, glutamine, phenylalanine and GABA in bran and other milling by-fractions. Due to the biological benefits of GABA and α-amino acids such as improving mild hypertension and the supply of essential amino acids, respectively, the extract can be applied successfully in food products.

Another possibility for the production of amino acids from wheat bran is the fermentation of acid bran hydrolysate using recombinant pentose-utilizing microorganisms such as *Corynebacterium glutamicum* (Gopinath, Meiswinkel, Wendisch, & Nampoothiri, 2011; Gopinath, Murali, Dhar, & Nampoothiri, 2012). *C. glutamicum*, which can utilize various carbon sources simultaneously, is commonly used for the fermentative production of amino acids like glutamate or lysine under aerobic conditions. After genetic engineering *C. glutamicum* is able to utilize also the pentoses xylose and arabinose, to produce not only glutamate and lysine, but also phenylalanine, aspartate, tryptophan, arginine and valine. Furthermore, it is resistant against inhibitors such as furfural or 5-HMF which are formed during pre-treatment processes (see 3.1.4). Thus, recombinant *C. glutamicum* can be used for the amino acid production from (hemi-)cellulosic biomass.

3.2.5. Wheat bran oil

Wheat bran contains approximately 3.5–3.9% total fat. However, only few data on the composition of wheat bran oil, its extraction or application can be found in the literature. Wheat bran oil has a yellowish color and a weak odor, and with regard to literature it can be assumed that the fatty acid composition of wheat bran oil is similar to wheat germ oil (Jung, Uddin, Kwon, & Chun, 2010; Kwon, Uddin, Jung, Sim, & Chun, 2010). The latter is used in pharmaceutical or cosmetic applications as well as an ingredient in the food and feed sector (Brandolini & Hidalgo, 2012). Wheat germ oil is usually extracted *via* solvent or supercritical CO_2 extraction techniques, reaching yields of 90 and 83%, respectively. Wheat bran oil extraction can also follow the same technology or even near critical CO_2 extraction, yielding 8.2 g oil/100 g wheat bran at 25 °C and 30 MPa (Jung et al., 2010).

The most abundant fatty acid in wheat germ and wheat bran oil is linoleic acid with about 57%, followed by oleic acid with 15% (El-Shami, El-Mallah, Hassanein, & Abdel-Razek, 2011; Jung, Uddin, Kwon, & Chun, 2010). Additionally, wheat bran oil contains relatively high contents of carotenoids (39.2 μg/g wheat bran oil), and thus, it can be regarded as oil with advanced nutritional quality.

4. Discussion

Currently, wheat bran is mainly used as a feed supplement, while the application in the food sector plays only a minor role. However, the general composition of wheat bran offers the potential to be used as a substrate in a biorefinery concept. Fig. 1 illustrates a mass flow based on published data, taking into consideration the natural range of possible compounds and derived products. Furthermore, the yields are demonstrated depending on varying technological feasibilities. The principle of a tailored concept will be dealt with below based on the relevant fractions.

4.1. Carbohydrate fraction

Starch, but also pre-treated hemicelluloses and cellulose can be degraded to C6- and C5-monomers *via* enzymatic liquefaction and saccharification, and consequently be fermented to LA, succinic acid or ABE. Moreover, wheat bran starch and hemicelluloses may be used as substrate for the production of furfural and 5-HMF, even if additional technological solutions are needed.

Arabinoxylans, extracted from destarched and deproteinized bran, have the potential to find new applications in the food sector due to their beneficial health effects such as prebiotic benefits associated with dietary fiber, the lowering of blood cholesterol and of post-prandial glycaemic response. Another approach is the production of xylitol from arabinoxylan *via* the monomer of xylose which is actually performed from hemicelluloses originating from hardwood or maize, but at high conversion costs.

Wheat bran is not a typical source of β-D-glucans, however its extraction is feasible and valuable due to its unique physical properties.

4.2. Non-carbohydrate fraction

Wheat bran is a rich source of ferulic acid. Ferulic acid is a valuable *per se*, since it possesses anti-oxidative, anti-microbial, anti-inflammatory, anti-thrombosis and anti-carcinogenic activities. Moreover, it can be easily converted to natural vanillin, one of the most important flavoring agents in the food industry.

Due to the relatively high amount of protein (13–18%), wheat bran is a good source for protein extraction, thereby providing a plant based protein for the food and feed area. Wheat bran proteins primarily consist of albumins and globulins, and possess a more

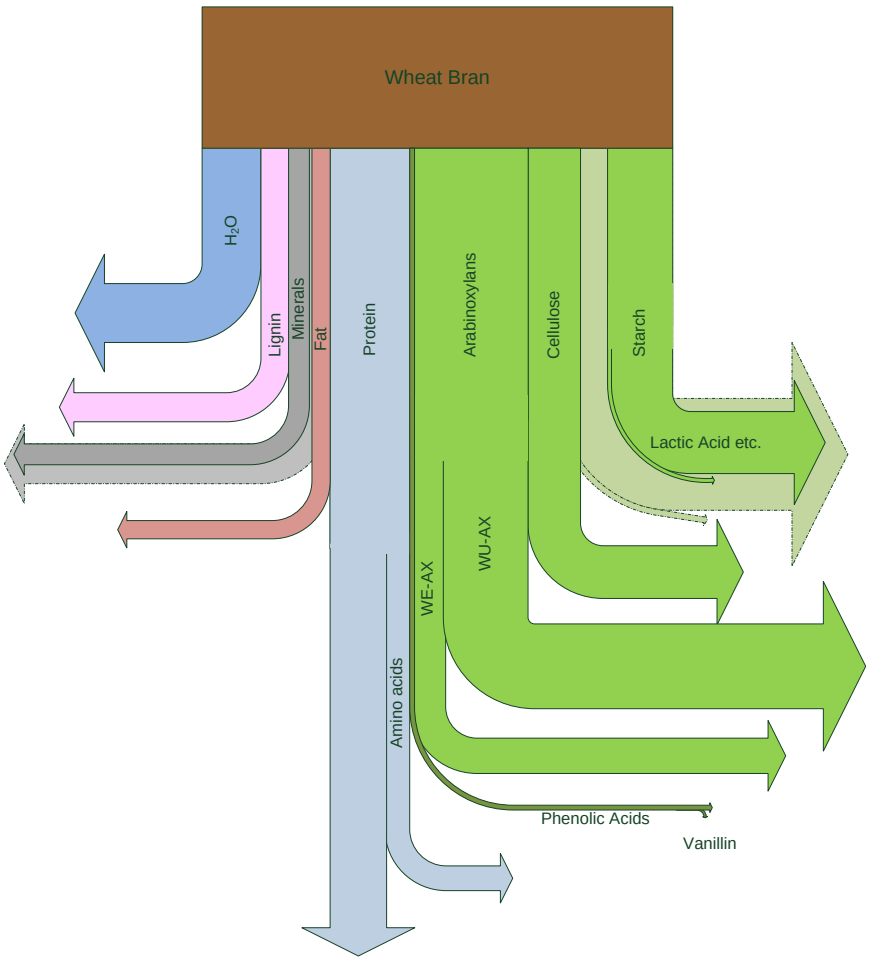

Fig. 1. Illustration of mass flows of wheat bran compounds and possible products.

equilibrated amino acid composition with a higher biological and nutritional value than endosperm proteins. They may be used as food ingredients or in special feeds. Last but not least, single amino acids can be produced from wheat bran protein, mainly the BCAA, arginine, lysine, glutamine, phenylalanine and GABA.

The composition of wheat bran oil is comparable to wheat germ oil, and can therefore find applications in the pharmaceutical, cosmetic or food and feed industry.

5. Conclusion

Based on this survey and on earlier published findings (Prückler et al., 2013) it is concluded that wheat bran can be regarded as a mass by-product of enormous versatility. A number of compounds, constituents and products can be produced out of this source offering a variety of applications in different branches of the food and feed area as well as in the fine chemicals business. According to a recently completed feasibility study (unpublished data) dealing with a comparative evaluation of the market potential of bran-based products, increased sales revenues may be envisaged if suited processing conditions and technologies are applied. This finding has to be evaluated more in-depth and in the light of hitherto traditional use of bran just as a feed supplement. However, further research activities will be necessary to increase the productivity of the extraction and to optimize the technologies at an economically satisfactory level.

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TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

Analytical techniques for the elucidation of wheat bran constituents and their structural features with emphasis on dietary fiber – A review

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Wheat bran is a by-product of white flour production available in abundance. It consists of nutritionally and technologically valuable constituents, above all around 50% of dietary fiber. Numerous applications ranging from functional dietary fiber to fine chemicals are currently being developed in order to valorize wheat bran beyond animal feed. This shift in utilization necessitates an adequate set of analytical tools. This review gives an overview over the analysis of wheat bran constituents in general and dietary fiber in particular with a focus on instrumental techniques. Gas chromatography, liquid chromatography, capillary electrophoresis, thin layer chromatography, size-exclusion chromatography, field-flow fractionation, nuclear magnetic resonance spectroscopy and infrared as well as near-infrared spectroscopy are presented and evaluated in detail for dietary fiber analysis. In addition, Association of Official Analytical Communities (AOAC) methods are discussed.

Introduction

Wheat bran makes up the outer layer of the wheat kernel and encompasses about 15% of the entire kernel. It is rich in dietary fiber, consisting of 13–18% protein, 14–25% starch, 3–4% fat, 3–8% minerals and 55–60% non-starch carbohydrates based on dry matter (Boudouma, 2009; DiLena, Vivanti, & Quaglia, 1997; Dornez, Gebruers, Wiame, Delcour, & Courtin, 2006; Hemery, Rouau, Lullien-Pellerin, Barron, & Abecassis, 2007). The non-starch carbohydrate fraction is made up of 52–70% arabinoxylan, 20–24% cellulose and about 6% β-(1,3)-(1,4)-glucan (Hemery *et al.*, 2007; Li, Cui, & Kakuda, 2006; Sun, Liu, Qu, & Li, 2008). These numbers are prone to large variation and can give a rough estimate only. Aside from values depending on the analytical method employed, wheat bran is also subjected to processing and natural fluctuations. Starch content is most heavily influenced by the degree of milling whereas the remaining components differ according to variety and seasonal changes.

As a by-product of white flour production, an annual amount of 112 million tons of wheat bran is amassed worldwide, the bulk of which is currently being underutilized as forage (FAOSTAT 2012). Considering the potential value of its components, a shift to more profitable applications is overdue. This shift brings about the need for analytical tools equipped to characterize wheat bran in detail.

Standard analyses of dietary fiber

As per the definition by the AACC International, dietary fiber is "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the

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human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation" (AACCI, 2001).

The current approaches to measure and categorize the non-starch carbohydrate fraction are issued by the Association of Official Analytical Communities (AOAC) and are largely based on sequential and gravimetrical analyses composed of one or more steps mimicking human digestion.

Of the established methods, Crude fiber (CF) determination is the most archaic. It measures fiber by weighing out a sample after refluxing in lye and acid (Henneberg & Stohmann, 1859). Even though it tends to underestimate the amount of indigestible material (Van Soest, 1994), it remains an official AOAC method (AOAC 962.09).

Neutral detergent fiber (NDF), not an AOAC method, is the most common routine and aims at extracting fiber as a whole. It uses a neutral detergent to extract non-fiber material and, like CF, determines the remainder gravimetrically (Van Soest, 1963b). However, it gives too low an estimation for samples rich in pectin and falsely includes insoluble, heat-treated protein (Van Soest, 1994). NDF is often followed up with acid detergent fiber (ADF), a method based on extraction with cationic detergents that aims to isolate cellulose and lignin, but also leaves some amounts of hemicelluloses in the residue (Van Soest, 1963a). ADF is an official AOAC method (973.18).

Another approach is the Prosky method for dietary fiber (DF; AOAC 985.29). It advances closer to the digestive system by combining enzymatic and chemical treatments followed by gravimetrical determination after precipitation of fiber in 80% ethanol (Prosky *et al*., 1984). Valid determination relies on thoroughly destarched and deproteinized samples.

The Uppsala method (AOAC 994.13) follows up the Prosky method with sulfuric acid hydrolysis and chromatographic sugar determination of the fiber while the acid-insoluble residue is determined as Klason Lignin (Theander, Aman, Westerlund, Andersson, & Petersson, 1995).

Being based on precipitation and gravimetric analysis of dietary fiber, these methods do not encompass non-digestible oligosaccharides, the bulk of which is soluble in 80% ethanol, and therefore underestimate the true amount of dietary fiber (McCleary, 2007). In order to close the gap, AOAC 2001.03 builds upon AOAC 985.29 (Prosky) and expands it by measurement of the ethanol filtrate with size-exclusion chromatography. This, of course, drastically increases the workload per analysis and makes liquid chromatography instrumentation mandatory. AOAC 2009.01 in turn adapts the enzymatic digestion step towards a more human-like model.

Kits for the standardized analysis of dietary fiber, e.g. available from Sigma Aldrich and Megazyme, conform to one of these protocols and provide the chemicals and enzymes required to meet the respective specifications.

This large number of approved methods gives the analyst an overabundance of valid choices to measure total dietary fiber. The fact that each method will give a different result can tempt to select the most profitable method for the task at hand. Either way, these methods put their emphasis on bulk quantification of dietary fiber and integrate chromatographic techniques only complementarily. The focus in science and application, however, is shifting from bulk dietary fiber to functional dietary fiber, since evidence is being amassed that type and structure of dietary fiber play a key role in health (Hopkins *et al*., 2003; Sapirstein, Wang, & Beta, 2013) and rheological implications (Berlanga-Reyes, Carvajal-Millan, Lizardi-Mendoza, Islas-Rubio, & Rascon-Chu, 2011). Additionally, consumers' perception is very critical when it comes to foodstuffs and therefore dietary fiber is required to be unobtrusive yet effective (Gurmeric, Dogan, Toker, Senyigit, & Ersoz, 2012).

Wheat bran features a complex matrix, which hampers selective extraction and calls for intricate analytical methods. Since custom chemical analyses tailored towards wheat bran do not exist, it is necessary to refer to methods employed for similar matrices, i.e. other cereals. Hence, not all applications mentioned have been utilized for wheat bran specifically, but as the basic composition of different grains is very comparable, adequacy of the adapted methods can be assumed for wheat bran, especially when the target compound is the same. The following is a brief account of instrumental techniques suitable for structural analysis of wheat bran constituents in general and wheat bran dietary fiber in particular, also discussing their scope and limitations.

Techniques for instrumental analysis of carbohydrates

Table 1 shows an overview of analytical methods for the analysis of wheat bran carbohydrates giving target substances, analytical purpose, advantages and disadvantages as well as select references.

Gas chromatography

Gas chromatography (GC) allows for the analysis of compounds that can be rendered volatile. The sample is passed through a capillary with silica-based coating with the help of an inert carrier gas, e.g. hydrogen, helium or nitrogen. Separation is achieved according to polar interactions between capillary coating (stationary phase) and analytes in the carrier gas (mobile phase), either at a fixed temperature (isothermal conditions) or by following a preset temperature program. GC can be coupled with a wide array of detectors, among them unspecific ones such as Flame Ionization Detector (FID), Electron Capture Detector (ECD) and Mass Spectrometer (MS) and highly specific ones such as Nitrogen–Phosphorus Detector (NPD), which only detects nitrogen and phosphorus.

Table 1. Analytical methods for wheat bran carbohydrates.

Analytical method	Target substance(s)	Analytical purpose	Advantages/disadvantages	Reference
GC	Monosaccharides	Quantification, linkage analysis	+ High separation capability + Low maintenance + Well established and robust + Selectivity by volatility − Derivatization required − Limited molecular weight	(Fischer *et al.*, 2004; Laine *et al.*, 2002; Willför *et al.*, 2009; Willför, Sundberg, Hemming, & Holmbom, 2005; Xu *et al.*, 2004)
Pyrolysis–GC–MS	Polysaccharides	Semi-quantitative composition, finger-print analysis	+ Analysis of solid samples + No sample preparation + Finger-print comparison − Quantification is delicate − High complexity of chromatograms − Strong matrix effects	(D'Antuono *et al.*, 1998; Galletti *et al.*, 1997; Galletti *et al.*, 1998; Peng & Wu, 2010)
HPLC	Oligosaccharides, monosaccharides	Quantification	+ No derivatization required + No molecular weight limitation + Well established and robust − Inferior separation (vs. GC)	(Bunzel *et al.*, 2005; Das, Gupta, Kapoor, Banerjee, & Bal, 2008; Simonetti *et al.*, 2002; Wood, Weisz, & Blackwell, 1991; Zhou *et al.*, 2010)
HPAEC-PAD	Polysaccharides, oligosaccharides, monosaccharides	Determination, quantification	+ Very good separation and detection of carbohydrates + High sensitivity (vs. HPLC) + Simultaneous separation of poly-, oligo- and monosaccharides − Limited flexibility	(Courtin *et al.*, 2009; Hollmann & Lindhauer, 2005; Pastell *et al.*, 2009; Verspreet *et al.*, 2012; Willför *et al.*, 2009)
CE	Oligosaccharides, monosaccharides	Determination, quantification	+ High separation capability + High sensitivity − Delicate setup − Not well established for carbohydrate analysis	(Li *et al.*, 2005; Volpi *et al.*, 2008; Westphal, Schols, Voragen, & Gruppen, 2010)
TLC	Oligosaccharides, monosaccharides	Determination	+ Cheap, robust method + Easily adapted selectivity + Wide range of selective detection methods − Mostly manual operation	(Burtseva & Terninko, 2010; Rantanen *et al.*, 2007; Wood & Fulcher, 1984)
HPSEC	Polysaccharides	Molar mass determination, size distribution	+ Well established system − High maintenance costs (vs. AsFlFFF)	(Katapodis *et al.*, 2003; Pitkänen *et al.*, 2010; Pitkänen *et al.*, 2009; Ragaee *et al.*, 2008; Rasmussen & Meyer, 2010)
AsFlFFF	Polysaccharides	Molar mass determination, size distribution	+ Low maintenance costs (vs. HPSEC) + Wide range of separation − Little literature available (vs. HPSEC)	(Lambo-Fodje *et al.*, 2007; Pitkänen *et al.*, 2010)
NMR	Arabinoxylan, β-glucan	Linkage pattern	+ Non-destructive + All-encompassing − Impurities severely impact sensitivity	(Cui *et al.*, 1999; Cyran, 2010; Ha, Jardine, & Jarvis, 1997; Sun, Cui, et al., 2011; Sun, Wen, et al., 2011; Tashpulatov, Khusanov, Usmanov, & Makhmudov, 1993)
IR/NIR	Starch, cellulose, arabinoxylan, monosaccharides	Determination, semi-quantification, functional groups	+ No sample preparation required + Rapid throughput + All-encompassing − Only relative comparisons possible − Extensive calibration required	(Blakeney & Flinn, 2005; Cyran, 2010; Pielesz, 2012; Robert *et al.*, 2011; Sun, Wen, Xu, & Sun, 2011)

The range of application in the analysis of carbohydrates from wheat bran is limited by the intrinsic prerequisite that an analyte be volatile. This can be overcome by pre-capillary derivatization, most commonly by converting the polar and intensely hydrogen-bonded hydroxyl groups into their acetyl or trimethylsilyl derivatives. But still a cut-off at roughly 1000 Da remains, often set by the employed MS detectors (Medeiros & Simoneit, 2007; Xu, Tian, & Pan, 2004). Thus, with the bulk of carbohydrates being present in the form of polysaccharides, an acidic cleavage into monosaccharides has to be performed prior to GC analysis. Here it is important to balance thorough cleavage and degradation of sugars. Willför *et al.* have proposed methanolysis using 2 M hydrochloric acid in methanol as the method of choice for the more easily degraded pentoses, which form a major part of wheat bran polysaccharides (Willför *et al.*, 2009).

When analyzing monosaccharides from polysaccharides after hydrolysis, it has to be taken into account that all structural information about linkages in the original polysaccharide is lost. In order to preserve such information, free hydroxyl groups can be derivatized to methoxy or ethoxy groups in the intact polysaccharide so that, after hydrolytic cleavage, monosaccharides can be classified according to their linkage (Ciucanu & Kerek, 1984; Watt, O'Neill, Percy, & Brasch, 2002).

Gas chromatography exhibits the highest separation capabilities, which is an asset that is required in order to meet the complexity of linkage analysis (Willför *et al.*, 2009). Fig. 1 shows a GC–MS chromatogram of arabinoxylan after methylation, methanolysis and silylation. Laine *et al.* have provided the complete spectrum of methylated monosaccharides for arabinose, galactose, glucose, mannose and xylose, which covers almost all polysaccharides present in wheat bran (Laine, Tamminen, Vikkula, & Vuorinen, 2002).

Pyrolysis–GC–MS

Pyrolysis–GC–MS is a modification of regular gas chromatography mass spectrometry (GC–MS). Instead of injecting and vaporizing a liquid sample, a solid sample is rapidly pyrolized at temperatures around 600 °C and the formed gasses are sucked onto the column of the GC. A well-defined heating rate and constancy of the final pyrolysis temperature are critical. Usually, a complex mixture of volatiles is generated (Peng & Wu, 2010). Separating and tracing them back to their compound of origin can be cumbersome and ambiguous. Fig. 2 gives an example of the complexity of a pyrolysis–GC–MS chromatogram of wheat bran. Since pyrolysis is hardly ever complete and the degree of pyrolysis depends heavily on the matrix of the sample, quantification is severely impeded. The drawbacks of pyrolysis–GC–MS are compensated by the capability to analyze solid samples and the fact that virtually no sample workup is required. The difficulty of quantification has been approached by monitoring marker substances that are characteristic pyrolysis products of a compound, which allows for a relative comparison within similar samples (D'Antuono, Galletti, & Bocchini, 1998). To our knowledge, pyrolysis–GC–MS has not been applied in the analysis of wheat bran in particular, but of comparable matrices. Galletti and Reeves could accurately follow changes in neutral detergent fiber and crude protein during maize stover growth by correlation with pyrolysis marker substances (Galletti, Reeves, & Bocchini, 1997). Galletti and D'Antuono have even used an internal standard to characterize differences in dietary fiber and lignin of pericarp layers of spelt, wheat and spelt/wheat crosses (Galletti, D'Antuono, Bocchini, & Rosolen, 1998). A similar approach should be feasible for wheat bran.

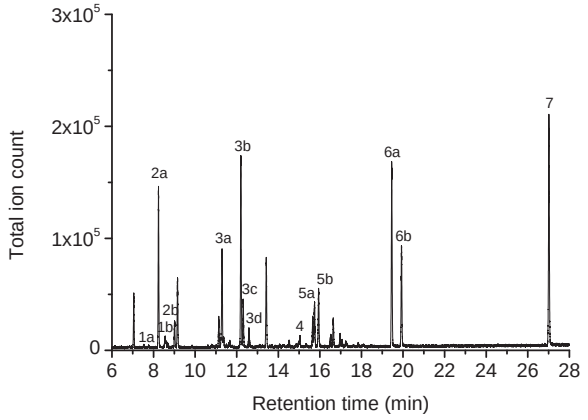

Fig. 1. Total ion chromatogram of the products of a methylation analysis of wheat arabinoxylan by GC–MS. 1a/b: The non-reducing xylopyranose end group as α- and β-*O*-methyl-2,3,4-tri-*O*-methyl xylopyranoside. 2a/b: The non-reducing arabinofuranoside end group as α- and β-*O*-methyl-2,3,5-tri-*O*-methyl arabinofuranoside. 3a/b/c/d: The 4-substituted xylose unit as α- and β-*O*-methyl-di-2,3-*O*-methyl-4-*O*-trimethylsilyl xylopyranoside and α- and β-*O*-methyl-di-2,3-*O*-methyl-5-*O*-trimethylsilyl xylofuranoside. 4: The 2,4-substituted xylose unit as α- and β-*O*-methyl-3-*O*-methyl-2,4-di-*O*-trimethylsilyl xylopyranoside (peaks not separated, furanose form negligible). 5a/b: The 3,4-substituted xylose unit as α- and β-*O*-methyl-2-*O*-methyl-3,4-di-*O*-trimethylsilyl xylopyranoside (furanose form negligible). 6a/b: The 2,3,4-substituted xylose unit as α- and β-*O*-methyl-2,3,4-tri-*O*-trimethylsilyl xylopyranoside (furanose form negligible). 7: The internal standard sorbitol as 1,2,3,4,5,6-hexa-*O*-trimethylsilyl sorbitol.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) separates analytes according to their distribution between a mobile liquid phase and a stationary solid phase. Among the most common detectors HPLC can be coupled with are UV/VIS, fluorescence and mass spectrometry.

Much like GC, HPLC can be used in the analysis of monosaccharides after cleavage of polysaccharides. Monosaccharides can be analyzed either directly (Zhou *et al.*, 2010) or, for improved separation and detection, after

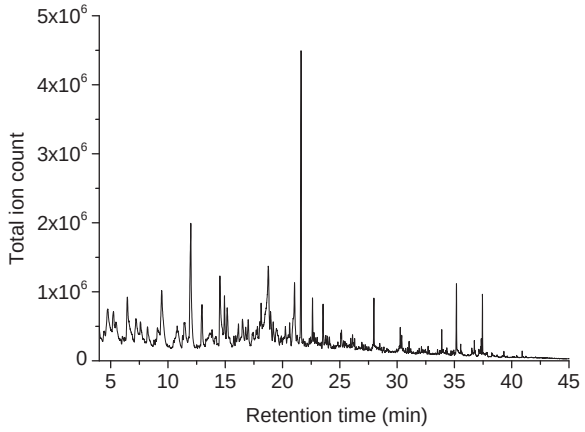

Fig. 2. Pyrolysis-GC–MS chromatogram of wheat bran.

reduction to alditols and derivatization with phenylisocyanate to their respective urethanes (Simonetti *et al*., 2002).

Unlike GC, HPLC is not hindered by a limit in molecular weight detection and therefore allows for analysis of oligosaccharides, which can be obtained by partial acidic hydrolysis or enzymatic treatment (Bunzel, Ralph, & Steinhart, 2005; Saulnier, Vigouroux, & Thibault, 1995). However, analysis of these oligosaccharides is far from trivial. Separation gets increasingly difficult with degree of polymerization and standards are often not commercially available, especially for branched hemicelluloses like arabinoxylan. Furthermore, oligomers usually cannot be differentiated reliably by the MS detector, since the ionization methods commonly employed in LC–MS (ESI, APPI, APCI) do not fragment the molecules and isomeric oligosaccharides of different sugar composition or linkage cannot be distinguished unambiguously by molecular mass alone.

High-performance anion-exchange chromatography with pulsed amperometric detection

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) makes use of the fact that carbohydrate hydroxyl groups are slightly acidic and separates them at a high pH on an anion-exchange column. Analytes are detected by the PAD as an increase in current flow at the working electrode as they are oxidized by pulses of voltage (Weitzhandler, Rohrer, Thayer, & Avdalovic, 2002). HPAEC-PAD is very well suited for the analysis of monosaccharides, (Yokota, Mori, Yamaguchi, Kaniwa, & Saisho, 1999), oligosaccharides and polysaccharides (Morales, Corzo, & Sanz, 2008) and has demonstrated capabilities as a fingerprint method for quality control (Ding, Shao, Ying, Liao, & Yang, 2011). In the analysis of wheat bran, HPAEC-PAD is generally used to measure monosaccharides after methanolysis/hydrolysis (Hollmann & Lindhauer, 2005) or oligosaccharides after enzymatic treatment or partial acidic hydrolysis (Pastell, Virkki, Harju, Tuomainen, & Tenkanen, 2009; Verspreet *et al*., 2012). E.g., Courtin *et al*. have used HPAEC-PAD to determine heat and pH stability of arabinoxylo- and xylooligosaccharides under varying conditions and have found that longer chain oligosaccharides are generally more stable than their short chain counterparts (Courtin, Swennen, Verjans, & Delcour, 2009). In comparison with GC, HPAEC-PAD cedes superior separation and is less forgiving towards impurities, but works without derivatization and allows for the analysis of oligosaccharides (Willför *et al*., 2009).

Capillary electrophoresis

Capillary electrophoresis (CE), sometimes synonymously called capillary zone electrophoresis (CZE), is a separation technique built upon the principle of electrophoresis, i.e. the movement of charged species in solution along an electric field gradient. Electrodes are applied to the ends of a glass capillary which is, depending on separation requirements, either packed with fused silica or an uncoated tubular system. When a so-called background electrolyte alkaline buffer is introduced into the capillary, the surface silanol groups are partially deprotonated and the charged species of the electrolyte generate an electroosmotic flow contingent upon pH towards one of the electrodes, which allows for separation of analytes according to charge, size and interaction with the electrolyte, i.e. their electrophoretic mobility (Baker, 1995). To confer charges upon mono- and oligosaccharides, often borate is added to the background electrolyte, which forms complexes with those analytes. The most common detectors are UV and MS.

The separation principle of CE is especially useful for protein analysis, but has also been directed towards the separation of oligo- and polysaccharides (Li, Wang, & Altman, 2005; Volpi, Maccari, & Linhardt, 2008). It is also frequently used for analysis of complex mixtures of carbohydrate degradation products, mainly containing organic acids and hydroxyacids (Bogolitsyna *et al*., 2011). CE has not been used in the analysis of wheat bran carbohydrates to our knowledge, but has much potential due to its powerful separation and sensitivity characteristics.

Thin layer chromatography

In light of the many high-tech instrumental techniques placed at the disposal of an analytical chemist today, thin layer chromatography (TLC), originally derived from paper chromatography, is sometimes regarded as a seemingly inferior veteran technique. TLC makes use of capillary forces in order to achieve a flow of the mobile phase. Samples are applied to a plate coated with a stationary phase, commonly silica gel, aluminum oxide or cellulose, and then separated according to the principles of solubility and adsorption as the mobile phase permeates the coating of the plate. Improvements such as automated sample application, controlled plate development and advanced detector coupling capabilities along with its intrinsic robustness and low costs have updated TLC to a modern technique that in some cases can be the method of choice. Modes of detection range from simple visual inspection, UV-absorption, IR, fluorescence with and without selective chemical derivatization to matrix-assisted laser desorption ionization with time-of-flight mass spectrometry (MALDI-TOF/MS) (Qureshi, Stecher, Huck, & Bonn, 2010) and desorption electrospray ionization (DESI) (Van Berkel, Ford, & Deibel, 2005).

TLC can be used for the analysis of monosaccharides (Gal, 1968) as well as oligosaccharides (Buckeridge *et al*., 1997), which makes it suitable for wheat bran carbohydrates. Fig. 3 shows the separation and detection of arabinose, glucose and xylose, which are the prevalent sugars found in wheat bran polysaccharides. Wood *et al*. have been able to separate (1,3)-β-D-glucan from cereal grains on TLC plates and selectively stain them with the

fluorescent label aniline blue (Wood & Fulcher, 1984). More recent studies focus on the separation and detection of arabinoxylo-oligosaccharides produced from enzymatic degradation of arabinoxylan (Pastell *et al*., 2009; Puchart & Biely, 2008; Rantanen *et al*., 2007).

High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC), also referred to as high-performance gel permeation chromatography (HPGPC), allows for the separation of (macro-) molecules by hydrodynamic volume. This is achieved by passing the sample through a column packed with a gel with defined pore-sizes. Accordingly, these pores admit less space to bigger molecules and therefore have them elute after a smaller volume of mobile phase. Classic detection is performed by measuring differences in refractive index (RI) in the eluant. However, RI detection can only measure concentration changes and therefore needs to rely on calibration with standards. Since polysaccharide standards with defined molecular weight are usually unavailable, RI detection is advantageously combined with a detector sensitive to molecular weight, such as light scattering detectors (RALLS, MALLS) (Trathnigg, 1995). For reporting specific properties of samples, UV and fluorescence are the detectors of choice. Fig. 4 shows an exemplary chromatogram of wheat arabinoxylan with dRI and MALLS detection.

HPSEC is the prevailing technique for molecular weight determination of polysaccharides in general and wheat bran arabinoxylan and β-glucan in particular. It is commonly applied to monitor processes of enzymatic degradation (Katapodis *et al*., 2003; Rasmussen & Meyer, 2010) or assess extractions with respect to their chain-degrading properties (Rose & Inglett, 2010). Li *et al*. (Li *et al*., 2006) have related chain length of wheat β-glucan to rheological properties finding that gelation rate and melting enthalpy decreased whereas melting temperature increased with increasing molecular weight.

It is noteworthy that the choice of mobile phase does not only affect the solubility of polysaccharides, but also their tendency to aggregate, which leads to an over-estimation of molecular weight. Pitkänen *et al*. (Pitkänen, Virkki, Tenkanen, & Tuomainen, 2009) have found cereal arabinoxylans to show a higher tendency to aggregate in aqueous solutions as compared to solutions in DMSO, concluding that DMSO as the eluant provides a more, albeit not absolutely accurate means of molecular size determination of arabinoxylans.

Asymmetric flow field-flow fractionation

Asymmetric flow field-flow fractionation (AsFlFFF), like HPSEC, separates molecules by size, but applies a different principle. Before passing the analytes through a channel, it concentrates them at the bottom at the beginning of the channel with a flow perpendicular to the channel and lets them diffuse for a while before the parallel flow is initiated. Bigger molecules will diffuse slower than smaller ones. With the flow being the fastest at the diametric center of the channel, these molecules will be eluted first. The range of detectors is identical to HPSEC.

Being a relatively recent technique, AsFlFFF is not as commonly used as HPSEC and therefore a smaller base of knowledge is available to draw from. However, maintenance costs are lower for AsFlFFF compared to HPSEC and analytical capabilities can be favorable. Pitkänen *et al*. have reported better separation of arabinoxylans in aqueous solution for AsFlFFF than HPSEC in a way that aggregates were discriminated (Pitkänen, Tenkanen, & Tuomainen, 2010).

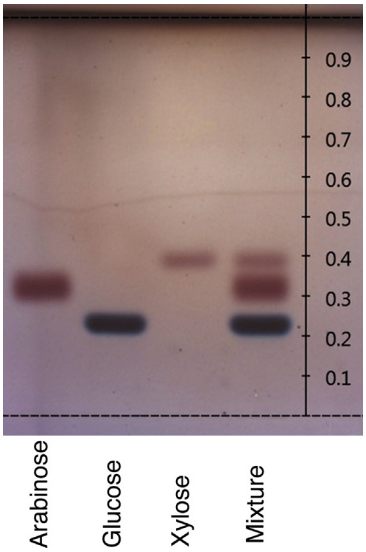

Fig. 3. Thin layer chromatography of arabinose, glucose, xylose and a mixture of these sugars after visualization with anisaldehyde in sulfuric acid.

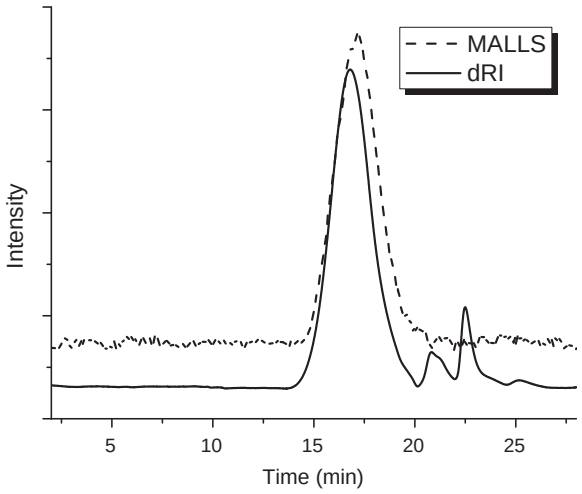

Fig. 4. High-performance size-exclusion chromatography of wheat arabinoxylan with dRI (concentration sensitive) and MALLS (molecular weight sensitive) detection.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) utilizes the spin properties of certain atomic nuclei, the most prominent being ^{1}H and ^{13}C. Exposed to a strong magnetic field, the spins will align either parallel or anti-parallel to the magnetic field. Since the anti-parallel orientation constitutes a state of higher energy, the parallel low-energy state is the more populated one. The difference in energy largely depends on the strength of the magnetic field and correlates to a frequency in the radio wavelength spectrum. Therefore, spins can be excited from the low to the high-energy state by irradiation at the transitional wavelength. This, however, would only give one signal per type of nucleus. To a minute but crucial degree, the difference between the two energy states is also influenced by the chemical environment of the nucleus, which can reinforce or weaken the magnetic field and thus give characteristic deviations in the excitation wavelengths. Neighboring spins can also reinforce or weaken the local magnetic field through parallel or anti-parallel orientation (spin coupling) and thus lead to a splitting of signals according to the number of neighbors. This renders vital information about connectivity between atoms (Keeler, 2010).

NMR is chiefly applied for structure elucidation and structure confirmation. This makes it an indispensable tool for linkage analysis of wheat bran polysaccharides (Kormelink *et al*., 1993; Sun, Cui, Gu, & Zhang, 2011; Swennen, Courtin, Lindemans, & Delcour, 2006). Fig. 5 shows a ^{1}H NMR spectrum of arabinoxylo-oligosaccharides in order to illustrate an example of linkage analysis. With NMR being an absolute technique, i.e. every carbon and hydrogen atom in the sample giving a signal of equal intensity, quantification can easily be performed with the addition of an internal standard. However, this also entails that impurities severely impact the measurement.

Cui *et al*. have compared wheat β-glucan to β-glucan from other cereals. By characterizing enzymatically generated fragments with the help of 2D NMR spectroscopy, they deduced a more regular structure predominantly based on repeating trisaccharide units for wheat β-glucan and relate that finding to improved gelling ability and lower solubility as compared to β-glucan from other cereals (Cui, Wood, Blackwell, & Nikiforuk, 1999).

Pastell *et al*. have investigated side chains of enzymatically produced arabinoxylo-oligosaccharides from different cereal brans and husks and discovered a novel sequence, namely β-D-xylopyranose (1 → 2)-linked to α-L-arabinofuranose (1 → 3)-attached to the xylan backbone. This structure, albeit present in corn cobs, barley husks, oat spelts, rice husks and even wheat straw, was not detected in wheat bran arabinoxylan (Pastell *et al*., 2009).

Infrared/near-infrared spectroscopy

Infrared spectroscopy (IR), more accurately referred to as mid-infrared spectroscopy, uses infrared light ranging from 4000 to 400 cm^{-1} to excite vibrations within a molecule and measures the absorption. Since different atomic weights and bond lengths effect different momenta of inertia and thus different vibrational frequencies, these absorptions can be related to functional groups and building blocks (Smith, 1999). Fig. 6 shows IR spectra of cellulose, starch, wheat arabinoxylan, a hydrothermal extract of wheat bran, bovine serum albumin and an ionic liquid extract of wheat bran.

Near-infrared spectroscopy (NIR) is based on the same principle as IR and covers the range from 14,000 to 4000 cm^{-1}. What is measured here, however, are molecular overtone and combination vibrations. These vibrations cannot be ascribed to parts of a molecule by wavelength, but need to be ascertained as a bulk. Therefore, NIR is commonly used as a black-box measurement in which parameters are determined by multivariate data analysis after calibration with large calibration sets predefined with respect to these parameters. These calibrations are cumbersome, but once established, NIR offers rapid sample throughput without any workup required (Siesler, 2002).

Pielesz gives a comprehensive account of how to identify monosaccharides after acid hydrolysis of plant wall polysaccharides using IR and provides a database of characteristic peaks for each carbohydrate (Pielesz, 2012).

Cyran has used IR as a complementary method to confirm his structural findings for feruloylated arabinoxylans from outer layers of rye (Cyran, 2010).

Using NIR, Blakeney *et al*. have calibrated upward of 70 grain samples on the basis of soluble, insoluble and total dietary fiber as well as arabinose, xylose and glucose (separated into cellulose and β-glucan) content for each of these fractions. For insoluble arabinoxylan and cellulose they achieved fits suited for analytical purposes. With the

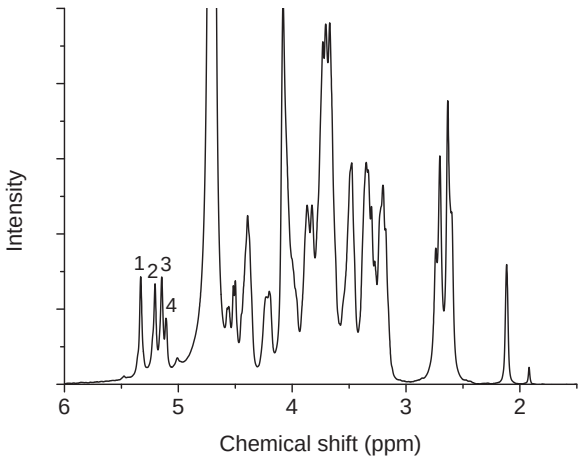

Fig. 5. ^{1}H NMR spectrum of arabinoxylo-oligosaccharides produced from arabinoxylan with xylanase treatment. 1: Anomeric proton of arabinose α(1 → 3) linked to disubstituted xylose. 2: Anomeric proton of arabinose α(1 → 3) linked to monosubstituted xylose. 3: Anomeric proton of arabinose α(1 → 2) linked to disubstituted xylose. 4: Anomeric proton of arabinose α(1 → 2) linked to monosubstituted xylose.

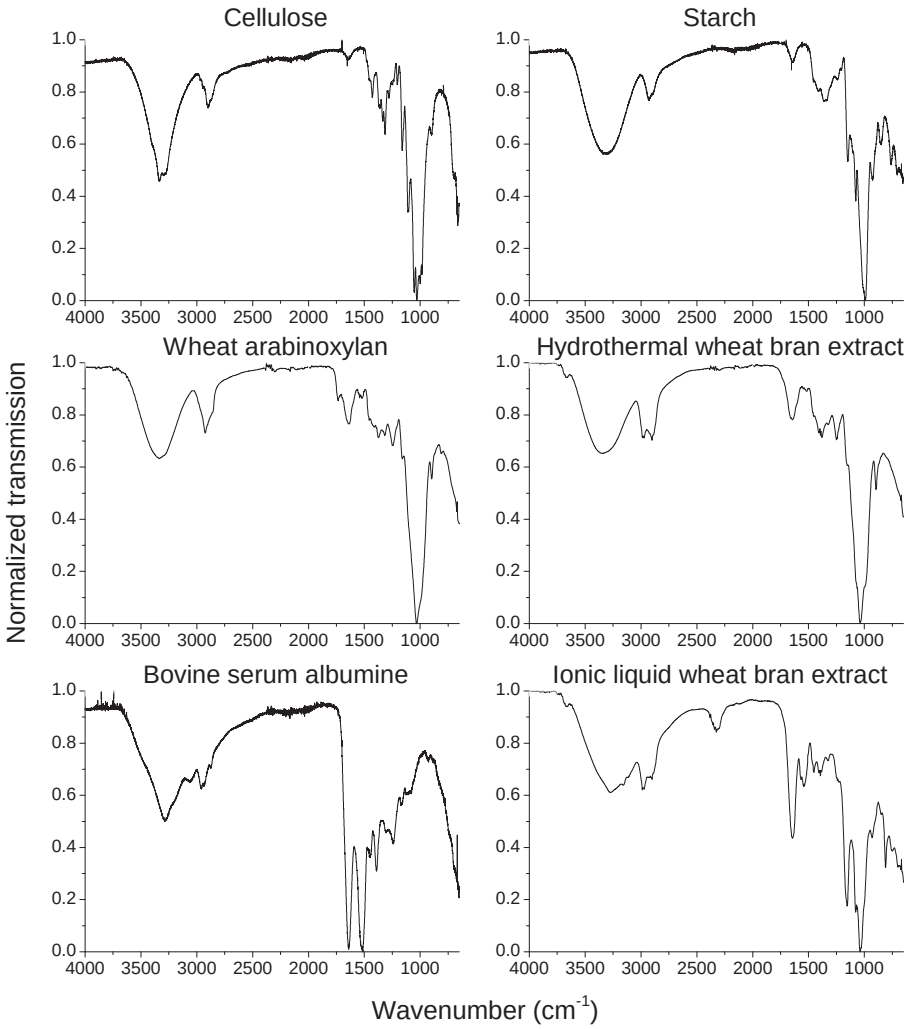

Fig. 6. Infrared spectra of cellulose, starch, wheat arabinoxylan, a hydrothermal extract of wheat bran, bovine serum albumin and an ionic liquid extract of wheat bran using 1-ethyl-3-methylimidazolium phosphinate. Starch and cellulose are the prevalent impurities for the hydrothermal extract. The ionic liquid extract shows substantial amounts of residual protein.

exception of the soluble fraction, which only makes up a small portion and is therefore more prone to error, all fits were at least suitable for screening purposes (Blakeney & Flinn, 2005).

IR and NIR can also be incorporated into two-dimensional image mapping of cell tissue by lining up single, adjacent scans and assigning colors to peaks of interest (Wetzel, 2012). Robert *et al*. (Robert *et al*., 2011) thus followed the development process of transfer and aleurone cells of wheat grain on the level of cell wall polysaccharides.

Techniques for the analysis of non-carbohydrate constituents

The analysis of non-carbohydrate constituents of wheat bran is largely dominated by standardized AOAC methods and therefore leaves little demand for discussion. On top of that, the focus of this work is on the analysis of wheat bran carbohydrates. Table 2 in brevity lists the non-carbohydrates along with the respective analytical method and a reference, preferably a standardized method. Where no standardized method was available, suitable literature citations are given.

Final considerations

The multitude of analytical techniques available makes for a handy toolbox in the analysis of wheat bran. Of course, acquisition costs for analytical instruments are often the limiting factor, but since many instruments essentially generate the same information and only differ in their drawbacks and advantages, a sound selection is sufficient to describe wheat bran in large parts.

Even though the starting material has already been thoroughly characterized, structural analysis is far from being

Table 2. Analytical methods for non-carbohydrates of wheat bran.

Target substance	Analytical method	Reference
Protein	Kjeldahl	ICC 105/2
Amino Acids	HPAEC-PAD	(Ding & Mou, 2004; Rombouts *et al.*, 2012; Yu & Mou, 2007)
	Amino Acid Analyzer	(Chiesl *et al.*, 2009; Kabaha, Taralp, Cakmak, & Ozturk, 2011)
Lipids	Soxhlet extraction	ICC 136
	GC–MS	(Durante, Lenucci, Rescio, Mita, & Caretto, 2012; Rout *et al.*, 2009)
Minerals	Ashing	ICC 104/1
	ICP-OES	(Araujo *et al.*, 2008; Ficco *et al.*, 2009)
Lignin	Klason-Lignin	AOAC 994.13
	Thermogravimetric Analysis (TGA)	(Cozzani, Lucchesi, Stoppato, & Maschio, 1997; Fujii, Mochidzuki, Kobayashi, & Sakoda, 2011)
Water	Oven-drying	ICC 110/1
Phytic acid	HPLC	(Amaro, Murillo, Gonzalez, Escalona, & Hernandez, 2009; Oberleas & Harland, 2007)
Ferulic acid	HPLC	(Dobberstein & Bunzel, 2010; Parker, Ng, & Waldron, 2005)

concluded. Advances in processing and ensuing health-related issues as well as physicochemical changes, especially regarding dietary fiber, can be expected to make structural analysis even more indispensable. With sample workup mostly being cumbersome and coherent information having to be put together manually from fragments collected in different analyses, there is still plenty of room to advance state-of-the-art technology in both instrumental capabilities and data evaluation. It is, however, rather unlikely that these techniques will be incorporated into official AOAC methods in the near future, since they are largely tailored to a specific matrix whereas AOAC methods are required to be more widely applicable. Either way, the strict divide between market-oriented quantification of dietary fiber according to AOAC methods and research-oriented structural elucidation, as it stands now, will have to give.

Acknowledgment

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Arabinoxylan Oligosaccharide Hydrolysis by Family 43 and 51 Glycosidases from *Lactobacillus brevis* DSM 20054

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Due to their potential prebiotic properties, arabinoxylan-derived oligosaccharides [(A)XOS] are of great interest as functional food and feed ingredients. While the (A)XOS metabolism of *Bifidobacteriaceae* has been extensively studied, information regarding lactic acid bacteria (LAB) is still limited in this context. The aim of the present study was to fill this important gap by characterizing candidate (A)XOS hydrolyzing glycoside hydrolases (GHs) identified in the genome of *Lactobacillus brevis* DSM 20054. Two putative GH family 43 xylosidases (XynB1 and XynB2) and a GH family 43 arabinofuranosidase (Abf3) were heterologously expressed and characterized. While the function of XynB1 remains unclear, XynB2 could efficiently hydrolyze xylooligosaccharides. Abf3 displayed high specific activity for arabinobiose but could not release arabinose from an (A)XOS preparation. However, two previously reported GH 51 arabinofuranosidases from *Lb. brevis* were able to specifically remove α-1,3-linked arabinofuranosyl residues from arabino-xylooligosaccharides (AXHm3 specificity). These results imply that *Lb. brevis* is at least genetically equipped with functional enzymes in order to hydrolyze the depolymerization products of (arabino)xylans and arabinans. The distribution of related genes in *Lactobacillales* genomes indicates that GH 43 and, especially, GH 51 glycosidase genes are rare among LAB and mainly occur in obligately heterofermentative *Lactobacillus* spp., *Pediococcus* spp., members of the *Leuconostoc/Weissella* branch, and *Enterococcus* spp. Apart from the prebiotic viewpoint, this information also adds new perspectives on the carbohydrate (i.e., pentose-oligomer) metabolism of LAB species involved in the fermentation of hemicellulose-containing substrates.

Hemicelluloses, in particular, heteroxylans, are among the most abundant polysaccharides in plant biomass. The basic configuration of arabinoxylans (AX), the major fraction of cereal cell wall heteroxylans (1), involves a linear xylan backbone of β-1,4-linked D-xylopyranose units with L-arabinofuranosyl side chains as the major substituents (1, 2). The xylose monomers can be substituted by up to two arabinose residues in α-1,2 and/or α-1,3 configuration (Fig. 1). The degrees of arabinose substitution, as well as occurrences of other substituents, most importantly ferulic acid, underlie high variability in plants and plant tissues (2).

AX depolymerization products obtained by *endo*-xylanase (EC 3.2.1.8)-catalyzed hydrolysis are denoted arabinoxylan-oligosaccharides. Following previously described criteria (1), the present paper uses the following acronyms: XOS for xylooligosaccharides, AXOS for arabino-xylooligosaccharides, (A)XOS for mixtures of XOS and AXOS, and (A)XOS as well for unspecified products of AX depolymerization. Complete (A)XOS hydrolysis requires the concerted action of α-L-arabinofuranosidase (EC 3.2.1.55) and β-D-xylopyranosidase (EC 3.2.1.37). Arabinofuranosidases with arabinoxylan arabinofuranohydrolase (AXH) activity are usually classified according to the nomenclature recommended by Van Laere et al. (3): the more common AXHm hydrolyze both α-1,2 and α-1,3 glycosidic bonds of single arabinose substitutions (Fig. 1). Enzymes that specifically catalyze the release of arabinose from disubstituted backbone residues are designated AXHd. So far, only a few examples of the latter class, denoted AXHd3 due to their selectivity for α-1,3 linked arabinose residues, have been reported (3–6). Arabinofuranosidases that would classify as AXHd2 (or AXHd2,3) have not been described so far.

AXs are recognized as important dietary fibers (1, 2), and (A)XOS have raised considerable interest regarding their potentially prebiotic effect on the intestinal microbiota. The bifidogenic effect of dietary (A)XOS was thoroughly studied and confirmed (1, 7). Furthermore, several bifidobacterial α-L-arabinofuranosidases (both AXHm and AXHd3) (3, 6, 8–10), as well as β-D-xylosidases (11, 12) required for complete (A)XOS degradation, have been identified and characterized, whereas all of the enzymes reported so far (both AXH and β-xylosidases) are members of glycoside hydrolase (GH) family 43 or 51 (13).

In contrast, information on the mechanisms of (A)XOS utilization by lactic acid bacteria (LAB), more specifically, members of the taxonomical order *Lactobacillales*, is still limited. The scientific literature available so far suggests that the ability to metabolize XOS *in vitro* may be a rare trait among LAB (14, 15), while evidence for AXOS utilization by LAB has, to our knowledge, not been presented at all. This knowledge gap is unfortunate, as LAB can be part of the beneficial intestinal microbiota as well (16). Further, LAB are ubiquitously present in fermentations of hemicellulose (AX)-rich cereals (17–19), and it is important to understand the LAB metabolism associated with such materials.

The aim of the present study was to expand on the knowledge of (A)XOS utilization by LAB by studying candidate (A)XOS-hydrolyzing enzymes of the heterofermentative species *Lactoba-*

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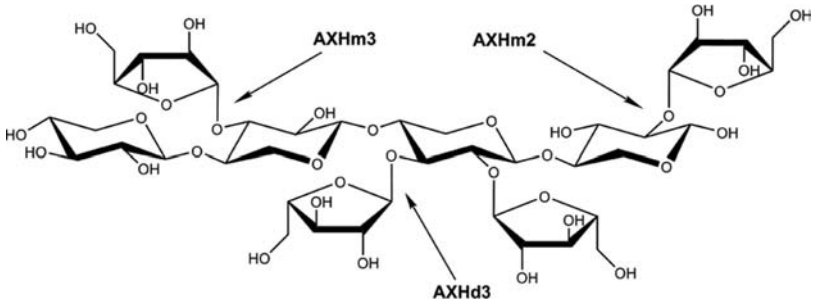

FIG 1 Schematic representation of an arabino-xylooligosaccharide (AXOS) molecule. The xylooligosaccharide backbone is substituted with arabinofuranosyl residues in α-1,2 or α-1,3 glycosidic links. With regard to their substrate specificity, arabinofuranosidases are classified as AXHm (specific for single arabinose substitutions) or AXHd (specific for residues from disubstitutions).

cillus brevis. So far, *Lb. brevis* is the only *Lactobacillus* species reported to efficiently utilize XOS (14, 20–22), and a XOS-hydrolyzing β-xylosidase was recently isolated from *Lb. brevis* NCDC01 after the strain was grown on XOS (23). The published genome of *Lb. brevis* (strain ATCC 367) contains three GH 43 glycosidase genes putatively designated β-D-xylosidases (24). Here, we report the heterologous expression of orthologs to these genes from *Lb. brevis* DSM 20054 and the subsequent characterization of the corresponding gene products. As recently reported (25), *Lb. brevis* further possesses two functional GH 51 arabinofuranosidases. We aimed to investigate whether these glycosidases (both GH 43 and GH 51) are capable of hydrolyzing (A)XOS and evaluated the presence of corresponding glycosidase genes in *Lactobacillales* genomes (as available at the time of writing).

MATERIALS AND METHODS

DNA manipulation and sequence analysis. PCR was performed with Phusion High-Fidelity PCR Master Mix (New England BioLabs, Ipswich, MA). Restriction enzymes and a Quick Ligation kit were also purchased from New England BioLabs. PCR products were purified with a Wizard SV Gel and PCR Clean-UP kit, and plasmid purification was done with a PureYield Plasmid Miniprep system (both from Promega, Madison, WI).

Oligonucleotide primers targeting the flanking regions of the genes encoding GH 43 hydrolases (all annotated as putative β-xylosidases) were designed based on the published genome of *Lb. brevis* ATCC 367 (GenBank accession no. NC_008497; loci LVIS_0375, LVIS_1748, and LVIS_2285 [24]). These primers (see Table S1, upper part, in the supplemental material) were used to amplify the respective open reading frames from the genomic DNA of *Lb. brevis* DSM 20054 (DSMZ GmbH, Braunschweig, Germany). The PCR products were ligated into pJET1.2 blunt cloning vectors (Thermo Fisher Scientific, St. Leon-Rot, Germany) and sequenced (LGC Genomics, Berlin, Germany). Based on the nucleotide sequences obtained, specific primers containing the necessary restriction sites (see Table S1, lower part) were designed to introduce the genes into pET21a expression vectors (Novagen, Madison, WI) in frame with a C-terminal His_6 tag. *Escherichia coli* T7 Express competent cells (New England BioLabs) were transformed with the plasmids obtained, and positive colonies were selected based on their ampicillin (100 μg ml^{-1}) resistance on LB agar plates.

Protein production, chromatography, and electrophoresis. Gene expression and subsequent protein purification by immobilized metal ion affinity chromatography (IMAC) and anion-exchange chromatography (AEC) were performed following established protocols and as recently reported (25). The binding buffer used for AEC was a 0.02 M citrate-phosphate buffer (pH 7.0) prepared according to the method of McIlvaine (26). Proteins were eluted with 1 M NaCl in the same buffer by applying a linear gradient in 10 column volumes; the purified enzymes were stored in 0.02 M McIlvaine buffer (pH 7.0).

Molecular masses were determined by size exclusion chromatography on a Sephacryl S-300 column (GE Healthcare, Uppsala, Sweden) (190 ml; column diameter, 16 mm) with 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl at a flow rate of 0.15 cm min^{-1}. The column was calibrated with protein standards (kit for molecular weights 29,000 to 700,000) from Sigma-Aldrich (St. Louis, MO).

SDS-PAGE (including Coomassie blue staining) was performed using a Mini-Protean system with precast gels (4% to 20%) from Bio-Rad (Hercules, CA); the molecular mass marker used was Precision Plus Protein Unstained (Bio-Rad) (10-to-250-kDa range).

Enzyme assays. The substrates used for chromogenic enzyme assays were *p*-nitrophenol (*p*NP)-linked glycosides (*p*NP-α-L-arabinofuranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-L-arabinopyranoside, and *p*NP-β-D-galactopyranoside) obtained from Sigma-Aldrich. The following were purchased from Megazyme International (Wicklow, Ireland): 1,5-α-L-arabinooligosaccharides (arabinobiose and arabinotriose) and 1,4-β-D-xylooligosaccharides (xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose).

Standard reaction conditions for all enzyme assays were 0.1 M McIlvaine buffer (pH 5.5) at 37°C and a 5-min incubation time (all determinations were performed in triplicate). Assays with *p*NP glycosides were stopped with 0.5 M Na_2CO_3 (2-fold volumetric excess), and the absorbance of *p*-nitrophenol was measured at 400 nm (ε_{400} = 18.300 M^{-1} cm^{-1} at pH 10.2) in a Beckman DU 800 spectrometer (Paolo Alto, CA). The temperature dependence of enzyme activity was determined by adjusting the temperature from 4 to 90°C, while pH dependence was determined by performing the above-described procedure with McIlvaine buffers ranging from pH 3.0 to 7.9 at 37°C.

Assays with oligosaccharides were conducted as described above but were stopped by heat inactivation at 80°C for 15 min. Arabinose, arabinooligosaccharides, xylose, and xylooligosaccharides were quantified by high-performance anion-exchange chromatography with pulsed amperometric gold electrode detection (HPAEC-PAD). The equipment used consisted of a Dionex DX500 system with a CarboPac PA 100 column (Dionex, Sunnyvale, CA) and an injection volume of 20 μl. Separation of saccharides was performed by applying a linear gradient from 100% eluent A (150 mM NaOH) to 28.8% eluent B (0.5 M sodium acetate–150 mM NaOH) over 25 min (12.5 ml) at a flow of 0.5 ml min^{-1} at 25°C and 115 × 10^5 Pa.

One unit (U) of reported glycosidase activity corresponds to the hydrolysis of 1 μmol of substrate per min under the investigated conditions. Kinetic constants (K_m, k_{cat}) were determined under standard assay conditions (pH 5.5, 37°C) by variation of the individual substrate concentrations in the assays (0 to 40 mM). The results (curve fits) were analyzed with SigmaPlot 11.0, the regression models used were those of Michaelis-

TABLE 1 Specific activities of the *Lb. brevis* GH 43 glycosidases for various substrates determined at 37°C and pH 5.5

Substrate	Specific activity (U mg^{-1})[a]		
	XynB1 (LVIS_0375) (10 mM)	XynB2 (LVIS_2285) (10 mM)	Abf3 (LVIS_1748) (10 mM)
p-Nitrophenyl-α-L-arabinofuranoside	0.059 ± 0.002	0.375 ± 0.005	1.79 ± 0.01
1,5-α-L-Arabinobiose	0.0014 ± 0.0001	0.081 ± 0.006	208 ± 8
1,5-α-L-Arabinotriose	ND	0.035 ± 0.001	95 ± 8
p-Nitrophenyl-β-D-xylopyranoside	0.097 ± 0.003	22.1 ± 0.1	ND
1,4-β-D-Xylobiose	ND	154 ± 6	ND
1,4-β-D-Xylotriose	ND	71 ± 4	ND

[a] Designations in parentheses represent the corresponding loci of *Lb. brevis* ATCC 367 (GenBank accession no. NC_008497). Values represent the average results of triplicate determinations ± standard deviations (SD). ND, not detectable.

Menten and Hill, and the k_{cat} was calculated as catalytic center activity, considering the theoretical molecular masses of the subunits.

endo-Xylanase and *endo*-arabinanase activities were assayed by determining the amount of reducing sugar by the dinitrosalicylic acid (DNSA) assay as described by Miller (27). Standard curves of arabinose and xylose were prepared in the range of 0 to 1 mg ml^{-1}.

Experiments with AX and (A)XOS. Wheat arabinoxylan (low viscosity, Megazyme) was dissolved in 100 mM McIlvaine buffer (pH 5.5) at a concentration of 2% (wt/vol) and treated with the *endo*-xylanase preparation Pentopan Mono BG (Sigma-Aldrich) at a dosage of 10 mg ml^{-1}. The reaction mixtures were incubated for 24 h at 50°C. Xylanase was heat inactivated (80°C, 30 min), and the (oligo)saccharides obtained were analyzed by HPAEC-PAD and methylation analysis as described below. This (A)XOS preparation served as the substrate for further enzyme assays. Assays were performed as described above but with incubation for 90 min at 37°C. The enzymes used as controls were a GH 51 α-L-arabinofuranosidase from *Aspergillus niger* (AFASE) and a GH 43 α-L-arabinofuranosidase (AFAM2) from *Bifidobacterium adolescentis*, both obtained from Megazyme. AFAM2 is identical to the AXHd3 enzyme from *B. adolescentis* (3, 6). All enzymes were used at a concentration of 0.25 mg ml^{-1}.

Methylation analysis. Methylation of oligosaccharides was performed according to references 28 and 29. Subsequent acidic methanolysis was performed according to Sundberg et al. (30).

Silylation. Samples were equilibrated in 400 μl of pyridine for 1 h. BSTFA [N,O-bis(trimethylsilyl)trifluoroacetamide] (200 μl) containing 10% TMCS (trimethylchlorosilane) (both from Sigma-Aldrich) was added, and the reaction mixture was incubated at 70°C for 2 h. Samples were diluted with 600 μl of ethyl acetate, filtered through a 0.45-μm-pore-size polytetrafluoroethylene (PTFE) syringe filter, and analyzed by gas chromatography.

Gas chromatography. A silylated sample (1 μl) was injected (260°C; split ratio, 1:50) into a HP-5 column (30 m by 0.25 mm; film thickness, 0.25 μm) in an Agilent 6890N Series gas chromatography (GC) system with an Agilent 5973 Series mass selective detector. The temperature program was 100°C (2 min); 4°C min^{-1}; 220°C (2 min); 15°C min^{-1}; and 300°C (2 min); the carrier gas was helium (1 ml min^{-1}, constant flow). Detector conditions were 70 eV with a scan range of from 40 to 600 Da. Data were acquired and processed with MSD Chemstation E.2.01.1177 software from Agilent Technologies.

Peaks were identified by comparison to the standards methylated xylose (2,3,4-linked xylose), methylated xylotriose (terminal xylose and 4-linked xylose), and nonmethylated xylose and arabinose (terminal xylose and terminal arabinose) as well as wheat arabinoxylan (2,4-linked and 3,4-linked xylose) according to the procedure described above. Relative retention times were in accordance with those in published literature employing a comparable setup (31).

Molar response factors were calculated from the standards listed above. Since standards for 2,4-linked and 3,4-linked xylose were not commercially available, their response factors were inversely calculated from the arabinose-to-xylose ratio of arabinoxylan after subtraction of the remaining constituents. Response factors for 2,4-linked and 3,4-linked xylose were assumed to be identical to those previously reported (31). The total ion count was used for calculations, with the exception of terminal xylose, whose data were obscured by silylation reagent products and whose value was calculated from its characteristic ion 101. A typical chromatogram obtained from arabinoxylan is shown in Fig. S1 in the supplemental material.

Nucleotide sequence accession numbers. Nucleotide sequences have been deposited in GenBank under the following accession numbers: KF305639 (*xynB1*), KF305641 (*xynB2*), and KF305640 (*abf3*). The sequences of *abf1* and *abf2* are accessible as HM363023 and HM363024, respectively.

RESULTS

Three genes encoding putative GH 43 glycosidases from *Lb. brevis* DSM 20054 (orthologous to loci LVIS_0375, LVIS_1748, and LVIS_2285 of *Lb. brevis* ATCC 367; GenBank accession no. NC_008497) were heterologously expressed. Initial assessment of the specific activities for various substrates (Table 1) indicated that the electrophoretically pure His_6-tagged proteins (Fig. 2) were functionally distinct. "LVIS_0375" showed low activities toward *p*NP-arabinofuranoside and *p*NP-xylopyranoside and was unable to hydrolyze arabino- and xylooligosaccharides; "LVIS_1748" displayed its main specificity for arabinosaccharides and "LVIS_2285" for xylooligosaccharides. Hydrolysis of *p*NP-α-L-arabinopyranoside, *p*NP-β-D-glucopyranoside, and *p*NP-β-D-galactopyranoside was not detectable for all three enzymes. According to these results, and based on sequence analyses (see

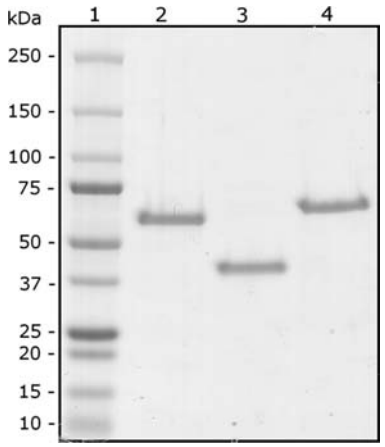

FIG 2 SDS-PAGE of the purified recombinant His_6-tagged GH 43 glycosidases from *Lb. brevis*. Lane 1, Precision Plus protein standard (Bio-Rad); lane 2, XynB1; lane 3, Abf3; lane 4, XynB2.

TABLE 2 Kinetic constants of the *Lb. brevis* GH 43 glycosidases determined at 37°C and pH 5.5

Glycosidase	Substrate	Kinetic constant[a]			
		V_{max} (U mg^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
XynB1	*p*NP-β-D-Xylopyranoside	0.109 ± 0.002	0.11	1.4 ± 0.1	0.083
	*p*NP-α-L-Arabinofuranoside	0.062 ± 0.002	0.065	0.57 ± 0.07	0.12
XynB2	*p*NP-β-D-Xylopyranoside	45 ± 2	46	11 ± 1	4.1
	1,4-β-D-Xylobiose	228 ± 8	233	4.8 ± 0.4	48
	*p*NP-α-L-Arabinofuranoside	0.471 ± 0.008	0.48	2.6 ± 0.1	0.19
Abf3	*p*NP-α-L-Arabinofuranoside	4.0 ± 0.3	2.5	12 ± 2	0.21
	1,5-α-L-Arabinobiose	246 ± 7	154	2.6[b] ± 0.2	59

[a] The values of V_{max} and K_m represent the average results of triplicate determinations ± SD.
[b] Value reflects $K_{0.5}$ of the Hill equation. The Hill coefficient (n_h) was determined to be 1.3.

below), the genes "LVIS_0375" and "LVIS_2285" were designated *xynB1* and *xynB2*, respectively. In continuation of the nomenclature presented in our previous study (Abf1 and Abf2 are the recently described GH 51 arabinosidases from *Lb. brevis* [25]), the arabinofuranosidase-encoding gene "LVIS_1748" was designated *abf3*.

Characterization of XynB1 and XynB2. *xynB1* and *xynB2* encode putative β-xylosidases of GH 43 with 32% amino acid sequence identities as determined through the BLASTP algorithm. According to the Conserved Domain Database (CDD) (32), both sequences are members of GH 43 subclass "Xyl1," listed as COG3507 in the Clusters of Orthologous Genes (COG) database (33). The distribution of putative GH 43 (subclass "Xyl1") family members in published *Lactobacillales* genomes (see Fig. S2A in the supplemental material) indicated the presence of putative orthologs to *xynB1* and *xynB2* in *Enterococcus* spp., *Pediococcus* spp., *Lactococcus lactis*, the *Leuconostoc*/*Weissella* branch, and several heterofermenting *Lactobacillus* spp. such as *Lb. brevis*, *Lb. fermentum*, and *Lb. pentosus*.

The molecular masses of the His_6-tagged proteins were determined to be 375 ± 3 kDa (XynB1) and 137 ± 1.5 kDa (XynB2) by size exclusion chromatography. The calculated molecular masses of the subunits (62.9 and 61.4 kDa, including a His_6 tag, respectively) suggest that XynB1 is a hexamer and XynB2 a dimer.

XynB2 had high catalytic efficiency with respect to xylobiose, having a k_{cat}/K_m value 10-fold higher than that obtained with *p*NP-β-D-xylopyranoside (Table 2), while the function of XynB1 could not be demonstrated with the substrates used. However, sequence alignment (see Fig. S3 in the supplemental material) implied that the amino acid residues identified to be catalytically significant in GH 43 xylosidases (34) are present in both XynB1 and XynB2. No *endo*-xylanase activities (of both XynB1 and XynB2) were detectable with the DNSA assay. XynB2 displayed its maximum activity when assayed at pH 6 and 50°C (Fig. 3), and the activity of XynB2 (determined with both *p*NP-arabinofuranoside and *p*NP-xylopyranoside) was inhibited by xylose and arabinose (Table 3). In contrast, XynB1 was inhibited less by xylose but much more by glucose. Apart from activity reduction by Cu^{2+}, the presence of divalent cations had little effect on XynB1 and XynB2 (Table 4).

Characterization of Abf3. Abf3 is assigned to the CCD subclass "1" of GH family 43, comprised of putative α-L-arabinofuranosidases (COG3940). Compared to XynB, the Abf3 sequence rather rarely occurs in LAB genomes (see Fig. S2B and Table S2 in the supplemental material). Abf3 shares low sequence similarity with AXHd3 from *B. adolescentis* (GenBank accession no. AAO67499.1), which belongs to a distinct GH 43 subclass ("Xyl2"; COG3507). Members of GH 43 "Xyl2" could not be identified in currently published *Lactobacillales* genomes.

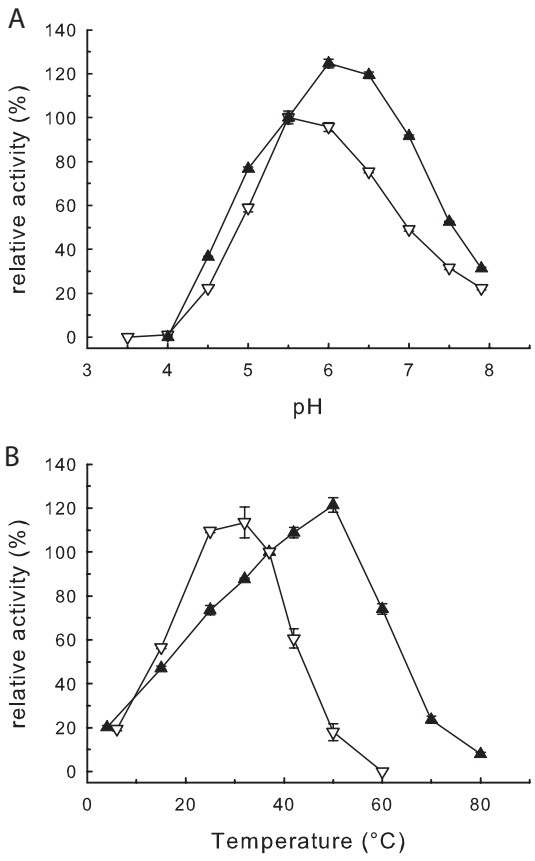

FIG 3 Influence of pH and temperature on XynB2 and Abf3 from *Lb. brevis* determined with *p*NP glycosides (1 mM) Abf3 (▽) and XynB2 (▲). (A) Assays (37°C, 5-min reaction time) were performed with 0.1 M McIlvaine buffers (pH 3.0 to 3.9). (B) Standard assay (pH 5.5, 5 min) performed at 4 to 80°C. All data represent the averages of triplicate determinations. Error bars indicate standard deviations (SD). Relative activity of 100% refers to standard assay conditions (37°C, pH 5.5).

TABLE 3 Inhibitory effect of glucose, xylose, and arabinose on the activities of the GH 43 hydrolases of *Lb. brevis* DSM 20054 determined with *p*NP-β-D-xylopyranoside and *p*NP-α-L-arabinofuranoside[a]

Compound and concn	% activity				
	XynB1		XynB2		Abf3
	*p*NPX	*p*NPA	*p*NPX	*p*NPA	(*p*NPA)
Glucose					
10 mM	83.0 ± 0.4	72 ± 4	96 ± 1	94 ± 6	95.3 ± 0.6
100 mM	29 ± 2	27 ± 1	76 ± 3	89 ± 5	96 ± 1
Xylose					
10 mM	99.2 ± 0.9	96 ± 2	98 ± 2	91 ± 6	94 ± 2
100 mM	80 ± 4	82 ± 2	33.8 ± 0.5	51.0 ± 0.6	89 ± 1
Arabinose					
10 mM	81.0 ± 0.6	97 ± 1	92 ± 2	101.8 ± 0.4	95.2 ± 0.6
100 mM	61 ± 1	85 ± 2	62 ± 2	72 ± 4	71 ± 1

[a] The values represent the average results of triplicate determinations ± SD. 100% activity was determined under standard assay conditions (pH 5.5, 37°C) without inhibitor. *p*NPX, *p*NP-β-D-xylopyranoside; *p*NPA, *p*NP-α-L-arabinofuranoside.

Abf3 eluted as a 143 ± 1 kDa tetramer (37.6 kDa subunit mass) which was highly selective for (linear) arabinooligosaccharides (Tables 1 and 2). In kinetic experiments performed with arabinobiose, Abf 3 showed slight positive substrate cooperativity, with an n_h (Hill coefficient) of 1.3. *Endo*-arabinanase (DNSA assay) could not be detected. The enzyme displayed its maximum activity at pH 5.5 and was found to be rather temperature labile, having maximal activity between 25 and 32°C and rapid inactivation at higher temperatures (Fig. 3). Inhibition by xylose and arabinose (Table 3) occurred at higher concentrations (100 mM). The activity of Abf3 was increased by the presence of Mn^{2+} and Zn^{2+} but reduced by the presence of Cu^{2+}.

Release of arabinose from (A)XOS by Abf1 and Abf2. An (A)XOS preparation was obtained by depolymerization of AX with Pentopan Mono BG, a xylanase preparation from *Thermomyces lanuginosus* that is widely used in baking. HPAEC-PAD analysis of this (A)XOS preparation (Table 5) indicated low concentrations of arabinose, xylose, and xylobiose. Structural information on (A)XOS was obtained by methylation analysis, and the arabinose/xylose ratio was determined to be 31:69 (0.45). Judged by the percentage of terminal (nonreducing end) xylose (T-Xyl), an average of 5 to 7 degrees of polymerization was estimated for the xylooligosaccharide backbone of (A)XOS (Table 6). The majority of the arabinose residues (65%) were α-1,3-linked to monosubstituted xylose; 8% occurred as a monosubstituted α-1,2-linkage and 26% on doubly substituted xylose. Using this preparation for further enzyme assays, release of arabinose by the GH 43 glycosidases XynB1, XynB2, and Abf3 could not be detected.

Experiments conducted with Abf1 and Abf2 of *Lb. brevis* and two commercial enzymes used as controls (Tables 5 and 6) indicated that the preparations AFASE (GH 51, *A. niger*), Abf1, and Abf2 (both GH 51) could release high quantities of arabinose from AXOS, accompanied by an increase in the levels of detectable unsubstituted xylooligosaccharides (Table 5). Although AFAM2 released large amounts of arabinose as well, the concomitant release of xylooligosaccharides was comparably low. Synergistic effects were observed when Abf1 and Abf2 were applied in combination with AFAM2. These results are in accordance with the known AXHd3 activity of AFAM2 and suggest that Abf1, Abf2, and AFASE possess AXHm specificity. Methylation analysis (Table 6) confirmed the AXHd3 specificity of AFAM2 and showed that the GH 51 arabinosidases AFASE, Abf1, and Abf2 are selective for α-1,3-linked arabinosyl residues of monosubstituted xylose molecules. The specific activities of Abf1 and Abf2 in this preparation were determined to be 4.5 and 4.3 U mg^{-1}, respectively.

TABLE 5 HPAEC-PAD analysis of an (A)XOS preparation treated with the GH 51 arabinofuranosidases Abf1 and Abf2 from *Lb. brevis* DSM 20054[a]

Prepn (20 mg ml^{-1})[b]	Concn (mg ml^{-1})						
	A	X	X_2	X_3	X_4	X_5	X_6
(A)XOS	0.21	0.49	0.62	ND	ND	ND	ND
AFASE	1.7	0.49	0.50	0.33	1.2	0.47	0.23
AFAM2	2.1	0.54	0.58	0.096	0.38	ND	ND
Abf1	1.6	0.57	0.57	0.43	1.2	0.49	0.24
Abf2	1.3	0.51	0.56	0.37	1.3	0.49	0.23
Abf1 + AFAM2	3.5	0.53	1.1	2.2	1.3	0.58	0.24
Abf2 + AFAM2	3.6	0.52	1.1	2.2	1.4	0.68	0.30

[a] The values represent the average results of triplicate determinations. A, arabinose; X, xylose; X_2 to X_6, xylooligosaccharides 2 to 6.
[b] AFASE, GH 51 α-L-arabinofuranosidase from *A. niger*; AFAM2, GH 43 α-L-arabinofuranosidase (AXHd3) from *B. adolescentis*. Both preparations were obtained from Megazyme International (Wicklow, Ireland).

DISCUSSION

Lb. brevis is recognized as one of the rather versatile members of the lactic acid bacteria, although the genetic basis for this ecolog-

TABLE 4 Influence of divalent cations on the activities of the GH 43 glycosidases from *Lb. brevis* DSM 20054 using *p*NP-β-D-xylopyranoside and *p*NP-α-L-arabinofuranoside as the substrates[a]

Cation (10 mM)	% activity			
	XynB1 (*p*NPX)	XynB1 (*p*NPA)	XynB2 (*p*NPX)	Abf3 (*p*NPA)
$CaCl_2$	105.6 ± 0.5	97 ± 2	100 ± 3	97 ± 8
$MgCl_2$	101 ± 5	100.9 ± 0.6	96 ± 2	96 ± 1
$MnCl_2$	100 ± 4	95.5 ± 0.9	99 ± 3	219 ± 8
$ZnCl_2$	106 ± 6	96.7 ± 0.6	69 ± 2	138 ± 3
$CuCl_2$	68.3 ± 0.8	69.1 ± 0.7	82.2 ± 0.6	27.9 ± 0.6

[a] The values represent the average results of triplicate determinations ± SD. 100% of activity refers to standard assay conditions (pH 5.5, 37°C) without additive. *p*NPX, *p*NP-β-D-xylopyranoside; *p*NPA, *p*NP-α-L-arabinofuranoside.

TABLE 6 Methylation analysis of (A)XOS treated with the arabinofuranosidases Abf1 and Abf2 of *Lb. brevis*[a]

Prepn[b]	% molar ratio				
	T-Xyl	4-Xyl	2,4-Xyl	3,4-Xyl	2,3,4-Xyl
(A)XOS	18 ± 2	46 ± 1	3.45 ± 0.04	27.8 ± 0.7	5.6 ± 0.2
AFASE	17.8 ± 0.3	61.2 ± 0.7	3.64 ± 0.04	10.9 ± 0.4	6.5 ± 0.6
AFAM2	13.0 ± 0.6	37.5 ± 0.4	24.7 ± 0.8	23.8 ± 0.9	0.97 ± 0.03
Abf1	18 ± 1	62.4 ± 0.3	3.6 ± 0.3	9.9 ± 0.6	6.2 ± 0.5
Abf2	18.1 ± 0.6	59.5 ± 0.4	3.6 ± 0.4	13.2 ± 0.1	5.5 ± 0.3

[a] The values represent the substitution patterns of the xylan backbone as molar ratios (%). T-Xyl, terminal nonreducing end xylose; 4-Xyl, 4-linked xylose; 2,4-Xyl, 2,4-linked xylose; 3,4-Xyl, 3,4-linked xylose; 2,3,4-Xyl, 2,3,4-linked xylose.
[b] AFASE, GH 51 α-L-arabinofuranosidase from *A. niger*; AFAM2, GH 43 α-L-arabinofuranosidase (AXHd3) from *B. adolescentis*.

ical flexibility is not completely understood (17). While *Lb. brevis* is usually classified as obligately heterofermentative, evidence for homofermentative hexose metabolism (i.e., through the Embden-Meyerhof-Parnas pathway [EMP]) was presented, and *Lb. brevis* may in fact be facultatively heterofermentative (20, 35). An interesting property of the species is that it reportedly lacks hierarchical control of carbohydrate utilization (carbon catabolite repression) and is therefore able to use a broad spectrum of sugars simultaneously (36). This fact has been shown to be of interest for lactic acid production from lignocellulosic biomass (37), especially because *Lb. brevis* is among the few LAB species known to efficiently utilize xylooligosaccharides, preferentially xylobiose (14, 20–22).

Lb. brevis is involved in the natural fermentation of hemicellulose-rich plant food/feed (e.g., cabbage, sourdough, and forage silage), and, despite the aspects mentioned below, it is also an important starter organism for the "overattenuation" process used to produce several beer varieties (18, 19, 38). However, next to *Pediococcus* spp., obligately heterofermentative *Lactobacillus* spp. (listed in group 3 according to reference 19) are commonly recognized in food spoilage, with *Lb. brevis* being one of the organisms primarily associated with wine and beer spoilage (19). Therefore, it seems to bear a certain irony that, in the context of enzymatic release of attractive wine aroma compounds from glycosylated precursors, we previously identified *Lb. brevis* as a versatile source of glycosidases (25, 39, 40).

As such, it is elucidating to discuss the occurrence of β-glycosidase genes that are putatively involved in hexose (glucose and galactose) and pentose (xylose and arabinose) metabolism in *Lactobacillales* (see Table S2 in the supplemental material). Based on molecular clock analysis and in comparison to ribosomal proteins, Makarova et al. (24) concluded that genes involved in carbohydrate metabolism and transport may have been acquired and duplicated early in LAB evolution. Nevertheless, such genes (including phosphotransferase system [PTS] components, β-galactosidases, GH 3 glycosidases, and GH 43 xylosidases) are also prone to violation of the (local) molecular clock, displaying high rates of horizontal gene transfer (HGT). Accordingly, it may not be surprising that genomic distribution and redundancy of (β-D/α-L) glycosidase genes (both PTS and non-PTS) appear to be heterogeneous among LAB genera/species, limiting the possibility of coherently inferring taxonomic or phenotypic relationships. In this context, it is of interest that the *Lb. brevis* GH 43 and GH 51 glycosidase sequences show high degrees of amino acid sequence identity to those of several intestinal bacteria (e.g., *Roseburia*, *Enterobacteriaceae*, *Clostridiales*, and *Bifidobacteriaceae*) (see Fig. S2). For example, putative xylosidases of *B. adolescentis* ATCC 15703 and *Roseburia intestinalis* M50/1 (see Fig. S2A) share 72% and 76% sequence identities, respectively, with XynB2, whereas the identities between XynB1 and XynB2 amount to only 32%. This could be a result of HGT between intestinal bacterial species, which, as previously reported (41), may be extensive. Furthermore, in *Lactobacillus* spp., GH 43 and GH 51 xylosidase/arabinosidase genes mainly occur in strictly heterofermentative *Lactobacillus* spp. of group 3 (among others, including *Lb. brevis*, *Lb. buchneri*, *Lb. fermentum*, *Lb. hilgardii*, and *Lb. reuteri*). While this is in agreement with the fact that obligately homofermentative *Lactobacillus* spp. (group 1) do not metabolize pentoses (19), the lack of corresponding genes in members of facultatively heterofermentative group 2 (including *Lb. plantarum*) is rather surprising. Despite the generally high levels of occurrence and redundancy of PTS-related GH 1 (phospho)glycosidases in *Lactobacillales* genomes, group 3 is distinguished by a complete absence of GH 1 phospho-β-glucosidase/phospho-β-galactosidase genes (except for one sequence in *Lb. brevis*). This correlates with the fact that sugar import and phosphorylation by PTS components is associated with homofermentative metabolism (EMP) whereas PTS components are not known to be directly involved in heterofermentative carbohydrate metabolism (phosphoketolase [PK] pathway), as phosphoenolpyruvate (PEP) does not occur as an intermediate in the PK pathway (35). However, evidence that mannose-specific PTS transporters (EIIB) facilitate xylose uptake in several group 2 members was previously presented (42).

Consistent with the results of the present study (see the supplemental material), apart from *Lb. brevis*, previous studies concerned with *in vitro* growth on XOS found little evidence for XOS utilization in the genus *Lactobacillus*. *Lb. fermentum* (syn. *Lb. cellobiosus*) (14, 21) and *Lb. acidophilus* (15) were found to have moderate grow rates on XOS, and Chapla et al. (43) reported that growth of *Lb. fermentum* and *Lb. acidophilus* was low compared to growth of *Bifidobacterium* spp. Furthermore, an intestinal *Lb. paracasei* strain was reported to be enriched by XOS (44). Interestingly, the *Leuconostoc/Weissella* branch (heterofermentative), as well as *Pediococcus* spp. (mainly homofermentative), both phylogenetically close to the genus *Lactobacillus* (19), show a high occurrence of both PTS and non-PTS (i.e., pentose-specific) glycosidases. Evidence for XOS utilization by *Leuconostoc lactis* (45) and *Weissella* sp. (46) has been previously reported.

With regard to the substrate selectivities of the enzymes presented here, we propose that GH 51 arabinosidases of LAB are principally involved (at least partially) in the debranching of arabinoxylan-oligosaccharides, while, depending on the subclass, the presence of GH 43 glycosidases may be indicative of the ability to utilize the resulting linear backbone oligosaccharides. Although putative orthologs to the GH 43 AXHd3 from *B. adolescentis* are so far not discernible in LAB genomes, AXHd3 activity was recently reported for a GH 51 arabinofuranosidase as well (8). Consequently, the ability to utilize AXOS could be restricted to only a few LAB species, as was indicated by the particularly low level of occurrence of GH 51 arabinosidases (see Fig. S2C and Table S2 in the supplemental material).

However, substrate selectivities (especially regarding the aglycone moiety) of glycosidases are still not sufficiently understood and the possibility cannot be excluded that, even within a GH (sub)family, significant differences in functionality may occur, as demonstrated in the case of XynB1 and XynB2. While XynB2 could be classified as a true xylan 1,4-β-D-xylopyranosidase with low α-L-arabinofuranosidase side activity (Table 1), the function of XynB1 could not be demonstrated with the substrates used in the present study. Although a recent study demonstrated marked activity enhancement of a GH 43 xylosidase in the presence of divalent cations (47), a similar effect could not be observed in the case of XynB1, and the results obtained with both XynB1 and XynB2 were consistent with the characteristics of an *Lb. brevis* β-xylosidase as reported in reference 23. XynB1 may therefore represent an inactive paralog of XynB2 or possess another, yet-to-be-identified function.

In the context of the structural genomics approach, there is at present sufficient evidence that the protein sequence and, thus, the resulting predicted three-dimensional fold of the catalytic domain (as implicit in GH classification) is a reliable indicator for the

glycone (nonreducing end sugar) selectivity of a glycosidase. Hence, amino acid residues recognizing the glycone moiety, as well as acid/base catalytic residues, were shown to be highly conserved within GH families (13, 48). In contrast, as was demonstrated best so far in the case of GH 1 glucosidases, residues known to be involved in aglycone recognition are much less conserved, and the resulting differences in the stereochemistry and physicochemical environment at the substrate binding pocket that determine substrate binding affinities (and selectivity for configuration of the glycosidic link) cause high flexibility regarding substrate selectivity, even within structurally closely related enzyme folds (48, 49). A resulting (though mainly empirical) distinction is that seen between GHs specific for oligosaccharides and aryl/alkyl-glycoside-specific hydrolases. Recently (25), we proposed that both GH 51 arabinofuranosidases (Abf1 and Abf2) of *Lb. brevis* would classify as aryl/alkyl-glycosidases. This was further confirmed by the fact that a related GH 51 enzyme from *Oenococcus oeni* could release both primary and tertiary terpene alcohols from natural glycosides whereas an *A. niger* arabinosidase of the same GH family (AFASE in the present study) that displayed high specificity for arabinobiose hydrolyzed only glycosides of primary terpenols (39). However, in light of the present findings, Abf1 and Abf2 (as well as AFASE) should be considered broad-specificity GH 51 arabinofuranosidases based on their propensity to hydrolyze *p*NP-arabinofuranoside and 1,5-α-L-arabinobiose as well as the α-1,3-linked arabinosyl residues of AXOS. This stands in clear contrast to the narrow specificity of the GH 43 enzyme Abf3, which was not able to remove arabinose residues of AXOS from either the 1,2 or 1,3 configuration and can therefore be classified as an arabinan 1,5-α-L-arabinofuranosidase.

In conclusion, this paper gives insights into the biochemical characteristics of arabinoxylan-oligosaccharide-degrading hydrolases of *Lb. brevis*. Further, by evaluating the occurrence of genes putatively encoding functionally similar enzymes in LAB genomes, new light was shed on lactic acid bacterial glycoside (oligosaccharide) metabolism in general. These results are indicative of the capability of other LAB species to utilize (A)XOS and warrant further studies. In particular, attention should be focused on obligately heterofermentative *Lactobacillus* spp., the *Leuconostoc/Weisella* branch, and *Enterococcus* spp. as well as the genera *Pediococcus* and *Lactococcus*.

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Effect of pretreatment on arabinoxylan distribution in wheat bran

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ABSTRACT

Arabinoxylan is one of the potential key products of a wheat bran based biorefinery. To develop a suitable process for the isolation of arabinoxylans, the effect of different processing approaches needs to be determined. In this work, chemical analysis was supplemented by immunolocalization of arabinoxylan by confocal microscopy, which proved valuable in the assessment of cell-structural changes occurring upon different chemical and mechanical bran treatments. The influences of acid, lye and hydrogen peroxide treatment, ball-milling, extrusion, fermentation and treatment with esterase, xylanase and a combination thereof were investigated.

Extensive ball-milling showed the best selectivity for harvesting arabinoxylan. Chemical treatments gave the highest yields, but did so at the cost of selectivity. Fermentative and enzymatic treatments were hampered by coextraction of other compounds.

1. Introduction

Wheat bran, the outer layer of the wheat kernel, is an abundant by-product of white flour production. Given its high content of nutritionally valuable and technologically desirable compounds, it is gaining interest as a raw material for biorefineries (Apprich et al., 2014). However, these components are either embedded in a complex and recalcitrant matrix or constitute this matrix themselves. This makes pretreatment imperative for down-stream processing. The choice of an appropriate pretreatment as well as suitable parameters is far from trivial and has to be adjusted according to the objective of the fractionation (Prückler et al., 2014).

Arabinoxylan is the most abundant valuable in wheat bran at about 32% of total dry matter (Maes & Delcour, 2002). As a dietary fiber substituted with hydroxycinnamates, such as ferulic and *p*-coumaric acid, arabinoxylan exhibits both nutritional and rheological benefits (Bauer, Harbaum-Piayda, Stockmann, & Schwarz, 2013; Berlanga-Reyes, Carvajal-Millan, Lizardi-Mendoza, Islas-Rubio, & Rascon-Chu, 2011; Hopkins et al., 2003). There is little consensus on the most advantageous methodology for selective extraction. Approaches range from chemical treatments, such as lye-based or oxidizer-assisted (Bataillon, Mathaly, Cardinali, & Duchiron, 1998; Maes & Delcour, 2001; Sun, Cui, Gu, & Zhang, 2011), over enzymatic treatments (Swennen, Courtin, Lindemans, & Delcour, 2006) to mechanical treatments, such as ball-milling (Van Craeyveld et al., 2009).

Few studies have been undertaken to compare the effectiveness of different treatments. Zhou et al. (2010) have compared peroxide-assisted alkaline extraction with enzymatic extraction and found the former to be more effective by 50%. However, they also found significant structural differences in the isolated products, which has to be taken into consideration for the intended application. Given the complex matrix of wheat bran, high yields usually only come at the cost of extensive treatment times or harsh conditions, which cause degradation of product and residual material, and challenges economic and ecological feasibility. Regardless whether the aim is to isolate arabinoxylan or to remove it in the purification of other target compounds, tracking its course is mandatory.

The aim of this study was to evaluate the efficacy of a wide spectrum of pretreatments with regard to their impact on arabinoxylan distribution and extractability. Evaluated pretreatments were acid, lye and hydrogen peroxide treatment, ball-milling, extrusion, fermentation and treatment with esterase, xylanase and a combination thereof. The effect of the pretreatments was assessed based on the carbohydrate content and distribution after

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thorough washing of the solid residue. An increase in extractability corresponds to a reduced amount of the solid sample. Three analytical approaches were chosen: quantification of the carbohydrate content by methanolysis, generating profiles of the carbohydrate distribution by enzymatic peeling and imaging of the arabinoxylan distribution by fluorescence labeling.

Carbohydrate content can be quantified after acidic cleavage of polysaccharides into monosaccharides. The favored method is methanolysis combined with GC–MS or GC–FID analysis, due to low degradation of the comparatively frail pentoses (Sundberg, Sundberg, Lillandt, & Holmbom, 1996; Willför et al., 2009). The composition of the carbohydrate fraction of the sample is gained and expressed as monosaccharide content. Being a mild method, methanolysis cannot decompose crystalline cellulose. Therefore, the glucose measured in this analysis is derived from residual starch, β-glucan and amorphous cellulose only. However, it cannot resolve arabinoxylan spatially which is a major requirement in the assessment of a pretreatment.

Hence, samples were also subject to an enzymatic peeling in order to obtain a cross-sectional profile of polysaccharide distribution (Sjöberg, Potthast, Rosenau, Kosma, & Sixta, 2005). The washed solids were treated with specific enzymes and the released monosaccharides in the supernatant were quantified. Thus, it was determined whether the effect of the pretreatment was only superficial or was affecting the whole sample.

The effects of the pretreatments were further elucidated by imaging the arabinoxylan distribution by immunolocalization in combination with confocal microscopy. Fluorescent-labeled monoclonal antibodies raised against specific polysaccharide epitopes allow for the localization of cell wall components, such as arabinoxylan, on a microscopic scale (McCartney, Marcus, & Knox, 2005). This technique has been used to map the polysaccharide distribution in cell walls of different wood types and to gain insight into the formation of cell walls (Donaldson, 2009; Donaldson & Knox, 2012). So far, immunolocalization has found little application in wheat bran research. To our knowledge it has been employed exclusively to follow xylanase-mediated degradation of arabinoxylan (Beaugrand et al., 2004a; Beaugrand, Reis, Guillon, Debeire, & Chabbert, 2004b).

2. Materials and methods

2.1. Sample preparation

Samples are classified in three treatment categories: chemical, mechanical and fermentation/enzymatical treatment. Chemical treatments were acid treatment (sample ID 1.1), lye treatment (1.2) and hydrogen peroxide treatment (1.3). Mechanical treatments were ball milling for 5 min (2.1) and 60 min (2.2) and extrusion (2.3). Fermentation and enzymatic treatments were fermentation with *Lactobacillus plantarum* (3.1); treatment with esterase (3.2); xylanase (3.3); a combination of esterase and xylanase (3.4); and a combination of *Lactobacillus brevis*, esterase and xylanase (3.5).

2.1.1. Chemical treatments

10 g of wheat bran were stirred at 60 °C for 4 h in one of the following: 200 mL of 1 M sulfuric acid, 1 M sodium hydroxide or 2% hydrogen peroxide solution adjusted with sodium hydroxide to pH 11.5 (all Sigma-Aldrich, St. Louis, MO, USA). The resulting slurries were filtrated and the residues were washed three times with 100 mL of water. Samples were freeze-dried before further analysis.

2.1.2. Mechanical treatments

Wheat bran was extruded using a Bühler Twin Screw Extruder BCTL 42/20D model (Bühler Group, Uzwil, Switzerland) without preconditioner. SME (specific mechanical energy) input was 179 Wh/kg at a maximum temperature of 133 °C.

Wheat bran was ball-milled with a Retsch MM 2000 ball-mill (Retsch, Haan, Germany) using 25 mL containers at about 10% loading with a single 25 mm stainless steel ball at 20 Hz. Milling times were 5 and 60 min.

2.1.3. Fermentation and enzymatic treatments

Wheat bran was inoculated with 2% w/w of bacterial culture for fermentation or 1% w/w of enzyme. After addition of 50% v/w of physiological sodium chloride solution samples were kept at room temperature for five days and then dried at 40 °C. Samples were treated with esterase Sternzym FSR 22010 (SternEnzym, Ahrensburg, Germany) and xylanase Pentopan Mono BG (Novozyme, Bagsvaerd, Denmark). Bacterial cultures were *L. brevis* DSM 20054 (DSMZ GmbH, Braunschweig, Germany) and *L. plantarum* WCFS1. For different combinations applied to samples see Table 1.

2.2. Methanolysis

In order to remove mobilized arabinoxylan before methanolysis, 200 mg of bran sample were stirred in 10 mL water for 1 h, filtrated, washed twice with 10 mL of water and dried in vacuo at room temperature.

2.2.1. Procedure and derivatization

Methanolysis was performed according to Sundberg et al. (1996). For silylation, dried samples after methanolysis were left to equilibrate in 400 μL of pyridine for 1 h. 200 μL of BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide) containing 10% TMCS (trimethylchlorosilane; both Sigma-Aldrich) were added and the samples kept at 70 °C for 2 h. Samples were diluted with 600 μL of ethyl acetate, filtrated through a 0.45 μm PTFE syringe filter and analyzed by GC–MS.

2.2.2. GC–MS

GC–MS was performed as follows. 0.2 μL of silylated sample was injected (260 °C, splitless) on a 30 m/0.25 mm HP-5 column (film thickness 0.25 μm) in an Agilent 6890N Series GC System with an Agilent 5973 Series Mass Selective Detector. The temperature program was 140 °C (1 min); 4 °C/min to 210 °C (0 min); 30 °C/min to 260 °C (5 min). The carrier gas was helium (0.9 ml/min, constant flow). Detector conditions were 70 eV with a scan range from 45 to 950 Da. Data was acquired and processed with MSD Chemstation E.2.01.1177 software from Agilent Technologies.

2.3. Enzymatic peeling

Procedure for enzymatic peeling was adopted from Sjöberg et al. (2005) with modifications to the enzyme mix to suit wheat bran polysaccharides.

2.3.1. Preparation of enzyme mix

The enzyme mix was composed of 0.5 g cellulase from *Trichoderma reesei* (Sigma-Aldrich), 3 g xylanase Pentopan Mono BG (Novozyme) and 2 mL of GH 43 α-L-arabinofuranosidase from *Bifidobacterium adolescentis* (Megazyme Internartional, Wicklow, Ireland). Cellulase activity is given as ≥1 U/mg, xylanase as 2.5 FXU/mg (farbe xylanase units) and α-L-arabinofuranosidase activity as 102 U/mg. The enzyme mix was filtrated through Whatman filter paper grade 4 and then desalted and concentrated by ultrafiltration to 1% of its original volume with a molecular weight cutoff at 1000 Da and then filled up to 50 mL with water.

2.3.2. Enzymatic peeling procedure

Twenty milligrams of washed sample (see Section 2.2) were stirred in a sodium acetate buffer adjusted to pH 4 at 40 °C for 1 h before the addition of 100 μL (20 mg/mL) of sorbitol as internal standard and 700 μL of enzyme mix. The suspension was kept at 40 °C and 0.2 mL aliquots were taken after 5, 10, 30, 60 and 120 min. Aliquots were filtrated through a 0.45 μM PTFE filter and incubated for 48 h at 40 °C for complete enzymatic hydrolysis. Samples were used for HPLC in a dilution of 1:10. A blank was recorded to correct for residual sugars in the enzyme mix.

2.3.3. HPLC analysis

Sugars were quantified on a Dionex UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA, USA) equipped with a Phenomenex 300 × 7.8 mm Rezex™ ROA-Organic Acid H+ (8%) column kept at 80 °C (Phenomenex, Aschaffenburg, Germany) and a HP 1100 Series G1362A RID Refractive Index Detector (Hewlett-Packard, Palo Alto, CA, USA). Mobile phase was 5 mM sulfuric acid at a flow of 0.4 mL/min. Calibration was performed externally relative to sorbitol, which was used as an internal standard. All standards were purchased from Sigma-Aldrich. Data was recorded with Dionex Chromeleon 6.8.

2.4. Immunolocalization

2.4.1. Sample preparation

Samples from Section 2.1 were prepared for immunolocalization by pouring the wheat bran flakes/powder into molten paraffin wax and letting it harden. The wax was trimmed to blocks and cut with a microtome so that cross sections of wheat bran were located at the surface of the block. Labeling with antibody, mounting and microscopy were performed on the block of wax.

2.4.2. Labeling

Immunolocalization of arabinoxylan was performed by placing a droplet of a 1:20 dilution of primary rat monoclonal antibody hybridoma cell culture supernatant LM11 (PlantProbes, Leeds, UK) in 0.1 M PBS (pH 7, containing 1% w/v acetylated bovine serum albumin as a blocking agent and 5 mM sodium azide) on a wax block over night at 4 °C so that the top of the wax block was entirely covered. Wax blocks were rinsed five times with PBS and dried before being covered with a droplet of fluorescently labeled secondary antibody Alexa647 (goat anti-rat; Invitrogen, Paisley, UK) at a 1:100 dilution in PBS (20 μg/mL) for 2 h under light exclusion at room temperature. Blocks were washed five times with PBS, mounted in SlowFade at pH 9 (Molecular Probes Inc., Eugene, Oregon, USA) and measured on the same day. A control sample was prepared accordingly with the omission of the primary antibody. Each sample was measured with and without immunolabeling. The overlap of fluorescence from the antibody and autofluorescence from the sample was negligible.

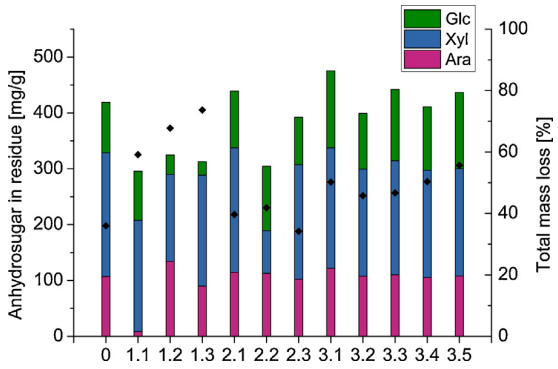

Fig. 1. Total mass losses and residual sugar content of samples after washing. **Glc** = glucose, **Xyl** = xylose, **Ara** = arabinose, ♦ = total mass loss. **0**: Untreated wheat bran; **1.1**: acid treatment; **1.2**: lye; **1.3**: hydrogen peroxide; **2.1**: 5 min ball-milling; **2.2**: 60 min ball milling; **2.3**: extrusion; **3.1**: *Lactobacillus plantarum*; **3.2** esterase; **3.3** xylanase; **3.4**: esterase + xylanase; **3.5**: *Lactobacillus brevis* + esterase + xylanase.

2.4.3. Confocal laser scanning microscopy

Confocal laser scanning microscopy was performed on a Leica SP5 II. Three channels were recorded in parallel. For autofluorescence, excitation was 488 nm (shown as green in Fig. 3) and 561 nm (shown as red) and emission was recorded at 500–535 nm and 570–620 nm. The fluorescent antibody was excited at 633 nm (shown as blue) and recorded at 650–750 nm. For all images, only overall exposure was adjusted when necessary. The relative exposure of the channels was kept constant throughout. All images are shown as maximum intensity projections.

3. Results and discussion

3.1. Methanolysis

Washing of untreated wheat bran resulted in a 36% weight loss and gave a residue composed of 11% arabinose-, 22% xylose- and 9% glucose-based polysaccharides. Due to the methodology, glucose values do not reflect cellulose content but only starch and β-glucan. For the assessment of cellulose accessibility and spatial distribution after treatments see Section 3.2. Total mass as well as specific polysaccharide losses of treated samples are given relative to untreated wheat bran and refer to solid residues after washing, i.e. negative values for residual sugars indicate an increase in solubilization. Methanolysis results and mass losses are shown in Fig. 1 (absolute values) as well as Table 1 (values relative to untreated wheat bran).

3.1.1. Chemical treatments

Acid treatment is a common pretreatment in the biorefinery of lignocellulosic biomass and is advantageously followed by

Table 1
Total mass losses and residual sugar contents of samples after washing as determined by methanolysis. Values are given relative to untreated wheat bran. Negative values for residual sugars indicate an increase in solubilization.

Sample ID	Type of treatment	Mass loss [%]	Arabinose [%]	Xylose [%]	Glucose [%]
1.1	Acid treatment	+64	−92	−10	−2
1.2	Lye treatment	+88	+25	−30	−61
1.3	Hydrogen peroxide treatment	+105	−16	−10	−73
2.1	Ball-milling 5 min	+10	+7	+1	+13
2.2	Ball-milling 60 min	+16	+5	−66	+28
2.3	Extrusion	−5	−5	−7	−6
3.1	*Lactobacillus plantarum*	+40	+13	−3	+53
3.2	Esterase	+27	+/−0	−13	+10
3.3	Xylanase	+30	+3	−8	+42
3.4	Esterase + Xylanase	+40	−2	−13	+26
3.5	*Lactobacillus brevis* + esterase + xylanase	+55	+1	−13	+51

enzymatic hydrolysis to yield monosaccharides (Eggeman & Elander, 2005; Gomathi et al., 2012; López-Arenas, Rathi, Ramírez-Jiménez, & Sales-Cruz, 2010). For wheat bran, acid treatment increased total solubles by 64%, but was not very specific towards polysaccharides. It only slightly increased the amount of soluble xylose- and glucose-based polysaccharides (−10% and −2%) but drastically solubilized arabinose (−92%). Arabinose occurs as singe-unit side chains in arabinoxylan and acid preferentially cleaves off such terminal monosaccharides.

Lye treatment is regarded as a selective method for the extraction of arabinoxylan from biomass (Cui, Wood, Weisz, & Beer, 1999; Sun et al., 2011). However, we found treatment with sodium hydroxide not to be specific towards arabinoxylan. Despite an increase of 88% in total solubles, it solubilized only average amounts of xylose-based polysaccharides (−30%), and also high amounts of glucose (−61%) were solubilized. The seeming increase in arabinose in the residue (+25%) is a relative increase due to the removal of other components. Still, it points to a favored extraction of non-substituted or lowly substituted over highly branched arabinoxylan.

Hydrogen peroxide is often combined with alkaline conditions in order to achieve simultaneous delignification and selective extraction of arabinoxylan (Hollmann & Lindhauer, 2005; Maes & Delcour, 2001). We found this treatment to give the highest increase in solubilization (by 105%), but could not attest good specificity towards arabinoxylan. Arabinose content in the residue decreased by 16%, xylose content by 10% and glucose content by 73%.

3.1.2. Mechanical treatments

Extensive ball-milling is known to lead to fragmentation of arabinoxylan into oligosaccharides and thus to an increase in solubility (Van Craeyveld et al., 2009). In our study, 5 min and 60 min of ball-milling increased overall solubility by 10% and 16%, respectively, but only after 60 min we observed a strong preference for xylan-based polysaccharides (−66%) over glucose (+28%). Five minutes of ball-milling showed no observable effect on sugar composition and the improved extractability was thus probably only caused by an increase in accessible surface area due to decreased particle size. According to microscopy, it was estimated that ball milling reduced the particle sizes to be generally smaller than 250 μm after 5 min and to be generally smaller than 20 μm after 60 min. the fragments of the employed bran are noticeably larger: 37% of the particles were smaller than 250 μm and only 17% were smaller than 125 μm.

Extrusion is commonly used for the preparation of convenience foods such as pasta or snacks from whole grain or white flour (Gajula, Alavi, Adhikari, & Herald, 2008). Despite comparatively low yields, the treatment holds potential for the isolation of arabinoxylan from wheat bran due to a smaller environmental footprint than alkaline extraction (Jacquemin, Zeitoun, Sablayrolles, Pontalier, & Rigal, 2012). After extrusion, we observed a slight decrease in overall extractability by 5%, but also a preference for polysaccharides: arabinose, xylose and glucose moieties decreased by 5%, 7% and 6%, respectively, in the residual material.

3.1.3. Fermentation and enzymatic treatments

L. plantarum is a lactic acid producing species that has been shown to possess strain-dependent probiotic properties (Goossens, Jonkers, Russel, Stobberingh, & Stockbrugger, 2006) as well as to be able to utilize wheat bran as a substrate (Naveena, Altaf, Bhadriah, & Reddy, 2005). In addition to carbohydrates, it can also ferment protein (Fadda, Vildoza, & Vignolo, 2010). After fermentation, we observed an increase of insoluble polysaccharides (arabinose +13%, xylose −3% and glucose +53%) despite an increase in overall solubility of 40% compared to untreated wheat bran. This points towards the conclusion that mainly protein and only small amounts of arabinoxylan have been solubilized, concentrating the remaining compounds.

Enzymatic extraction of arabinoxylan is considered to be the mildest form of pretreatment and thus best suited for food and feed applications (Swennen et al., 2006). Esterase and xylanase treatment showed a comparable increase in total solubility (27% and 30%). In the residual polysaccharide analysis, they showed similar amounts of arabinose moieties (0% and +3%). Esterase treatment seemed to be slightly more efficient for solubilization of xylose (−13% against −8%) and showed a smaller increase in residual glucose-based polysaccharides (+10% against +42%).

When combined, however, esterase and xylanase brought overall solubility to 40%. The synergistic effect is presumed to be due to esterase being able to cleave ester bonds between arabinoxylan and phenolics as well as between two arabinoxylan chains, which can be ester-linked through ferulic acid at arabinose side chains. This gives xylanase access to more substrate for depolymerization (Lewis & Yamamoto, 1990). However, the fact that residual polysaccharides stayed within the same range (arabinose −2%, xylose −13% and glucose +26%) implies a concomitant increase in extractability of untracked plant constituents, for example protein.

The addition of *L. brevis* to the enzyme mix of esterase and xylanase caused total solubilization to rise to 55%. *L. brevis* has been shown to be able to utilize xylooligosaccharides, which might account for the observed increase (Michlmayr et al., 2013). Yet again, extraction was accompanied by other compounds so that polysaccharides in the residual material remained to make up a comparable percentage with only glucose-based polymers showing some discrimination (arabinose +1%, xylose −13% and glucose +51%).

3.2. Enzymatic peeling

It is important to note that wheat bran is too resistant to undergo complete enzymatic hydrolysis. Even for isolated, insoluble arabinoxylan, enzymatic hydrolysis rates do not exceed 45% (Sørensen, Pedersen, & Meyer, 2007). Furthermore, only the aleurone layer is readily accessible for enzymes. Thick cell walls and lignification shield the outer layers against enzymatic attack (Benamrouche, Cronier, Debeire, & Chabbert, 2002). Therefore, it must be assumed that the application of an enzymatic peeling to wheat bran can only profile a limited layer of the sample surface.

Untreated wheat bran showed a comparatively low amount of accessible cellulose, especially so in the outer layers (corresponding to the first 30 min of peeling). However, values for xylose release were amongst the highest and showed an enrichment of arabinoxylan in the deeper layers (after 30 min of peeling, see Fig. 2).

Enzymatic peeling profiles for glucose from cellulose and xylose are given in Fig. 2.

3.2.1. Chemical treatments

The chemical treatments showed by far the most pronounced effect regarding the enzymatic release of glucose from cellulose with peroxide and lye treatment exceeding acid treatment. This points towards an improved accessibility of cellulose, which might be due to the removal of inhibitory or protective substances such as lignin (Rahikainen et al., 2011).

In the release of xylose, all chemical treatments fared substantially worse than untreated bran. Peroxide treatment gave an increase in the outer layers, but its release over time was rather stagnant so that little more xylose was accessible in deeper layers. Lye treatment released the smallest amount of xylose of all samples whereas acid treatment performed slightly below average.

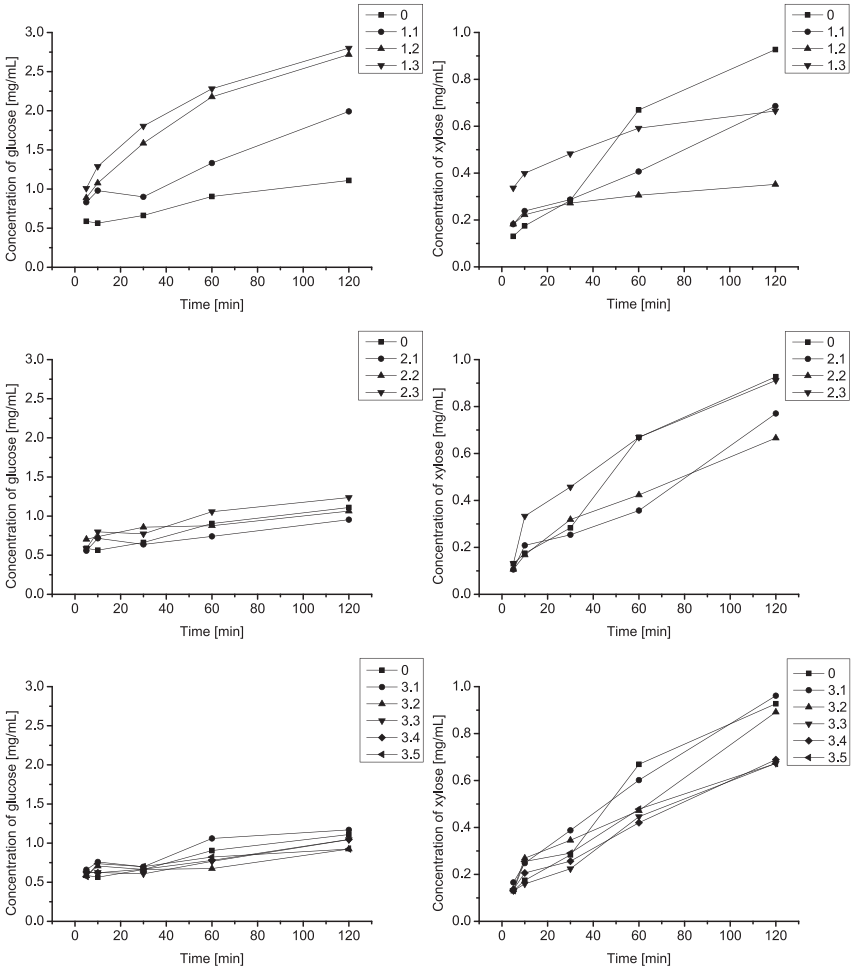

Fig. 2. Release of glucose (left) and xylose (right) over time during enzymatic peeling. **0**: Untreated wheat bran; **1.1**: acid treatment; **1.2**: lye; **1.3**: hydrogen peroxide; **2.1**: 5 min ball-milling; **2.2**: 60 min ball milling; **2.3**: extrusion; **3.1**: *Lactobacillus plantarum*; **3.2** esterase; **3.3** xylanase; **3.4**: esterase + xylanase; **3.5**: *Lactobacillus brevis* + esterase + xylanase.

3.2.2. Mechanical treatments

Ball-milling for 60 min released slightly more glucose over time than 5 min of treatment, but both gave overall release values marginally lower than untreated wheat bran. The 60 min treatment seemed to expose cellulose in the outer layers and therefore most of the glucose was released within the first 30 min of peeling. As for xylose, both ball-milling treatments revealed substantially less accessible substrate than untreated wheat bran. Again, 60 min of ball-milling led to increased exposure of xylose moieties in the outer layer whereas 5 min of treatment led to a delayed, albeit eventually more pronounced release of xylose during peeling (see Fig. 2).

Extrusion showed an increased accessibility of cellulose in the outer layers and released overall more glucose than untreated wheat bran. Also for xylose a high solubilization was observed in the first 30 min and overall levels were comparable to untreated wheat bran.

3.2.3. Fermentation and enzymatic treatment

Glucose release for fermented and enzymatically treated samples suggested a distribution of cellulose very similar to that of untreated wheat bran. *L. plantarum* and esterase treatment framed the upper and lower limit of effect reached after 60 min of peeling.

Differences were more prominent for the release of xylose. Here, *L. plantarum* showed high amounts of accessible arabinoxylan throughout the layers with the largest total amount of released xylose of all samples. Esterase treatment showed an enrichment of arabinoxylan in the very outer (10 min) as well as the very deepest layers (120 min) and reached overall values comparable to untreated wheat bran. Similar amounts of total xylose were released during peeling for the treatments with xylanase; the combination of esterase and xylanase; and *L. brevis* in combination with esterase (−8% to −13%). Xylanase treatment resulted in rather low concentration of arabinoxylan in the outer layers (up to 30 min) while *L. brevis* in combination with esterase and xylanase suggested high concentrations in the outer layers (10 min) and lower amounts in deeper layers (60–120 min). The combination of esterase and xylanase gave a rather even distribution throughout.

3.3. Immunolocalization

Fig. 3 shows all three channels recorded with confocal microscopy as an RGB overlay to provide a spatial context for arabinoxylan. Fig. 4 shows only the signal of the arabinoxylan label fluorescence in black on white.

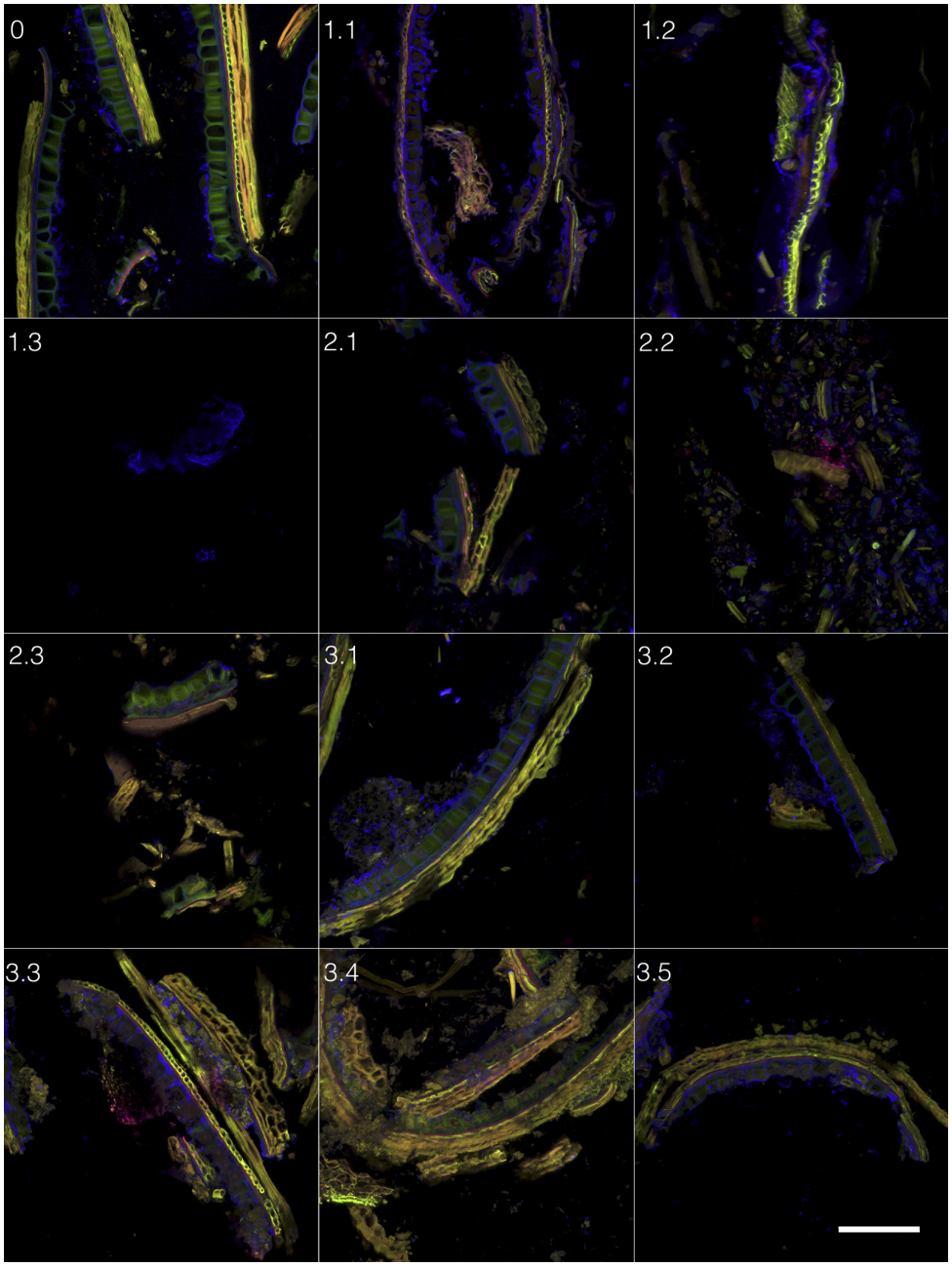

Fig. 3. Immunolocalization images of arabinoxylan (blue) and autofluorescence (red and green) as overlay images. **0**: Untreated wheat bran; **1.1**: acid treatment; **1.2**: lye; **1.3**: hydrogen peroxide; **2.1**: 5 min ball-milling; **2.2**: 60 min ball milling; **2.3**: extrusion; **3.1**: *Lactobacillus plantarum*; **3.2** esterase; **3.3** xylanase; **3.4**: esterase + xylanase; **3.5**: *Lactobacillus brevis* + esterase + xylanase. Bar = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.1. Sample preparation

The sample preparation employed proved drastically more time efficient and substantially less laborious than the commonly used fixation and subsequent embedding in acrylic resin (Fasseas, Roberts, & Murant, 1989). Ten samples could be prepared within about 2 h instead of several days. Furthermore, only a few drops of antibody solution were required to cover a block of wax as compared to several hundred μL for soaking samples, while washing could be performed by simple rinsing instead of having to pipette or filter off supernatant multiple times. Whether this technique is limited to softer samples such as cereals or whether it could as well be applied to wood samples remains to be tested.

No overlap of fluorescence from the antibody and autofluorescence from the sample were detected. Omission of the primary antibody accordingly resulted in the absence of fluorescence according to the specificity of the secondary antibody.

3.3.2. Untreated wheat bran

Wheat bran arabinoxylan is mainly located in the aleurone layer (ca. 25%), nucellar epidermis (ca. 25%) and pericarp (ca. 38%) (Benamrouche et al., 2002). Since its occurrence is limited to cell

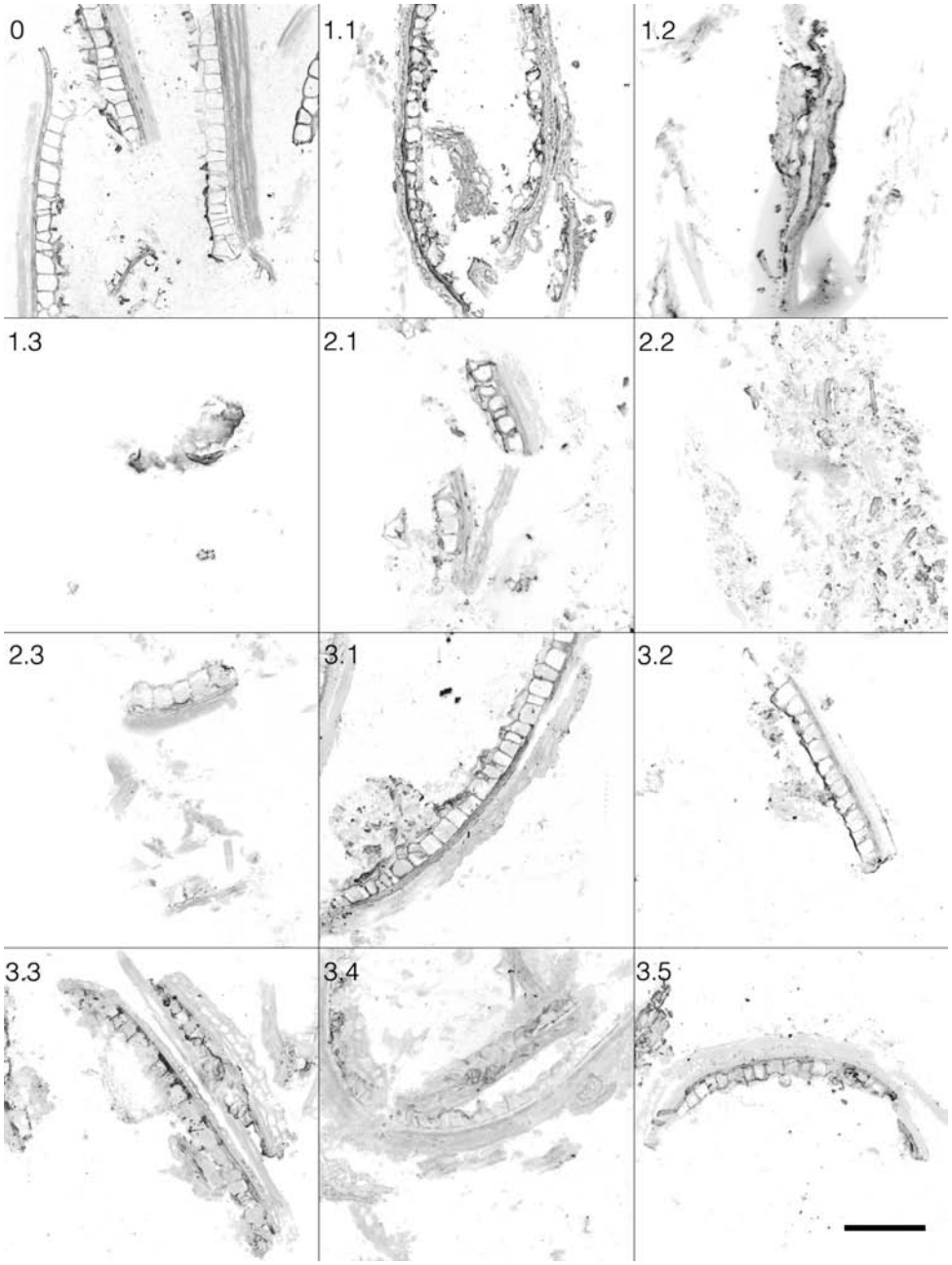

Fig. 4. Immunolocalization images of arabinoxylan shown as single channel recordings. Colors have been inverted and rendered black and white for better discernibility. **0**: Untreated wheat bran; **1.1**: acid treatment; **1.2**: lye; **1.3**: hydrogen peroxide; **2.1**: 5 min ball-milling; **2.2**: 60 min ball milling; **2.3**: extrusion; **3.1**: *Lactobacillus plantarum*; **3.2** esterase; **3.3** xylanase; **3.4**: esterase + xylanase; **3.5**: *Lactobacillus brevis* + esterase + xylanase. Bar = 200.

walls, arabinoxylan is more concentrated in the aleurone layer due to its comparatively thin cell walls, as can be deduced from the distribution of fluorescence from the immunolabel in Figs. 3 and 4.

3.3.3. Chemical treatments

Acid treatment caused the cell walls of the aleurone layer to swell so that the distribution of arabinoxylan was accordingly broader. Only little arabinoxylan seems to have been mobilized which is in agreement with the findings from methanolysis (see Section 3.1.1). Given that the enzymatic peeling could digest merely an outer layer of the sample, the smaller release of xylose as compared to untreated wheat bran is probably due to the wider distribution of arabinoxylan within the cell walls of the aleurone layer.

Lye treatment effected a complete disintegration of the aleurone layer, which accounts for most of the decrease in arabinoxylan content, and a swelling of the nucellar epidermis. Since the aleurone layer is by far the most accessible for enzymatic hydrolysis (Benamrouche et al., 2002), removal of the layer should lead to poor solubilization of additional arabinoxylan as has been observed during enzymatic peeling.

Peroxide treatment caused the strongest disruption of wheat bran cells as expected after 74% of total mass loss. Since peroxide is able to degrade lignin (Sun, Sun, & Wen, 2001), treatment resulted

in a near obliteration of auto-fluorescence. The fragments that are found with fluorescent microscopy are difficult to assign to distinct cell layers due to their deformation, but the presence of arabinoxylan, especially in the outer layers, is evident, as has been confirmed by sugar analysis and enzymatic peeling.

3.3.4. Mechanical treatments

Five minutes of ball-milling only exhibited a reduction in particle size affecting neither cellular structures nor the distribution of arabinoxylan. This increase in surface area is in accordance with the increase in overall solubility and the similarity in residual sugar composition with untreated wheat bran.

After 60 min of ball-milling, however, cells are broken up beyond recognition and arabinoxylan is scattered loosely. The rather selective extractability of arabinoxylan observed by methanolysis (see Section 3.1.2) can be deduced from the image. The relatively low release of xylose during enzymatic peeling is probably due to depletion of arabinoxylan after washing.

Extrusion showed a small increase in residual material with a slight decrease in arabinose and xylose moieties compared to untreated wheat bran, which implies selectivity towards arabinoxylan. Enzymatic peeling released twice the amounts of xylose compared to native wheat bran and indicated enrichment of arabinoxylan in the outer layers (see **2.3** in Fig. 2). The immunolocalization images are in accordance with these findings, revealing a cell structure that is intact but slightly depleted of arabinoxylan.

3.3.5. Fermentation and enzymatic treatment

Fermentation and especially enzymatic treatments showed a surprisingly low selectivity.

Fermentation with *L. plantarum* did not affect the cell wall integrity and the distribution of arabinoxylan appeared virtually identical to that of untreated wheat bran. This supports the results of methanolysis, namely that *L. plantarum* mainly solubilized protein. The removal of protein might have increased the enzymatic accessibility to arabinoxylan which is reflected in the slightly increased release of xylose during enzymatic peeling.

Esterase treatment caused a light swelling of aleurone cell walls, the only layer effectively accessible to enzymatic treatment (Benamrouche et al., 2002), shifting the arabinoxylan distribution more towards the very border of cell walls. This observation is substantiated by the profile obtained during enzymatic peeling.

Xylanase treatment effected an overall more pronounced swelling of aleurone cell walls as well as damage to inward-facing cell walls so that the distribution of arabinoxylan was increasing towards the nucellar epidermis. This change can be expected to render arabinoxylan less accessible to further enzymatic attack as was shown by the lowered release of xylose during enzymatic peeling.

The combination of esterase and xylanase showed the strongest impact on aleurone cells. Cell walls were swollen, perforated or even partially disintegrated. Arabinoxylan was distributed in broader zones of smaller intensity across the aleurone layer. Keeping in mind that the increased overall solubilization gave a residual sugar ratio comparable to other less effective enzymatic treatments, extraction of further cell compounds must be concommitant to arabinoxylan extraction. As a consequence of the broader distribution of arabinoxylan, enzymatic peeling gave a profile with lower arabinoxylan concentrations.

Fermentation with *L. brevis* in combination with esterase and xylanase lead to swollen cell walls in the aleurone layer. The overall reduced amount of arabinoxylan was found to be highest in the outer layer where it could be detected by enzymatic peeling. Since the cells appeared to be less damaged compared to the treatment without *L. brevis*, the further increase in total mass loss must have come at the cost of compounds not made visible by fluorescence microscopy.

4. Conclusion

Little is known about the association of arabinoxylan and cellulose in wheat bran cells. The data obtained from enzymatic peeling and methanolysis after diverse treatments suggest a rather loose affiliation allowing for the manipulation of one polysaccharide without affecting the other.

Yet, defining a selective pretreatment for the isolation of arabinoxylan has proven difficult. Chemical methods, especially lye and peroxide treatment, solubilized by far the biggest percentage of wheat bran but did so at the cost of substantial coextraction of other compounds. Given their severe conditions, degradation of other valuables as well as formation of inhibitory and hazardous substances take place. Lye treatment has displayed the best selectivity among chemical treatments.

Extensive ball-milling resulted in the most promising results with a strong specificity for xylan-based polysaccharides. Since it applies mere mechanical force, the results point towards a less rigid integration of arabinoxylan into the cell wall or a greater disposition for depolymerization compared to cellulose. Extrusion, which combines heat and mechanical energy input, resulted in a slightly more selective extraction of arabinoxylan, but overall extractability was low so that the return is unlikely to justify the costs for this application.

Enzymatic extraction with or without fermentation showed little selectivity for arabinoxylan and mostly influenced degrees of overall solubilization. Ostensibly, enzymatic removal of arabinoxylan is always concomitant with the extraction of other cell compounds. Treatment with *L. plantarum* even showed a strong preference for non-carbohydrate material.

The application of immunolocalization to visualize the cell-structural changes perpetrated by pretreatments on arabinoxylan was helpful to interpret the findings of the chemical analyses. As a complementary method, it is very helpful in taking stock and pointing towards optimization strategies. For example, the depletion of arabinoxylan from the aleurone layer, which would mark a ceiling for enzymatic extraction, is difficult to measure in chemical analysis but easily observed by immunolocalization. Our simplified sample preparation for confocal laser fluorescence microscopy further facilitates rapid measurement.

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A comparison between near-infrared (NIR) and mid-infrared (ATR-FTIR) spectroscopy for the multivariate determination of compositional properties in wheat bran samples

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ABSTRACT

The objective of this study was a comparison of the performance of Fourier transformed near-infrared (NIR) with Fourier transformed mid-infrared (MIR) spectroscopy using attenuated total reflectance for the multivariate determination of compositional parameters in wheat bran samples. These parameters were the contents of water, protein, ash, starch, soluble as well as insoluble dietary fibers, and lipids. Partial least squares (PLS) regression was used to construct a calibration model. NIR was found to perform better for ash, starch and soluble as well as insoluble dietary fiber, while MIR was superior only for protein. The scores for water and fat were about equal. The prediction results for ash, insoluble dietary fiber and fat were good. The analysis of soluble dietary fiber suffered from the inaccuracy of the underlying wet-chemical reference method, which had a negative impact on the calibration. Starch was prone to a large relative error despite a good coefficient of determination. Protein and water gave acceptable relative errors but suboptimal goodness of fit. From the precision achieved with a limited sample set, it can be concluded that infrared spectroscopy is an appropriate method to establish a rapid analysis of wheat bran. In general, NIR seemed to be the superior and more robust method.

1. Introduction

Wheat bran is the major byproduct of white flour production. It contains the bulk of dietary fiber, minerals, and vitamins of wheat. However, consumer acceptance is rather low due to its bitterness, fibrous texture and mouth-feel (Delcour & Poutanen, 2013; Saricoban, Yilmaz, & Karakaya, 2009). Consequently, wheat bran has found wide application as a cheap ingredient in animal feeding (Fuller, 2004).

For monogastrics, wheat bran delivers about 60% of the net energy per kg compared to whole wheat (Taylor-Pickard & Spring, 2007). The nutritional value relies on a relatively high starch content and is limited by the amount of dietary fiber. Wheat bran is mainly used to lighten energy-dense feed formulations. Pretreatments, such as fermentation and extrusion, can be applied in order to increase digestibility (Kraler, Schedle, Domig, Heine, Michlmayr, & Kneifel, 2014). Ruminants are superior to monogastrics at digesting dietary fiber. Therefore, wheat bran is of higher value in their feed and can be incorporated at a larger percentage of up to 50% (Singh, Garg, Malik, & Agrawal, 1999).

Since the composition of wheat bran is heavily influenced by the milling process as well as the wheat variety and seasonal conditions, the analysis of key constituents is essential for proper intake control. However, the wet-chemical analyses according to industry-standard protocols are laborious, especially in the case of dietary fiber and starch, and require specialized facilities, which are costly to establish on site. Acquiring all the analytical parameters that were tested in this study takes about a week for ten samples, and working time scales unfavorably with sample number.

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For a large number of similar samples with a predictable variation in composition, chemometry applied to infrared spectra has proven to be an apt method for rapid screening or even analytical purposes (Benito, Ojeda, & Rojas, 2008). The chemometric approach relies on the computational correlation of spectral information with data derived from chemical analysis. This is based on a multivariate analysis of the spectrum, which takes into account not only a single, representative band, but overall changes in the spectrum that correlate with the analyte. The necessity for a unique spectral absorbance is thus eliminated by deconvolution of the spectrum through multivariate linear regression analysis. Given a representative set of samples and an influence of the measure on the spectral data, a calibration and prediction model can be calculated.

Most commonly, spectra recorded in the near-infrared range (ca. 13400–4000 cm^{-1}) are used. They record convoluted overtone and high frequency combinations of molecular vibrations (McClure, 2003). Spectra in this range reveal little information to the naked eye, but are sensitive to a multitude of compounds and molecular interactions (Blanco & Villarroya, 2002). To give some examples, Blakeney and Flinn (2005) have determined non-starch polysaccharides in cereal grains. Fontaine, Schirmer, and Horr (2002) have created a model for protein and its amino acid composition in various grains. Yang et al. (2009) have measured fatty acid concentrations in maize grain, while Viljoen, Brand, Brandt, and Hoffman (2005) have predicted ash, dry matter, protein, acid detergent fiber and neutral detergent fiber in grains.

In mid-infrared spectroscopy (ca. 4000–400 cm^{-1}) most functional groups can be observed since their vibrational resonances fall within this range of excitation frequencies (Gordon, 2011). This makes it a valuable tool in compound identification. Even more complex and very similar structures, such as polysaccharides, have been differentiated by mid-infrared spectra (Pielesz, 2012; Robert, Marquis, Barron, Guillon, & Saulnier, 2005). The chemometric approach is taken less frequently in combination with MIR compared to NIR, but is not without examples. Kim, Himmelsbach, and Kays (2007) have determined *trans*-fatty acids in cereal products. Suchowilska, Kandler, Wiwart, and Krska (2012) could differentiate between species of wheat by applying principle component analysis to MIR spectra. Tamaki and Mazza (2011) quantified different types of polysaccharides, ash, and extractives in straw, albeit with mixed results.

Comparisons of the performances of NIR and MIR for cereal samples are rare. In fact, the closest related topic found was a single comparison of mixtures of triticale and pea regarding acidic detergent fiber, neutral detergent fiber, and total nitrogen (Calderon, Vigil, Reeves, & Poss, 2009). Therefore, this study establishes a comparison of the efficacy of NIR vs. MIR for the determination of nutritional properties in wheat bran samples: native, extruded, fermented, and enzymatically treated wheat bran. Evaluated compositional properties were the contents of water, protein, ash, starch, soluble and insoluble dietary fiber as well as lipids.

2. Material and methods

2.1. Materials

A total of 44 different wheat bran samples were analyzed. All native samples were obtained from VonWiller Mill (Schwechat, Austria) produced from a roller mill. The set contained 15 types of feed bran, 24 types of food bran, one extruded and four sour dough samples: untreated; treated with cellulase (Celluclast, Novozyme, Bagsvaerd, Denmark); xylanase (Pentopan, Novozyme, Bagsvaerd, Denmark); and with both enzymes. Sour doughs were produced through spontaneous fermentation at 38 °C for 48 h without agitation. Enzymes were dosed at 1wt%. Different degrees of milling using a CUM 300/CGM Condux mill (Netzsch, Hanau, Germany) were applied to nine of the food brans, the resulting particle sizes ranged from 95 μm to 250 μm. All samples were ground to pass at least a 500 μm screen for chemical as well as NIR and MIR analysis.

2.2. Water

Water content was measured indirectly through dry mass determination at 105 °C, according to International Association for Cereal Chemistry (ICC) Standard No. 110/1 (ICC website).

2.3. Ash

Ash was determined gravimetrically after a two-step ashing process in a fast incinerator and a muffle furnace, according to ICC Standard No. 104/1 (ICC website).

2.4. Protein

Protein was determined titrimetrically as total nitrogen by the Kjeldahl method, according to ICC Standard No. 105/2 (ICC website).

2.5. Starch

Starch was measured photometrically after enzymatic hydrolysis, according to AACCI Method 76-13.01 (AACCI 1999). Glucose oxidase, peroxidase and ABTS are used to give a color reaction with the produced glucose.

2.6. Soluble and insoluble dietary fiber

Soluble and insoluble dietary fiber were determined according to the Prosky method of AOAC 985.29 (Prosky et al., 1984). This method is based on a gravimetric determination after multiple enzymatic treatments and has to be corrected for residual ash and protein.

2.7. Lipids

Lipids were determined gravimetrically after Soxhlet extraction, according to ICC Standard No. 136 (ICC website).

2.8. Spectroscopy

NIR spectra were recorded on a Bruker MPA FT-NIR spectrometer (Bruker, Billerica, MA). For each spectrum, 20 scans from 12400 cm^{-1} to 4000 cm^{-1} were accumulated using Bruker's Opus software version 7.0. Spectra were recorded in duplicate, of which one was used for calibration and the other for test set validation.

MIR spectra were recorded on a Perkin Elmer Frontier ATR-FTIR spectrometer equipped with a Frontier UATR Diamond/ZnSe with 1 Reflection Top-Plate (Perkin Elmer, Waltham, MA). For each spectrum, four scans from 4000 cm^{-1} to 550 cm^{-1} were accumulated using Spectrum software version 10.03.02. Spectra were recorded in duplicate, of which one was used for calibration and the other for test set validation.

2.9. Chemometrics

NIR and MIR spectra were processed using Bruker's Opus software version 7.0. Preprocessing algorithms that correct for variation in the spectra unrelated to analyte compounds were chosen as

suggested by the software after optimization. Opus computes all possible preprocessing and ranks the results according to the relative mean square error of prediction (RMSEP). In order to avoid overfitting, latent variables were kept between six and ten. Partially least squares (PLS) regression was used as a calibration model. Outliers were not excluded since there was no reason to do so from an analytical point of view.

3. Results and discussion

Table 1 shows the distribution of the contents of water, protein, ash, starch, soluble dietary fiber, insoluble dietary fiber and lipids in wheat bran as determined by wet chemical analysis. Range, mean, standard deviation and relative error of the wet-chemical method are given. Table 2 presents the mathematical preprocessing and the spectral range selected for optimization for NIR and MIR. Table 3 gives the calibration and test set validation results for NIR as well as the number of latent variables. Table 4 does so for MIR. Fig. 1 shows an example of what was considered a poor and a good fit for validation plots. Since it is the test set validation that expresses the model's actual capability to determine unknown samples, which is the main interest of this study, the text will focus on these results.

Water gave a rather poor coefficient of determination (R^2) for both NIR (0.78) and MIR (0.79). This is surprising, since water has sharp, characteristic absorption bands in NIR as well as MIR and therefore would have been expected to be predicted more accurately. Accordingly, the relative error of validation (REV) is quite high for NIR and MIR (both 11.6%). The error might lie in the methodology as two weeks passed between the wet chemical analysis and the infrared measurement, during which the samples were stored in screw cap containers in a cooling room. Finely ground bran is a hygroscopic material and might have accumulated additional moisture during that time giving way to error in prediction.

The coefficients of determination for protein were even poorer, but here a strong advantage of MIR over NIR was observed for both R^2 (0.75 vs. 0.66) and REV (7.1% vs. 8.4%). The lower R^2 but smaller REV compared to the determination of water shows that a large coefficient of determination does not automatically infer a small error. A good fit to a statistical model does not imply that the model itself has to be a good predictor of the given variable (Kleinbaum, Kupper, & Muller, 2007). Protein absorption bands in MIR are clearly observed at characteristic wavenumbers, e.g. NH and OH stretching at 3400–3000 cm^{-1}, CO stretching at 1653 cm^{-1}, CN stretching, NH bending at 1550 cm^{-1} and CN bending at 1450 cm^{-1} (Chalmers & Griffiths, 2002). Since some of these bands are often well separated from the rest of the spectrum, calibration is likely to be more selective in MIR than in NIR, where protein bands are convoluted.

Ash determination showed good predictability. With an R^2 of 0.91 and an REV of 5.7%, NIR clearly surpassed MIR (0.85 and 7.1% respectively). This difference is difficult to deduce from spectral properties. Tamaki and Mazza (2011) also struggled at accurately predicting ash contents in wheat straw using MIR calibration. Their prediction model returned an R^2 of 0.81 and an RMSEP of 0.40. There are numerous papers that predict ash using NIR and report R^2 values of 0.88 up to 0.99 (Bruun, Jensen, Magid, Lindedam, & Engelsen, 2010; Dahl, Christensen, Munck, Larsen, & Engelsen, 2000; deAldana, Garcia Criado, Garcia Ciudad, & Perez Corona, 1996). It is a facile argument, however, to infer superiority of NIR from a literature comparison since the results heavily depend on sampling size, sample selection, matrix, instrumentation and wet-chemical accuracy. Our findings suggest that in the case of wheat bran, NIR is indeed the favorable technique for ash determination.

In the prediction of polysaccharides, NIR fared better than MIR for all three categories put to the test. R^2 values achieved by NIR were 0.88, 0.77 and 0.93 for starch, soluble dietary fiber, and insoluble dietary fiber, respectively, whereas MIR attained 0.82, 0.60 and 0.82. REV values (same order) were 17.3%, 12.9% and 3.8% for NIR and 20.9%, 16.8% and 5.9% for MIR. This stands in contrast to the findings of Calderon et al. (2009) who observed no considerable difference between NIR and MIR in the determination of acid detergent and neutral detergent fiber in triticale and peas. In the presented study, the prediction of soluble and insoluble dietary fiber with NIR showed promising results and would be sufficient for intake control.

Starch content showed good correlation, but the prediction was heavily scattered, resulting in a high REV. Our RMSEP of 1.92% (NIR) compares well with results found in the literature. Using NIR, Li, Chen, Li, Singh Brown, and Danao (2014) achieved an RMSEP of 1.23% for starch in sorghum. Katayama, Komaki, and Tamiya (1996) attained an RMSEP of 1.91% in sweet potatoes. Since the amount of starch present in wheat bran is rather small compared to whole cereals or potatoes, a bigger relative error is inferred.

The inferior performance for soluble compared to insoluble dietary fiber is likely due to a carry-over of the larger error in the wet-chemical method (RE of 16.1% compared to 0.56% for insoluble dietary fiber). Prediction with NIR as well as MIR could match this level of error, which suggests that the model reaches the precision of the wet chemical analysis and probably could be improved with more accurate data from the external calibration. We would expect a calibration of total dietary fiber to give results as good as those for insoluble dietary fiber. Since the analysis of dietary fiber is the biggest contributor to the workload in wet-chemical analysis, it is most crucial to have a good prediction model for this parameter.

Polysaccharides comprise highly hygroscopic material. With most of the samples containing around 10% water and more than 60% polysaccharides, we expect the water to be mainly adsorbed to polysaccharides. This adsorption might interfere with the MIR signal of polysaccharides, since water absorbs strongly in the same range and might mask characteristic bands of polysaccharide partially. The effect, however, is less pronounced in the vibrational overtones recorded in NIR, which could explain the advantage seen for NIR over MIR in the analysis of polysaccharides (Burns & Ciurczak, 2008).

Lipids were determined quite accurately. With an R^2 of 0.88 and an REV of 6.3%, MIR performed slightly better than NIR (0.87 and 6.6%). Data from the literature is mixed regarding lipids in cereal grains. Comparable results have been reported using NIR for lipids in ground sorghum with an R^2 of 0.91 and an REV of 5.8% (Figueiredo et al., 2006). For oats, which are significantly higher in lipids, an R^2 of 0.97 and an RE of about 3.8% were achieved (Liu, Zhou, Ren, 2014). On the other hand, Dowell et al. (2006) struggled to predict lipids in wheat. At best they achieved an R^2 of 0.74 and SECV of 3.24% (standard error of cross validation; this does not represent a relative, but an absolute error). Calderon et al. (2007)

Table 1
Wet-chemical sample data. SD = Standard deviation of range. RE = Relative error of wet-chemical analysis (Relative mean square error/Mean*100).

	Range [%]	Mean [%]	SD	RE [%]
Water	2.9–11.9	9.5	2.4	0.91
Protein	9.3–15.5	11.5	1.7	2.5
Ash	2.2–7.2	5.8	1.1	1.9
Starch	1.2–37.5	11.1	5.5	2.8
Soluble DF	1.9–6.4	4.3	1.2	16.1
Insoluble DF	25.6–61.5	47.5	6.9	0.56
Lipids	2.6–6.0	3.9	0.72	3.2

Table 2
NIR and MIR preprocessing method and selected spectral range.

	Preprocessing NIR	Spectral range NIR [cm^{-1}]	Preprocessing MIR	Spectral range MIR [cm^{-1}]
Water	Line subtraction	9403.7–5446.3; 4601.6–4246.7	None	3870–3216.5; 1583.5–1256
Protein	1st derivative + Vector normalization	9403.7–7498.3; 6102–4597.7	Multiplicative scatter correction	3217–2563; 1910–603
Ash	Multiplicative scatter correction	6102–5446.3	Line subtraction	3870–3543; 3217–2889.5; 2563.5–1909.5; 1583.5–929.5
Starch	1st derivative	9403.7–6098.1; 5450.1–4246.7	Offset correction	3870–2889.5; 2236.5–929.5
Soluble DF	1st derivative + Multiplicative scatter correction	9403.7–5446.3; 4601.6–4246.7	Min/max correction	3543.5–2889.5; 1910–1256
Insoluble DF	1st derivative	7502.1–6098.1	2nd derivative	2890–2563; 1583.5–929.5
Lipids	Line subtraction	9403.7–6098.1	Multiplicative scatter correction	3870–3543; 3217–2236.5; 1910–929.5

Table 3
NIR statistics. Cal = Calibration. Val = Validation. RMSEC = Relative mean square error of calibration. RMSEP = Relative mean square error of prediction. LV = Latent variables. REV = Relative error of validation (RMSEP/Mean*100).

	Cal R^2	Val R^2	RMSEC [%]	RMSEP [%]	LV	REV [%]
Water	0.87	0.78	0.95	1.10	9	11.6
Protein	0.87	0.66	0.70	0.96	10	8.4
Ash	0.96	0.91	0.25	0.33	9	5.7
Starch	0.94	0.88	1.52	1.92	10	17.3
Soluble DF	0.84	0.77	0.52	0.55	8	12.9
Insoluble DF	0.98	0.93	1.15	1.79	10	3.8
Lipids	0.91	0.87	0.25	0.26	10	6.6

Table 4
MIR statistics. Cal = Calibration. Val = Validation. RMSEC = Relative mean square error of calibration. RMSEP = Relative mean square error of prediction. LV = Latent variables. REV = Relative error of validation (RMSEP/Mean*100).

	Cal R^2	Val R^2	RMSEC [%]	RMSEP [%]	LV	REV [%]
Water	0.91	0.79	0.84	1.10	10	11.6
Protein	0.82	0.75	0.78	0.82	8	7.1
Ash	0.95	0.85	0.27	0.41	10	7.1
Starch	0.86	0.82	2.38	2.32	10	20.9
Soluble DF	0.66	0.60	0.76	0.72	9	16.8
Insoluble DF	0.98	0.82	1.04	2.79	6	5.9
Lipids	0.94	0.88	0.20	0.25	8	6.3

compared NIR and MIR for the determination of individual fatty acids in forage samples. For NIR validation, they reported an average RE value of 21%, for MIR 27%. They further questioned the robustness of their method due to a large discrepancy between calibration and validation. All these results eventually suggest that the determination of lipids is highly matrix-dependent. We have found wheat bran to be a suitable matrix that marginally favors MIR over NIR calibration.

The results presented so far are based on individual optimizations of the spectroscopic method and data pretreatment for each analyte. We were interested whether we could find a unified preprocessing method and spectral range suitable for all parameters in NIR and MIR, respectively. This would facilitate the measurement even more and speak to the robustness of the method. Therefore we ran the entire set of calibrations through all preprocessing methods, which had been chosen for at least one of the single parameters. A spectral range was chosen that encompassed most of the previously selected ranges, so that all relevant spectral data were included.

Spectral ranges of 9404–5446 cm^{-1} for NIR and 3500–900 cm^{-1} for MIR were chosen. Best results were obtained with the first derivative as overall universal preprocessing method. These results are presented in Table 5. While all parameters saw a decrease in overall fit and an increase in relative error compared to individual optimization, these negative effects were drastically more pronounced for MIR than for NIR. R^2 values decreased by 0.01–0.10 for NIR, whereas the difference for MIR was between 0.07 and 0.38. NIR relative errors of validation only increased from 0.2 to 2.9% compared to 1.3–8.9% for MIR. The biggest increase was observed for starch, which already had the highest REV from the beginning. The other parameters showed less than a 1.2% increase in REV for NIR and less than 6.2% for MIR.

The fact that NIR performed drastically better than MIR in a unified approach demonstrates superior robustness of the method. In contrast to NIR, MIR seems to rely heavily on characteristic bands even in a multivariate calibration. This selectivity can be observed in the small windows of spectral ranges chosen for individual parameter optimization (Table 2). We surmise therefore that MIR is more error-prone than NIR in a one-size-fits-all approach that spans a wider spectral range.

4. Conclusion

NIR was shown to be the generally superior technique for the

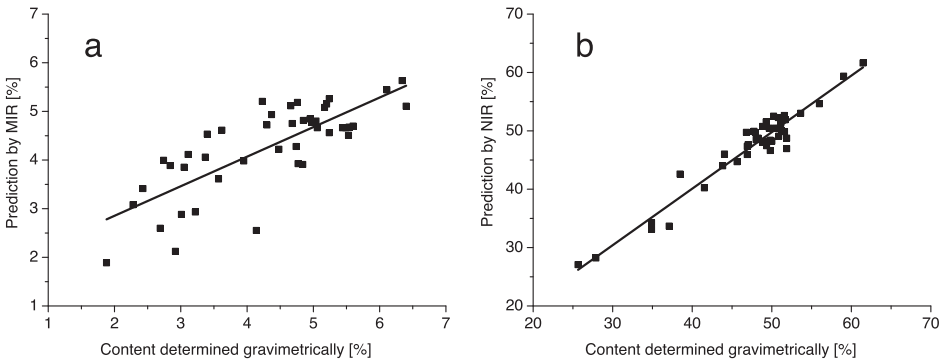

Fig. 1. Example validation plots for a poor (a) and a good fit (b). a) Soluble dietary fiber determined by MIR. $R^2 = 0.60$. b) Insoluble dietary fiber determined by NIR. $R^2 = 0.93$.

Table 5
NIR and MIR statistics with unified preprocessing and fixed spectral range. Preprocessing was 1st derivative. Spectral range was 9403.7–5446.3 cm^{-1} for NIR and 3500–900 cm^{-1} for MIR. REV = Relative error of validation (RMSEP/Mean*100).

	R^2 NIR	REV NIR [%]	R^2 MIR	REV MIR [%]
Water	0.75	12.8	0.50	17.7
Protein	0.57	9.5	0.37	11.3
Ash	0.88	6.5	0.78	8.6
Starch	0.83	20.1	0.63	29.8
Soluble DF	0.77	13.1	0.29	22.4
Insoluble DF	0.90	4.6	0.74	7.2
Lipids	0.82	7.7	0.65	10.7

analysis of compositional parameters in wheat bran. It performed better for ash, starch and soluble as well as insoluble dietary fiber, whereas MIR only got the upper hand for protein. The scores for water and fat were about equal. Furthermore, NIR demonstrated better robustness under a universal parameter setting.

Overall, ash, insoluble dietary fiber and fat showed good prediction results. The analysis of soluble dietary fiber suffered from imprecision of the wet-chemical method, which translated into the calibration. The determination of starch content was prone to a large relative error despite a good coefficient of determination. Protein and water gave acceptable relative errors, but suboptimal goodness of fit. They would still be suited for screening for strong deviations.

It has to be taken into consideration that while the sample set employed in this study was relatively small, it was sufficiently large to reveal the suitability of either spectroscopic method to replace time consuming wet-chemical analyses. For a serviceable application, a more precise calibration would need to be established based on the presented findings.

Acknowledgment

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7 Synthesis of a label for the determination of oxidative damage in water-soluble polysaccharides

7.1 Introduction

Pretreatment and processing of wheat bran can lead to depolymerization and degradation of polysaccharides, the major constituents of wheat bran (Reisinger, Tirpanalan, Huber, Kneifel, & Novalin, 2014; Reisinger et al., 2013). While depolymerization can be the aim of a process, degradation, which usually occurs as oxidative damage, is the unwanted result of too harsh processing conditions. We set out to develop a method that realizes the simultaneous determination of both factors in a single analysis.

Oxidative damage usually results in the introduction of carboxyl groups into a polysaccharide. We tried to synthesize a fluorescent label that binds specifically to those groups to allow their quantitative determination. Combined with the determination of molecular size distribution by gel permeation chromatography, the labeled carboxyl groups would be detected by a fluorescence detector. Thus the oxidative damage in relation to the polysaccharide chain length would be measured.

Such a label, called 9*H*-fluoren-2-yl-diazomethane (FDAM), has already been developed in our group for cellulosic materials (Bohrn et al., 2006). However, labeling and measurement with FDAM is carried out in *N*,*N*-dimethylacetamide, which does not dissolve arabinoxylan. Consequently, we needed to transfer the process to a water-based system. Unfortunately, due to its low polarity, FDAM labeling proved to render water-soluble polysaccharides insoluble. Therefore, we had to synthesize a new label.

Such a fluorescent label has to meet specific requirements.

One, its fluorescent properties must not interfere with the wavelength of the light scattering detector employed for molecular size distribution.

Two, its synthesis should be efficient, scalable, green and from affordable starting materials. FDAM is synthesized from fluorene-2-carboxaldehyde. The aldehyde is converted with hydrazine to a hydrazone and then oxidized to the respective diazo compound. Our goal was to adopt this synthetic route, i.e. start from an aldehyde or introduce one.

Three, the label must be water soluble to allow for homogeneous labeling and it must not render polysaccharides insoluble through binding. This presented a particular challenge, since fluorescence is effected by conjugated ring structures that are nonpolar by nature, demanding the introduction of polar functional groups. Unfortunately, most polar functional

groups either react with the carboxyl binding diazo group or one of its precursors, or are susceptible to the chemicals used in the synthesis.

Four, the resulting diazo compound needs to be stable under labeling conditions, but reactive enough to bind carboxyl groups. This property could not be ascertained from literature and had to be realized in a trial and error approach once the final step of a synthetic route was reached.

These requirements severely limited the choice of molecules. We investigated syntheses based on fluorene-2-carboxaldehyde, vanillin, quinoxaline, fluorescein, Rhodamine, quinine, indole-3-carboxaldehyde and pyridoxal. Eventually, none of them could be brought to fruition. What follows is a story of failure, but we believe that our findings might serve as a basis for further approaches.

7.2 Results and Discussion

7.2.1 Synthesis analogous to FDAM

The diazotation procedure for FDAM as depicted in Figure 2 (Bohrn et al., 2006) was adopted for all our synthetic routes from the point where an aldehyde had been introduced into the label.

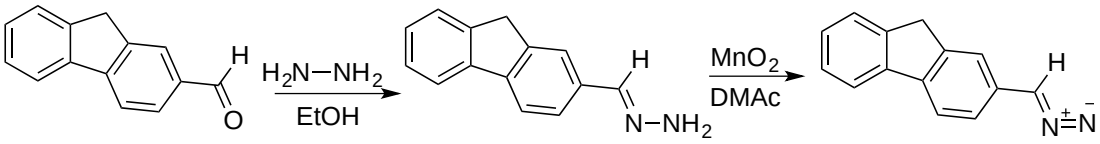


Figure 2. Synthesis of FDAM

7.2.2 Modification of fluorene-2-carboxaldehyde

Since the FDAM label based on fluorene-2-carboxaldehyde rendered polysaccharides water-insoluble, our first approach was to make fluorene more polar by the introduction of nitro groups and their subsequent reduction to amino groups as detailed in Figure 3.

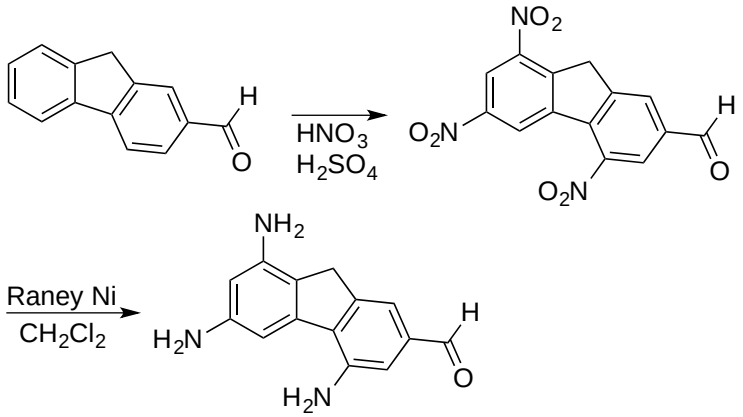


Figure 3. Synthetic pathway of a more polar derivative of fluorene-2-carboxaldehyde

We observed the formation of a precipitate when a solution of the product was left to stand over night. Since we deemed the reaction to have been successful from thin-layer chromatography (TLC) observations (fluorescence was extinguished upon nitration and restored upon reduction to the respective amino compound), we assumed the polymerization to be caused by polymerization due to Schiff base formation between the newly introduced amino and the aldehyde group. Unfortunately, the reaction conditions were not flexible enough to be adjusted so that the formation of the precipitate could be prevented. Hence, the approach was discarded.

7.2.3 Vanillin

Vanillin is a cheap aldehyde that exhibits fluorescent properties, albeit not very strong (Ishikawa, Kuwano, & Matsumoto, 2007). Since it is a small molecule, we assumed it to bear little impact on solubility upon binding to a polysaccharide.

TLC of the reaction of diazo vanillin with glucuronic acid as a model compound showed a rather strong fluorescent band separate from both glucuronic acid and the labeling reagent. This band, however, did not elute at all with any given mobile phase. Since this cannot be explained by the expected polarity of the resulting compound, we had to assume that, again, polymerization must have taken place, which is initiated by the reaction with a carboxyl group. Figure 4 proposes a possible structure for this polymer, which would also explain the intensification of fluorescence. As this polymerization was both undesirable and uncontrollable, we decided to abandon the approach.

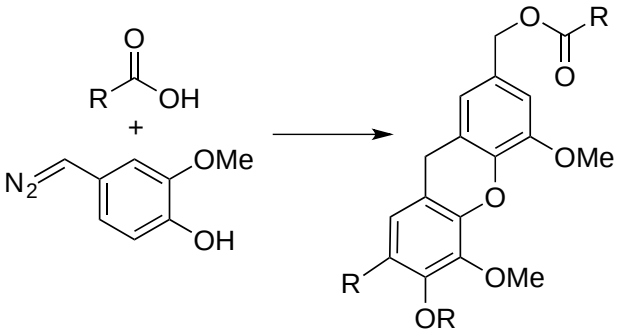


Figure 4. Proposed structure for the polymer from diazovanillin and a carboxyl group

7.2.4 Synthesis of quinoxaline-6-carboxaldehyde

Quinoxaline derivatives are fluorophores known for their good solubility in polar solvents (Perez-Melero et al., 2004). Furthermore, due to a simple and flexible synthesis from diaminobenzonitrile, their properties are quite adaptable.

Unfortunately, we had to observe that under all parameters tested for the reduction of quinoxaline-6-carbonitrile to quinoxaline-6-carboxaldehyde with DIBAL-H in a tubular flow reactor following a design of experiment approach, the reduction of the nitrile occurred always together with the reduction of the heterocycle as shown in Figure 5.

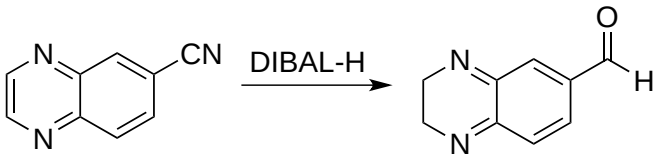


Figure 5. Reduction of quinoxaline-6-carbonitrile with DIBAL-H

A second route was tried to synthesize quinoxaline-6-carboxaldehyde from 3,4-diaminobenzoic acid and glyoxal *via* quinoxaline-6-carboxylic acid and quinoxaline-6-hydroxymethyl by reduction with lithium aluminum hydride and re-oxidation with Dess-Martin periodinane in a batch reaction. Still, we were facing the same side reaction of the heterocycle being reduced concomitantly to the functional group making further steps impossible.

7.2.5 Modification of fluorescein and Rhodamine B

Since we wanted to keep the synthetic approach we had taken with quinoxaline, we were looking for a molecule with a more robust core structure. Fluorescein is a cheap and highly conjugated compound with intense fluorescence and good polarity. Our aim was to use its carboxyl group along the lines of quinoxaline-6-carboxylic acid, i.e. reduce the carboxyl group to an alcohol and oxidize it subsequently to an aldehyde.

When reducing the ester of fluorescein to a hydroxyl group, one inadvertently also reduces the keto group of the xanthene ring system to a hydroxyl. This corollary severely affects the fluorescent properties and thus necessitates rearomatization of the xanthene ring structure. The accordant procedure had already been published by Santra et al. (2010). However, in our attempts to follow the protocol, rearomatization of the xanthene structure only led to the formation of a small amount of spiro ether instead, which renders our targeted functional group inaccessible. The reaction is detailed in Figure 6. The ratio of spiro ether to fluorescein alcohol obtained was 0.065:1.

Neither a method using elemental sulfur (Nguyen, Ermolenko, Dean, & Al-Mourabit, 2012) nor one using iodine (Gogoi & Konwar, 2005) resulted in rearomatization or the formation of an aldehyde, but in a complex mixture of products we did not identify further.

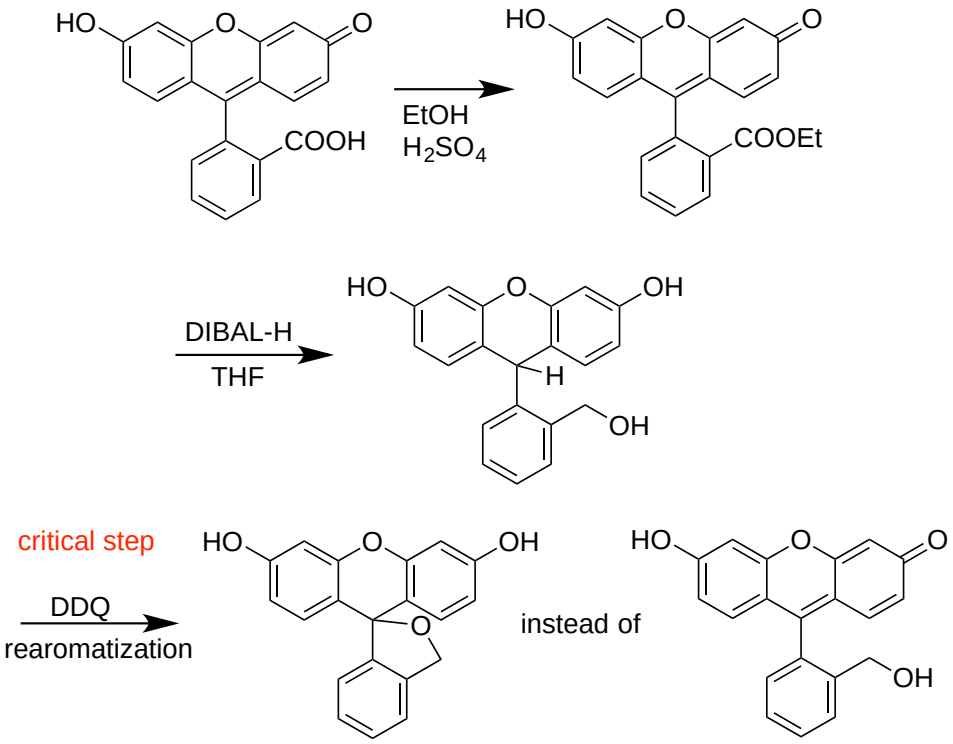


Figure 6. Synthetic route for fluorescein alcohol

Equal measures were taken with Rhodamine B, which is similar to fluorescein except for the substituents on the xanthene ring system. Therefore, a different electron density is imbued, but the change in properties did not make rearomatization possible.

Since the reduction of fluorescein introduced obstacles we could not overcome, we tried to circumvent this step and instead introduce an aldehyde precursor via an amide bond to the carboxylic acid. Upon formation of an amide bond with e.g. 3-amino-1,2-propanediol, the diol should then be easily converted into an aldehyde using sodium periodate (Van Brabandt,

Vanwalleghem, D'hooghe, & De Kimpe, 2006). Amide bonding of fluorescein is well described in the literature. However, for the three protocols we tried, we could never observe a reaction taking place with NMR and TLC and therefore abandoned the approach.

7.2.6 Modification of quinine

Our further quest for an appropriate, yet cheap and safe starting material led us to quinine. Quinine is a readily available compound rendered from the bark of the cinchona tree. It has a long history of being used as a drug in the treatment of malaria (Achan et al., 2011), but has also found widespread application as a bitter flavoring agent in tonic water.

Quinine does not contain an aldehyde group per se, but it posesses a terminal olefin, which can be converted into an aldehyde in numerous ways. A classic route is the oxidation of the double bond to a diol and the subsequent cleavage of diol to aldehyde.

Our approach was to oxidize quinine with potassium permanganate in an alkaline acetone solution according to Yanuka et al. (1981) and then cleave the diol with sodium periodate according to Van Brabandt (2006). However, the initial step of oxidation resulted in a mixture of compounds that did not give an aldehyde signal in NMR even after fractionation through column chromatography. The reaction could not be brought to fruition by varying either temperature or reaction time.

Having had to abandon this route, we further investigated ozonolysis as a one-step oxidation from olefin directly to the desired aldehyde. We resorted to Schiaffo and Dussault's method as a basis for our investigation (Schiaffo & Dussault, 2008). After various adjustments, we managed to produce quinine aldehyde *N*-oxide. We considered the formation of the *N*-oxide, which occurred concomitantly to the formation of the aldehyde, as beneficial, since it drastically increased the desired water-solubility of the compound and facilitated the purification and separation from triphenylphosphine and its reaction products.

Quinine aldehyde *N*-oxide could be further converted to the hydrazone as a precursor to the diazo label according to the FDAM protocol. However, oxidation of the hydrazone to the final diazo compound yielded a product that was too unstable to be characterized and resulted in the immediate formation of nor-quinine through elimination of nitrogen and the formation of a double bond towards C5. The reaction is detailed in Figure 7. Unfortunately, try as we might with lower temperatures and inert atmosphere, we could not find reaction conditions that initiated reduction of the hydrazone but stopped at the diazo compound.

The stability of diazo compounds depends on the substituent(s) of the carbon. Since the carbon is electron-rich, an electron-withdrawing substituent will increase stability (Regitz &

Maas, 1986). Therefore, an alkyl substituted diazo group, as in our case of quinine, will be less stable than most aryl substituted ones.

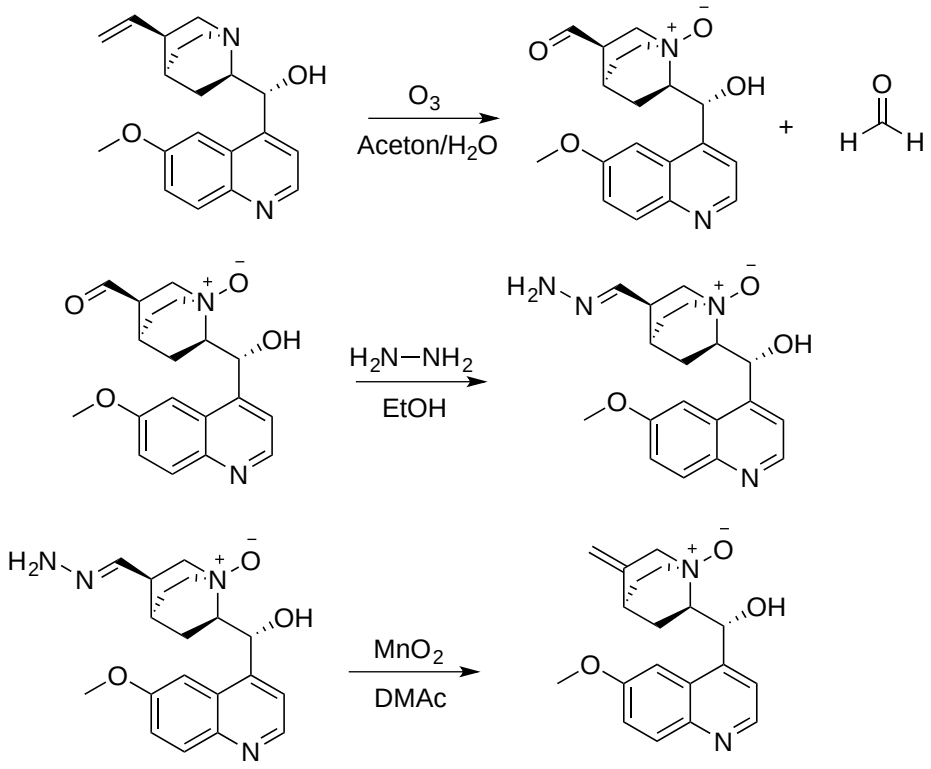


Figure 7. Reaction pathway for quinine

Having realized a technique that converts olefins into aldehydes, we mused over possible applications to arrive at an aromatic instead of an alkylic aldehyde and happened upon a paper that describes the *de novo* synthesis of a fluorescein derivative from resorcinol and glycerol carrying an aryl olefin in the form of a vinyl group in place of benzoic acid (Sen & Sarkar, 1925). The reaction is shown in Figure 8. However, the proposed procedure resulted in an unidentifiable mixture of fluorescent compounds and variation of reaction time and temperature did not amend the outcome. Yet again, we abandoned the approach.

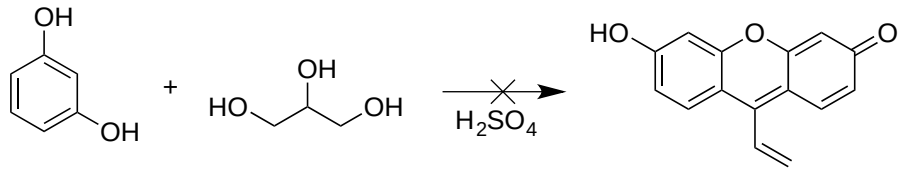


Figure 8. *De novo* synthesis of fluorescein derivative 9-ethenyl-6-hydroxy-3H-xanthen-3-one

7.2.7 Indole-3-carboxaldehyde

Indole-3-carboxaldehyde was another starting material with promising properties: polar, fluorescent, affordable and an aromatic aldehyde is already present. Yet, upon conversion to

the hydrazone, we observed near-quantitative dimerization as illustrated in Figure 9. Since this made impossible the follow-up reaction, we did not conduct further investigations.

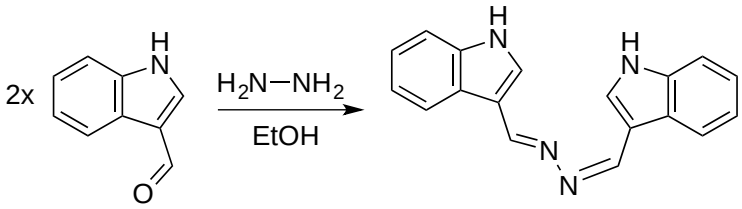


Figure 9. Dimerization of indole-3-carboxaldehyde upon conversion to the hydrazone

7.2.8 Pyridoxal

Pyridoxal is part of the water-soluble vitamin group B_6. Interestingly, it exhibits fluorescent properties despite its single-ring structure (Honikel & Madsen, 1972). As it is an aldehyde, no modification was necessary prior to the diazotation analogous to FDAM.

The hydrazone could be prepared from the free base in near-quantitative yield. Unfortunately, the backbone of pyridoxal did not prove stable against manganese dioxide and resulted in a mixture of degradation products.

Since we deemed the hydroxymethyl group in C5 to be the most susceptible element, we tried pyridoxal phosphate in hopes of better stability. However, the increased polarity due to the phosphate group rendered the compound insoluble in most organic solvents and only poorly soluble in acidic and neutral water. Alkaline aqueous conditions are required for complete dissolution. While conversion to the respective hydrazone could still be partially achieved in a heterogeneous reaction with hydrazine in ethanol, reduction with manganese dioxide could not be performed since it would have entailed a solid-solid reaction.

7.3 Conclusion

Despite having been unsuccessful at synthesizing the targeted labeling compound, we can draw some conclusions from our erroneous attempts. One, the presence or introduction of amino groups is undesirable, since it will eventually lead to the formation of Schiff bases with the formyl group. Two, even though the label will ultimately have to be water-soluble, too high a polarity can lead to solubility issues during the conversion from aldehyde to diazo compound as was observed for pyridoxal phosphate. Three, the diazo group has to be aryl-substituted or neighboring an electron-withdrawing group in order to be stable under labeling conditions. Our trials with quinine have shown that an alkyl-substituted diazo group decomposes rapidly upon formation.

There would be more compounds, synthetic pathways and optimization strategies to try and - we are sure of it - one of them would eventually be successful. But given the limited time frame of this work, we had to draw the line at some point. In believing that there are a thousand noes for every yes, we hope that this catalogue of failures inspires further research and helps point the vigilant chemist in the right direction.

7.4 Experimental

7.4.1 Diazotation analogous to FDAM

5.4 mmol of fluorene-2-carboxaldehyde (or the respective aldehyde-bearing compound) was dissolved in 110 mL of absolute ethanol. After the addition of 11 mL of a 1 M solution of hydrazine in THF (2 eq.), the mixture was refluxed for 30 minutes under inert atmosphere. Upon cooling, solvents were evaporated and the solid residue of the respective hydrazone was dissolved in 20 mL of dimethylacetamide. 5 g of manganese dioxide were suspended and stirred in the solution for 1 h at room temperature. Finally, the suspension was filtrated through a layer of Celite/magnesium sulfate 1/1 to yield deeply red FDAM. The reaction is shown in Figure 2. (Bohrn et al., 2006)

7.4.2 Amination of fluorene-2-carboxaldehyde

0.5 g of fluorene-2-carboxaldehyde were added slowly to a mixture of 2 mL of 99.5% nitric acid and 2.55 mL concentrated sulfuric acid cooled on ice. The reaction was stirred for 1.5 h at 0 °C and then stopped by the addition of 5 g of ice. The aqueous phase was extracted twice with an equal amount of dichloromethane. The pooled organic phases were washed twice with 20 mL of water and dried over magnesium sulfate before being evaporated to dryness. The residue was purified over a silica column with a 4:3 mixture of hexane and dichloromethane.

The subsequent reduction was performed in an H-Cube® continuous-flow hydrogenation reactor (ThalesNano, Budapest, Hungary) 1 mM in dichloromethane over a Raney Nickel catalyst with a flow of 0.3 mL/min at 2 bar and 40 °C.

7.4.3 Vanillin

Vanillin was converted to the respective diazo compound according to the protocol for FDAM (Bohrn et al., 2006) with the modification that the conversion of the hydrazone to the diazo compound was performed in ethyl acetate instead of dimethylacetamide due to solubility issues. The reaction yielded an intensely dark-yellow liquid indicating the formation

of a diazo compound. 1 mL of this product was used to label 10 mg of glucuronic acid as a model compound in 3 mL methanol for three days at 40 °C. The reaction was monitored with TLC (hexane/ethyl acetate 5:1 v/v).

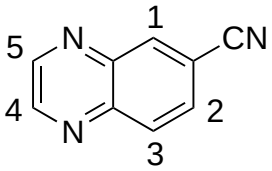
7.4.4 Synthesis of quinoxaline-6-carboxaldehyde

Quinoxaline-6-carbonitrile

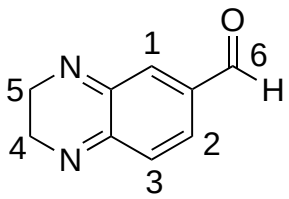
Quinoxaline-6-carbonitrile was synthesized from 3,4-diaminobenzonitrile and glyoxal according to (Ishikawa, Sugiyama, Kurita, & Yokoyama, 2012) with a few modifications: 7.0 mmol of 3,4-diaminobenzonitrile were refluxed for 2 h with 1.5 eq. of a 40% solution of glyoxal in 40 mL of methanol. The resulting suspension was mixed with 100 mL of water acidified to pH 1 and extracted three times with equal volumes of chloroform. The pooled organic phases were washed two times with equal volumes of neutral water and evaporated to yield quinoxaline-6-carbonitrile as a grey powder.

Quinoxaline-6-carboxaldehyde

Then, quinoxaline-6-carbonitrile was to be reduced to the corresponding aldehyde with diisobutylaluminium hydride (DIBAL-H). Since this reaction is quite sensitive and prone to overreduction, it was carried out in a tubular flow reactor for precise control of reaction time and temperature. A steel tube with 1 mm in diameter and 1 m in length was fitted to a T-piece and fed from two lines, a solution of quinoxaline-6-carbonitrile in chloroform and one of DIBAL-H in dichloromethane. The efflux was directed into a beaker of water in order to be quenched immediately. A design of experiment approach was taken to determine the most suitable parameters for temperature, reaction/dwell time, DIBAL-H equivalents and DIBAL-H concentration. We tested temperatures of 0 °C, 20 °C and 40 °C, dwell times of 10, 30 and 50 seconds, DIBAL-H equivalents of 1, 1.5 and 2 and DIBAL-H concentrations of 0.05 M and 0.5 M. The concentration of quinoxaline-6-carbonitrile was kept at 0.05 M throughout. Products, which were of yellow to brown color, were analyzed by TLC and NMR.



^{1}H-NMR ($CDCl_3$, 400 MHz): δ(ppm) = 8.98 (s, 2H, 4/5), 8.52 (d, J = 2 Hz, 1H, 1), 8.22 (d, J = 8.8 Hz, 1H, 3), 7.93 (dd, J_1 = 2 Hz, J_2 = 8.8 Hz, 1H, 2).



^{1}H-NMR ($CDCl_3$, 400 MHz): δ(ppm) = 9.63 (s, 1H, 6), 7.11 (dd, J_1 = 1.6 Hz, J_2 = 8.0 Hz, 1H, 2), 7.01 (d, J = 1.6 Hz, 1H, 1), 6.48 (d, J = 8.0 Hz, 1H, 3), 3.49 (m, 2H, 5), 3.41 (m, 2H, 4).

Quinoxaline-6-carboxylic acid

Quinoxaline-6-carboxylic acid was synthesized from 3,4-diaminobenzoic acid and glyoxal in the following way: 7.0 mmol of 3.4-diaminobenzoic acid were dissolved in 10 mL of 0.1 M sodium hydroxide. 2.25 equivalents of a 40% aqueous glyoxal solution were added dropwise to the solution and the mixture was stirred at room temperature for 10 h. Quinoxaline-6-carboxylic acid was precipitated by the addition of hydrochloric acid to a pH of 4, filtrated and washed with 0.1 M hydrochloric acid prior to being dried.

Quinoxaline-6-carboxaldehyde

Then, quinoxaline-6-carboxylic acid was to be reduced to the corresponding alcohol with lithium aluminum hydride and subsequently oxidized to quinoxaline-6-carboxaldehyde using Dess-Martin periodinane. The reduction was carried out according to (Takatori, Nishihara, Nishiyama, & Kajiwara, 1998): To 7.5 mmol lithium aluminum hydride suspended in 4 mL of dry THF was added dropwise a solution of 5 mmol quinoxaline-6-carboxylic acid in 20 mL of dry THF at 0 °C. The suspension was stirred at room temperature for 3 h before being quenched carefully with 1 mL of 40% sodium hydroxide at 0 °C. The precipitate was filtered off and washed with 100 mL of dichloromethane and 100 mL of ethanol. Combined filtrates were evaporated and analyzed by NMR and TLC (ethyl acetate/methanol 5:1 v/v).

7.4.5 Modification of fluorescein and Rhodamine B

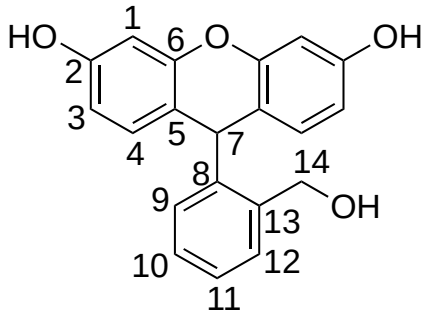
Fluorescein ethyl ester

Fluorescein exists in organic solvents in the lactone form. Therefore it was necessary to convert it to an alkyl ester prior to reduction. We performed the reaction according to (Adamczyk, Grote, & Moore, 1999): 30 mmol of fluorescein were suspended in 30 mL of absolute ethanol. 5 mL of concentrated sulfuric acid were added dropwise to the suspension. The mixture was refluxed for 48 h under inert atmosphere with a dripping funnel filled with

3 Å molecular sieve in between reflux condenser and reaction flask to trap water formed in the reaction. Then, the reaction was cooled to room temperature and 10 g of ice were added. The suspension was filtrated and washed with water until the filtrate was neutral. The residue was dried to yield fluorescein ethyl ester.

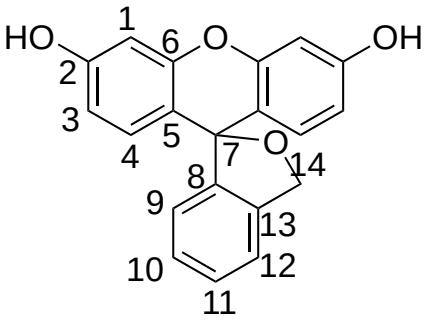
Reduction of fluorescein ethyl ester

The reduction of fluorescein ethyl ester to the respective alcohol and subsequent rearomatization of the xanthene ring structure was carried out according to Santra et al. (2010): 4.02 mL of a 1 M solution of DIBAL-H in dichloromethane were added dropwise to a solution of 1 mmol fluorescein ethyl ester in 20 mL THF over 15 min at -78 °C under nitrogen atmosphere. The resulting solution was stirred at this temperature for 10 min and then brought to room temperature. After stirring for 2 h, 5 mL of diethyl ether were added to the solution at 0 °C and then 3.5 mL of a saturated solution of ammonium chloride were added dropwise to the mixture at the same temperature. This mixture was brought to room temperature again and stirred for 1 h. Another 5 mL of diethyl ether and 1.1 mmol of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) were slowly added to the mixture at 0 °C. After stirring for 2 h at 25 °C, the mixture was filtered through a 2 cm thick layer of Celite, and the pad was rinsed with ethyl acetate. The filtrate was dried over sodium sulfate, filtrated, and evaporated to yield a red powder.



^{1}H-NMR (DMSO, 400 MHz): δ(ppm) = 9.49 (s, 2H, 2), 7.37 (m, 1H, 12), 7.12 (m, 2H, 10/11), 6.94 (m, 1H, 9), 6.79 (d, J = 8.7 Hz, 2H, 4), 6.48 (s, 2H, 1), 6.39 (d, J = 8.7 Hz, 2H, 3), 5.47 (s, 1H, 7), 5.22 (dd, J_1 = 5.1 Hz, J_2 = 5.1 Hz, 1H, 14-OH), 4.59 (d, J = 5.1 Hz, 1H, 14-CH_2).

^{13}C-NMR (DMSO, 400 MHz): δ(ppm) = 156.9 (s, 2), 150.9 (s, 6), 145.5 (s, 8), 138.6 (s, 13), 130.4 (s, 4), 130.1 (s, 9), 127.9 (s, 12), 127.6 (s, 10), 125.9 (s, 11), 115.1 (s, 5), 111.2 (s, 3), 102.3 (s, 1).



^{1}H-NMR (DMSO, 400 MHz): δ(ppm) = 9.76 (s, 2H, 2), 7.37 (m, 1H, 12), 7.12 (m, 2H, 10/11), 6.94 (m, 1H, 9), 6.71 (d, J = 8.5 Hz, 2H, 4), 6.55 (d, J = 2.4 Hz, 2H, 1), 6.51 (dd, J_1 = 8.5 Hz, J_2 = 2.4 Hz, 2H, 3), 5.19 (s, 2H, 14-CH_2).

^{13}C-NMR was not sensitive enough to detect the small amounts.

Rearomatization with elemental sulfur

Rearomatization with elemental sulfur was performed according to (Nguyen et al., 2012): 50 mg of fluorescein alcohol were mixed with 30 mg of elemental sulfur and heated under vacuum and solvent-free conditions to 160 °C for 30 min. Upon cooling, 5 mL of ethanol and 0.4 mL of 37% hydrochloric acid were added prior to 10 mL of water. The mixture was extracted three times with 100 mL of chloroform. Pooled organic phases are evaporated to yield a black solid that showed too complex a mixture in NMR and TLC to be purified further.

Rearomatization with iodine

Rearomatization with iodine was done according to (Gogoi & Konwar, 2005): 1 mmol of fluorescein alcohol were dissolved with 1.5 mmol of potassium carbonate, 0.5 mmol of potassium iodide and 2 mmol of iodine in 4 mL of water containing 30% acetonitrile. The solution was heated to 90 °C for 20 min. After cooling to room temperature, the mixture was extracted three times with equal volumes of ethyl acetate. Organic phases were pooled and evaporated to yield a brown solid that showed too complex a mixture in NMR and TLC to be purified further.

Amide formation from fluorescein and diethylamine

Three protocols, starting from either the lactone or the ethyl ester of fluorescein and diethylamine as a simple model compound were tried to achieve the formation of an amide bond.

One: 0.1 mmol of fluorescein were dissolved in 3 mL dimethylformamide (DMF). 0.14 mmol of *N*-hydroxysuccinimide as well as 0.14 mmol of *N,N'*-dicyclohexylcarbodiimide

were added and the mixture stirred at 50 °C for 2 h. The mixture was let cool down to room temperature before 0.15 mmol of diethylamine were added and the reaction was stirred at room temperature for 12 h (Redy, Kisin-Finfer, Sella, & Shabat, 2012). TLC was used to monitor the reaction (hexane/ethyl acetate 1:1 v/v).

Two: 56 mg of potassium *tert*-butoxid were dissolved in 3 mL THF. 0.25 mmol of fluorescein ethyl ester as well as 0.25 mmol of diethylamine were added to the mixture. The reaction was stirred at room temperature for 24 h and periodically checked with the same TLC system (Kim et al., 2012).

Three: 2 mmol of fluorescein were dissolved in 15 mL dioxane. 24 mmol of *p*-nitrophenol followed by 4 mmol of *N,N'*-dicyclohexylcarbodiimide and 0.1 mmol of 4-dimethylaminopyridine were added under stirring at room temperature. The solution was stirred for 2 h at the same temperature prior to the addition of 2 mmol of diethylamine and another 4 mmol of *N,N'*-dicyclohexylcarbodiimide. The reaction was stirred at room temperature for 24 h and periodically checked with the same TLC system (Shukla, Mishra, Watal, & Misra, 2005).

7.4.6 Modification of quinine

Permanganate oxidation of quinine

We set out to oxidize quinine according to Yanuka et al. (1981) and then cleave the diol according to Van Brabandt (2006). The oxidation was conducted as follows:

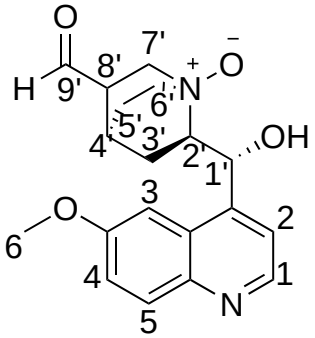
1 mmol of quinine and 2.1 mmol of potassium hydroxide were dissolved in 20 mL of water containing 30% acetone and cooled to 0 °C. A solution of 1 mmol potassium permanganate in 10 mL water was added dropwise under stirring. The mixture was stirred for 30 minutes while warming up to room temperature. Precipitated manganese dioxide was filtrated off and the filtrate was stirred with 5 g of Amberlite IRN-77 cation exchange resin for 2 h. The resin was filtrated off and solvents were evaporated from the filtrate to yield a dark brown solid, which was analyzed by TLC (ethyl acetate/ethanol 9:1 v/v) and NMR spectroscopy.

Ozonolysis of quinine

Quinine aldehyde *N*-oxide was produced according to a variation of the protocol by Schiaffo and Dussault (Schiaffo & Dussault, 2008):

0.71 mmol of quinine were dissolved in 200 mL of acetone containing 5% water. A few drops of Sudan III in acetone were added as an indicator to give a slightly red color and the solution was cooled to -10 °C in an ice bath with sodium chloride. A BBC ozone generator

(BBC Brown Boveri & Company, Baden, Switzerland) fed by an oxygen stream was used to bubble ozone through the solution via a glass frit under stirring. The generator was set to 0.1 A and a gas flow of 0.15 L/min was passed through the solution until the red color of Sudan III began to fade (about 40 min). Generation of ozone was stopped, but the flow of oxygen was continued for another five minutes to drive out remaining ozone, upon which the red color faded completely and a white precipitate of quinine aldehyde *N*-oxide formed. Residual peroxides were quenched by adding 200 mg of triphenylphosphine and stirring the solution for 1 h at room temperature. A peroxide test was performed with potassium iodide and starch. Then, water was added until the precipitate dissolved and acetone was evaporated from the solution under vacuum. The watery phase was extracted thrice with equal volumes of ethyl acetate to remove triphenylphosphine and triphenylphosphine oxide and evaporated to dryness to yield quinine aldehyde *N*-oxide.



^{1}H-NMR (DMSO, 400 MHz): δ(ppm) = 9.51 (s, 1H, 9'), 8.69 (d, J = 4.3 Hz, 1H, 1), 7.84 (d, J = 2.7 Hz Hz, 1H, 5), 7.60 (d, J = 4.3 Hz, 1H, 2), 7.58 (s, 1H, 3), 7.18 (d, J = 2.7 Hz, 4), 6.88 (s, 1H, 1'), 4.18 (m, 2H, 6'), 3.69 (dd, J_1 = 6.2 Hz, J_2 = 6.1 Hz, 2H, 7'), 3.36 (m, 1H, 2'), 3.21 (m, 3H, 6), 3.14 (m, 1H, 8'), 2.49 (m, 1H, 4'), 2.29 (m, 2H, 3'), 1.95 (m, 2H, 5').

The preparation of the respective diazo compound was done according to the FDAM protocol. The resulting nor-quinine *N*-oxide was identified by the disappearance of an aldehyde signal and the appearance of two singlets at 4.95 ppm and 4.72 ppm in ^{1}H-NMR, which were found to long-range couple to the 4' and 7' position in both COSY and HMBC 2D NMR experiments, which strongly suggested the formation of a terminal olefin.

De novo synsthesis of fluorescein derivative

The *de novo* synthesis of the fluorescein derivative 9-ethenyl-6-hydroxy-3H-xanthen-3-one as a precursor for ozonolysis was carried out according to (Sen & Sarkar, 1925):

5.0 mmol of glycerol and 10.0 mmol of resorcinol were dissolved in 5 mL of concentrated sulfuric acid and heated to 150 °C for 5 h. Upon cooling, 10 mL of ice-water was slowly

added to the mixture. The resulting suspension was filtrated and the retentate was washed with water until the filtrate was neutral. A black mixture of compounds was obtained that could not be characterized further with TLC and NMR.

7.4.7 Indole-3-carboxaldehyde

The preparation of indole-3-diazomethane from Indole-3-carboxaldehyde was performed according to the FDAM protocol.

7.4.8 Pyridoxal

Pyridoxal was purchased as the respective hydrochloride and therefore had to be stirred in absolute ethanol for 2 h at 50 °C under inert atmosphere with an equimolar amount of sodium bicarbonate in order to liberate the free base and be able to dissolve in ethanol. The precipitated salt was filtered off and the reaction was continued according to the FDAM protocol.

Pyridoxal phosphate did not dissolve in absolute ethanol, but was still reacted as a suspension as described in the FDAM protocol.

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8 Curriculum vitae

Personal information

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Work experience

01/2012 - 12/2015	Scientific staff and PhD thesis “Advanced analytical strategies for in depth characterisation of wheat bran fractions” Institute: Department of Chemistry and Department of Food Science within a Christian Doppler Laboratory for Innovative Bran Biorefinery at BOKU University, Vienna
04/2010 - 12/2011	Quality assurance for the home banking app iOutBank for iPhone, iPad and Mac at stoeger it GmbH, Dachau, Bavaria
03/2006 - 08/2006	Tutor for German, English, math and physics at “Best for Kids”, Schrobenhausen, Bavaria
07/2005 - 03/2006	Civil service in care at the nursing home of Kühbach, Bavaria

Technical skills

Methods	Chromatography (GC-MS/FID, HPLC, GPC, TLC), spectroscopy (NMR, NIR/MIR), microscopy (confocal-, fluorescence-, scanning-electron-, bright-field-), organic synthesis, realtime-qPCR, cell culture, anti-body labelling
Software	MS Office, Apple iWork, Origin Pro, Adobe Photoshop, Final Cut Pro

Studies

10/2006 - 04/2011	Food chemistry at Technical University of Munich (degree: 1st state examination, grade 1.6)
10/2010 - 04/2011	Final Thesis “Effect-structure correlation of ingredients of selected alcoholic beverages on the function and mechanism of acid secretion in human gastric cells” Institute: Institute for Nutritional and Physiological Chemistry at University of Vienna

International experience

- 13th European Workshop on Lignocellulosics and Pulp (EWLP), 2014, Seville, Spain
- COST Workshop on Valorisation of Vegetable Waste, 2014 Novi Sad, Serbia
- Training School Food Waste Processing in the Frame of a Biorefinery Concept, 2014, Lisbon, Portugal
- Final conference of COST Action FP0901, 2013, Turku, Finland
- Short-term scientific mission through COST Action FP0901, 2013, Rotorua, New Zealand
- Summer School in Biorefinery and Biocomposite Analysis, 2012, Åland, Finland
- 5th International Dietary Fiber Conference, 2012, Rome, Italy

Language skills

English - Fluent, working language, C1; Latin - Advanced proficiency certificate; Italian - Basic skills; Serbian - Basic skills (currently improving)

Scientific record

Hell, J., Prückler, M., Danner, L., Henniges, U., Apprich, S., Rosenau, T., Kneifel, W., & Böhmdorfer, S. (2015). A comparison between near-infrared (NIR) and mid-infrared (ATR-FT-IR) spectroscopy for the multivariate determination of compositional properties in wheat bran samples. *Food Control* (Accepted for publication).

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Hobbies

Movie projects; writing short stories; sports (boxing, basketball, tennis); music (violin, piano, guitar)