Formation and Ecotoxicity of N-Heterocyclic Compounds on Ammoxidation of Mono- and Polysaccharides

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ABSTRACT: Ammoxidation of technical lignins under mild conditions is a suitable approach to artificial humic substances. However, carbohydrates as common minor constituents of technical lignins have been demonstrated to be a potential source of N-heterocyclic ecotoxic compounds. Ethyl acetate extracts of ammoxidation mixtures of the monosaccharides glucose and xylose exhibited considerable growth inhibiting activity in the OECD 201 test, with 4-methyl-1H-imidazole, 4-(hydroxymethyl)-1H-imidazole, and 3-hydroxyypyridine being the most active compounds. The amount of N-heterocyclic compounds formed at moderate ammoxidation conditions (70 °C, 0.2 MPa O₂, 3 h) was significantly lower for the polysaccharides cellulose and xylan (16–30 μg/g of educt) compared to glucose (15.4 mg). Ammoxidation at higher temperature is not recommendable for carbohydrate-rich materials as much higher amounts of N-heterocyclic compounds were formed from both monosaccharides (100 °C: 122.4–160.5 mg/g of educt) and polysaccharides (140 °C: 5.52–16.03 mg/g of educt).

KEYWORDS: ammoxidation, Maillard reaction, lignin, N-modified lignin, artificial humic substances

INTRODUCTION

Ammox (or “ammox”), that is the simultaneous treatment of a given organic substrate with ammonia and oxygen, has been established and advanced as an appropriate method to convert technical, virtually nitrogen-free lignins into nitrogenous soil-improving materials. In particular lignins ammoxidized under comparatively “mild” reaction conditions (T ≤ 100 °C; P O₂ ≤ 0.2 MPa; 5% aqueous ammonia) have been demonstrated to share similarities with natural humic substances. They act as soil amendments with slowly nitrogen releasing, water storing, ion exchanging, and texture improving properties, and are hence beneficial for plant growth. Positive effects of ammoxidized lignins on the growth of agricultural crops or trees have been reported, such as in forest plant breeding, ornamental horticulture, or rehabilitation of degraded soils.

However, there are still some uncertainties with regard to the application of ammoxidized lignocellulosic materials in soil improvement and crop cultivation. Besides insufficient knowledge about different types of nitrogen bonding and the resulting fertilizing properties, the major concern is about possible formation of ecotoxic, carbohydrate-derived nitrogenous compounds under ammoxidation conditions.

The reaction of mono-, oligo-, and polysaccharides under aqueous alkaline conditions in general, and of sugars with ammonia in Maillard-type reactions in particular, have been comprehensively studied in the past decades. Base-catalyzed degradation of cellulose and monosaccharides was reviewed by Knill and Kennedy, while Fay and Bernard gave a more recent overview of the Maillard reaction. To the best of our knowledge, no study investigated the types of nitrogenous products obtained by a joint treatment of carbohydrates with aqueous ammonia and oxygen, even though a considerable number of papers on technical aspects of biomass ammoxidation and ammoxidative pretreatments (ammonia recycle pretreatment, ARP) have been published.

This paper investigates the reaction behavior of cellulose, xylan, D-glucose and D-xylose under ammoxidative conditions at different temperatures (70 °C, 100 °C, 140 °C), oxygen pressures (0.2 MPa, 1.0 MPa), but constant initial ammonia concentration (5%, w/v) and pH of about 11.9. In addition to the weight of the product fractions (solid phase, aqueous phase, ethyl acetate extracts), the composition of the different phases was studied by GC/MS, in part after derivatization. Authentic samples of the main identified N-heterocyclic reaction products were synthesized. These pure compounds were used in ecotoxicity studies with regard to growth inhibition of the fresh-water algae Pseudokirchneriella subcapitata (OECD ecotoxic test 201), and as analytical standards in GCMS. As phytotoxicity correlated mainly with the content of monosaccharides, gel permeation chromatography was additionally used to study the degradation of polysaccharides under ammoxidation conditions.
MATERIALS AND METHODS

Beech xylan (97.4% xylose, 0.8% glucose, 0.2% mannose, 0.2% rhamnose, 0.2% cellulose, 1.1% 4-O-Me-glucuronic acid; MW 10.210 kg/mol) was provided by Lenzing AG (Lenzing, Austria). All other chemicals, including cellulose (acid-washed and milled cotton linters for column chromatography), were purchased from Sigma-Aldrich Handels GmbH (Vieenna, Austria) in the highest purity available, and were used, unless otherwise noted, without further purification. Ethyl acetate (EtOAc) was dried over anhydrous calcium chloride, distilled once with acetic anhydride and sulfuric acid, and subsequently over potassium carbonate in order to remove water, ethanol and acetic acid, respectively. Pyridine and ethyl acetate for derivatization were stored over molecular sieves, and filtered through a 0.45 μm syringe filter prior to use.

Ammoxidation. was accomplished in a 100 mL 4566 C Series laboratory-scale pressure vessel (Parr Instruments, Frankfurt, Germany) equipped with a glass liner and a septum valve, and heated with an oil bath. Monosaccharides: 10 mL of 10% aqueous ammonia were placed inside the reactor. After flushing three times with oxygen, the reactor was closed and heated under continuous stirring to the respective temperature and pressure for 3 h, the reaction vessel was pressurized with 0.2 and 1 MPa of O2, respectively, and left cooling to room temperature, 900 °C, 100 or 140 °C, 1.39 MPa (100 °C) and 1.80 MPa (140 °C). Polysaccharides: Deviating from the above-described procedure, a suspension of the respective polysaccharide in 10 mL of water was first pretempered in the reactor before the reaction was initiated by addition of 10 mL of 10% aqueous ammonia.

Workup Procedures. After stirring the reaction mixtures at the respective temperature and pressure for 3 h, the reaction vessel was cooled and depressurized. Workup procedure prior to optimization: The reaction mixture was extracted three times with 20 mL ethyl acetate. The combined extracts were dried over Na2SO4 and evaporated under reduced pressure to dryness. Optimized workup procedure: The reaction mixture was evaporated at 3.5 kPa (35 °C) to remove ammonia, solids were separated by centrifugation, washed with water, and freeze-dried for SEC and elemental analysis. The pH value was adjusted to 8.5 using 2% aqueous ammonia in order to ensure complete deprotonation of imidazole and pyrazine functionalities. Higher concentrations of ammonia should be avoided in order to prevent acetamide formation from ethyl acetate during extraction. After filling up to 20 mL, the aqueous phase was extracted four times with 100 mL of water-saturated ethyl acetate. The combined organic phases were evaporated to approximately 1/10 of their volume under reduced pressure, whereupon the water was removed by azotropic distillation. The residue was redissolved in 10 mL of dry EtOAc and evaporated at 35 °C (9 kPa) affording a yellowish oil. Aliquots of the extract were used for GC/MS analyses, and for phytotoxicity testing. The aqueous phase was freeze-dried and weighed.

Silylation. An aliquot containing approximately 1 mg of dry matter was lyophilized overnight at −25 °C and 10−20 Pa. Then, 200 μL of dry pyridine, containing 1.5 g/L of the catalyst 4-(N,N-dimethylamino)pyridine (DMAP), and 10 μL of a 10 g/L solution of the internal standard phenyl α-glucopyranoside in pyridine were added. The mixture was vortexed for a minimum of 30 s, before 100 μL of the silylation mixture (BSTFA containing 10% trimethylchlorosilane) was added. Silylation was performed at 70 °C for 2 h. After cooling to room temperature, 900 μL of dry ethyl acetate were added, the mixture was vortexed and analyzed by GC/MS within 24 h. For reference spectra of silylated compounds, a 200 μg aliquot of the respective substance was subjected to the above procedure.

Synthesis of Authentic Samples. (5-Methyl-pyrazin-2-yl)-methanol. Mild oxidation of 2,5-dimethylpyrazine (2.16 g, 20 mmol) with N-chlorosuccinimide (3.19 g, 23.9 mmol) and dibenzoylperoxide (123.6 mg, 0.68 mmol) in 50 mL of CCl4 as described elsewhere.15 Yield after purification by column chromatography: 936 mg (38%) and 143 mg (5.1%) of the byproduct 2,5-bis[(hydroxymethyl)pyrazine]. The product was stored in a dark place at 4 °C.

1H NMR (CDCl3): δ 2.59 (s, 3H, CH3), 3.16 (s, 1H, OH), 4.18 (s, 2H, CH2OH), 8.43 (s, 1H, pyrazine-H6), 8.53 (s, 1H, pyrazine-H3).
13C NMR (CDCl3): δ 21.1 (CH3), 62.6 (CH2OH), 141.6 (C3), 143.1 (C6), 152.2 (C5), 151.6 (C5).

2-(Arabino-tetrahydroxybutyl)-5-(erythro-2,3,4-trihydroxybutyl)pyrazine (Deoxyfructosazine). Synthesis by portionwise addition of D-glucosamine hydrochloride (2.16 g, 10 mmol) to a solution of 3.05 g (25 mmol) of phenylboronic acid in aqueous sodium hydroxide as described elsewhere.16 Yield: 1.31 g (86%).

1H NMR (DMSO-d6): δ 2.71 (1H, dd, CH3, J = 13.8 Hz, 9.3 Hz), 3.04 (1H, dd, CH2, J = 2.5 Hz, 13.8 Hz), 3.36 − 3.45 (3 μ, 3H, J = 6.3, 3.63 μ, 3H, J = 3.72 − 3.77 (1H, 1H), 4.45 and 4.68 (7H, br, OH), 4.92 (1H, J = 3.4, tetrahydroxybutyl-H1), 8.38 (1H, J = 1.2, 8.61 (1H, J = 1.2). 13C NMR (DMSO-d6): δ 18.97 (CH3), 56.42, 63.62, 63.98, 71.68, 71.74, 74.24, 75.38 (7C, CH2O), 142.70, 143.67 (2C, CH3), 153.62, 156.15 (2C, C=C).

4-(Arabino-tetrahydroxybutyl)-imidazole. Synthesis by condensation of D-fructose (5.40 g, 30 mmol), formamide acetate (5.03 g, 36 mmol) and liquid ammonia (20 mL) at 75 °C using a Parr 4566 C pressure reactor.15 Yield: 2.04 g (36%).

1H NMR (CD3OD): δ 3.60 − 3.66 (1H, m, CH2OH), 3.68−3.75 (2H, dd, tetrahydroxybutyl-H2/H3), 3.77−3.82 (1H, m, CH2OH), 4.97 (1H, tetrahydroxybutyl-H1, d, J = 1.7 Hz), 7.04 (1H, s, imidazole-H5), 7.61 (1H, d, imidazole-H2, J = 1.0 Hz). 13C NMR (DMSO-d6): δ 64.8 (tetrahydroxybutyl-C4), 68.0 (tetrahydroxybutyl-C1), 73.1 and 75.67 (tetrahydroxybutyl-C2/3), 136.0 (imidazole-C2).

Instrumental Analysis. NMR spectra were recorded on a Bruker Avance II 400 (resonance frequencies 400.13 MHz for 1H and 100.63 MHz for 13C) equipped with a 5 μm observe broadband probe head (BBFO). Standard Bruker pulse programs were used.

GC-MS was performed on a GC 6890N/MSD 5975B instrument equipped with a 30 m x 0.25 mm i.d., 25 μm, capillary HP-5 ms column (Agilent, Germany). Helium total flow was 27.5 mL/min at 46.9 kPa; the column flow was 0.9 mL/min. Temperature program: 50 °C (2 min), 5 °C/min to 280 °C (20 min). 0.2 μL aliquots of the dissolved samples were injected at 230 °C inlet temperature in splitless mode, or with a 1:2 split, depending on the concentration. Ionization was performed in El mode at 70 eV. Data were acquired and processed with the Agilent Chemstation E02.02.1431 software package. Unless otherwise noted, tentative peak assignment was accomplished by comparison with the NIST MS library, version 2.06.

Size exclusion chromatography (SEC): Dionex DG-2410 online degasser; Kontron 420 pump with pulse damper; HP 1100 auto sampler; Gynkotek STH 585 column oven; Shodex RI-71 refractive index and a Wyatt Dawn DSP and a Wyatt DAWN DSP, 1488 nm laser multiple-angle light scattering detector. Data evaluation: ASTRA and GRAMS/2 software. Separation was accomplished using four 300 mm × 7.5 mm i.d., 20 μm PL gel mixed A LS columns (Agilent, Germany), an injection volume of 100 μL, eluent flow of 1.00 mL/min and run time of 45 min. Mobile phase: N,N-dimethylacetamide/lithium chloride (0.9% v/v), filtered through a 0.2 μm filter.

Activation of cellulose: 20 mg of dry cellulose was subsequently soaked in a large excess of deionized water, ethanol, and N,N-dimethylacetamide and left in the latter under shaking overnight. After filtration, 2 mL of N,N-dimethylacetamide/LiCl 9% (v/w) was added and the sample was left for dissolution on a vertical shaker overnight. Prior to measurement, 0.3 mL of the respective samples were diluted with 0.9 mL of N,N-dimethylacetamide and filtered. Carboxyl and carboxyl group contents were determined after labeling with the fluorescent markers carbazole-9-carboxylic acid [2-(2-amino-oxyethoxy)ethoxy]amid [CCOA; carboxyl groups]16 and 2-diazotio methyl fluorene (FDAM; carboxyl groups), respectively.17

Elemental analyses were performed at the Microanalytical Lab of the University of Vienna.
per-trimethylsilylation. Dichromate was used as a positive reference. All measurements (all
Aqueous Phases, and Water-Insoluble Fraction
Table 2. Amounts of Ammoxidation Products (mg Product/100 mg of Educt) in the EtOAc Extracts (Optimized Protocol),

Table 1. Summary of Nitrogenous Compounds Present in Ammoxidized D-Glucose, D-Xylose, Cellulose, and Xylan

ammonia salts of
acids glycolic acid (36), glycric acid (42), erythronic acid (49), threonic acid (50), ribonic acid (56), arabinonic acid (57), mannoic acid (66), gluconic acid (66), lactic acid (35), 3-hydroxypropanoic acid (38), 5,4-dihydroxybutyric acid (46), 2-deoxypentonic acid (53), 3-deoxyhexonic acid (60)

amino acids glycine (41), alanomalic acid (47), alanine (74), serine (75)
amino sugars glucosamine (62), fructosamine (65), di(glucopyranosyl)amine (73), aminohexopyranoside and aminoglycosides of unknown constitution (69), amides lactamide (6), glycolamide (7), urea (10), formamide (32), oxamic acid (39), acetamide (86)
imidazoles 1H-imidazole (2), 4-methyl-1H-imidazole (5), 4-hydroxymethyl-1H-imidazole (43), 4-(1,2-dihydroxyethyl)-1H-imidazole (55), 4-(o-arabino- tetrahydroxybutyl)-1H-imidazole (68), 2-acetyl-4-(tetrahydroxybutyl)-1H-imidazole (77)
pyridines 3-hydroxypyridine (4), 2-hydroxymethylpyridin-5-ol (20)
pyrazines 2-pyrazinol (1), 2-hydroxy-5-(dihydroxyethyl)-5-methyl-pyrazine (3), 2-pyrazinyl-methanol (7), 2-hydroxy-3-methyl-pyrazine (8), 2-hydroxymethyl-6-methyl-pyrazine (12), 2-(dihydroxyethyl)-pyrazine (18), 2-(dihydroxyethyl)-5-methyl-pyrazine (21), 2, 5- and 2,6-(dihydroxyethyl)-pyrazine (22), 2-(dihydroxy-ethyl)-5-(hydroxymethyl)-pyrazine (23), 2-(tetrahydroxybutyl)-pyrazine (24), 2-(tetrahydroxybutyl)-5-methyl-pyrazine (25), 2-(tribhydroxypropyl)-pyrazine (28), 2-(tribhydroxybutyl)-5-methyl-pyrazine (29), 2-hydroxy-6-(dihydroxyethyl) pyrazine (79), 2, 5- and 2,6-deoxy-fructosazine (84), 2,5- and 2,6-fructosazine (85), and 7 further pyrazine derivatives

“Numbers in parentheses refer to peak assignments for Figures 1 and 4.

Table 2. Amounts of Ammoxidation Products (mg Product/100 mg of Educt) in the EtOAc Extracts (Optimized Protocol),

| EtOAc extract | water soluble | water insoluble | ammoxidation conditions | EtOAc extract | water soluble | water insoluble |
|---------------|---------------|----------------|------------------------|---------------|---------------|----------------|
| Glucose       |               |                |                        |               |               |                |
| 0             | 104           | 0              | RT, ambient pressure   | 0.1           | 2             | 93.7           |
| 1.8           | 67.5          | 0              | 70 °C                  | 0.4           | 2.1           | 89.6           |
| 14.0          | 77.2          | 0.6            | 100 °C                 | 0.9           | 2.1           | 91.9           |
| 13.0          | 65.6          | 6.8            | 140 °C                 | 1.2           | 9.8           | 89.9           |
| Xylose        |               |                |                        |               |               |                |
| 0             | 71.0          | 0              | RT, ambient pressure   | 0.1           | 39.0          | 59.1           |
| 3.4           | 91.1          | 0              | 70 °C                  | 0             | 77.4          | 11.7           |
| n.d.          | 82.5          | 0              | 100 °C                 | 0.2           | 26.3          | 68.5           |
| 17.7          | 71.9          | 2.3            | 140 °C                 | 3.9           | 43.5          | 41.4           |

“Aqueous ammonia (5 w%), 0.2 MPa oxygen partial pressure, 3 h of reaction time.

Ecotoxicity Screening. Dilution series (dilution factors 1, 2, 4, 8, 10, 16, 32, 64, 100, 256, 1000, 1024, and 10 000) of all studied compounds were prepared from respective stock solutions containing 10 mg of sample in 20 mL of water, adjusted to pH 7.0 ± 0.3 with 1 M NaOH. Two mL of this solution was transferred to well plates, inoculated with the exponentially growing test organisms Pseudokirchneriella subcapitata (OECD test 201, approximately 20,000 cells per well) and cultivated for 72 h under following conditions: Illuminated shaker, 6,500–7,000 lx, day/night = 16h/8h, 22 °C. Potassium dichromate was used as a positive reference. All measurements (all
concentration levels of samples and reference, blank) were performed in triplicate. Photometric measurements at 485 nm were performed at 0, 24, 48, and 72 h of incubation time.

Quantitation was based on the areas of the growth curves that can be calculated according to the following equations where M_i is the extinction at the time of the n-th measuring t_n, and A the integral of the growth curve. Inhibition was calculated from the integrals of the growth curves of both the blank (A_b) and that of the sample (A_s).

\[ A_1 = (M_b - M_s) t_{1/2} \]
Ammonoxidation of both monosaccharides (D-glucose, D-xylan) and polysaccharides (cellulose, xylan) at 70–140 °C (0.2–1.0 MPa O₂) afforded a broad spectrum of lower-molecular compounds with distinctly higher total amounts in the case of the monosaccharides as compared to polysaccharides, especially at lower ammonoxidation temperature. GC/MS analysis revealed that N-heterocyclic compounds of low H₂O/EtOAc partition coefficients (log P ≈ 0) were the main constituents of the organic phases (Figure 2). Therefore, the extraction protocol was optimized towards virtually quantitative (≥97%) extraction of the key compound 4-methyl-1H-imidazole. Following this approach a significantly higher yield of extractives was isolated, indicating only a very minor extent of oxidative damage along the chain as well as little participation in reactions with ammonia. A significant order of magnitude higher for the monosaccharides than for the polysaccharides ammonoxidized under comparable conditions. The two monosaccharides afforded nearly the same amount of extractives, with xylose giving slightly higher yields. The amount of extractives from xylan was generally higher than that obtained from cellulose.

The fraction of moderately polar to nonpolar compounds that was extractable with EtOAc increased with the ammonoxidation temperature for all studied substrates, and ranged about 14–17% for the monosaccharides and 1.2–3.9% for the polysaccharides at 140 °C, relative to the mass of the respective starting material. The amount of dark-colored, water-insoluble products increased correspondingly with temperature, which is indicative of polymerization reactions as shown previously. Cellulose was increasingly degraded upon ammonoxidation beyond 100 °C, which is evident from the amounts of water-soluble products that increased 4-fold when raising the reaction temperature from 100 to 140 °C. For xylan, which was partly soluble in aqueous ammonia (39% relative to the amount of educt) due to its glucuronic acid side groups, the weight of the water-soluble product fraction increased when raising the ammonoxidation temperature from room temperature to 70 °C (77.4 mg/100 mg xylan). The water solubility was lower at 100 °C (26.3 mg/100 mg xylan) which is assumed to be due to a loss of glucuronic acid groups. At 140 °C, the fraction of water-soluble products increased up to 43.5%, and the optical appearance changed from white fibrous flakes (70 °C) to brown syrup, suggesting that decomposition of the xylan was so pronounced that water-soluble products of low molecular weight formed.

Compared to the influence of temperature during ammonoxidation on the weight of the different fractions, the effect of oxygen pressure was rather small. At least for xylose and xylan (140 °C), a higher oxygen pressure favors the formation of water-soluble products (likely by generation of carboxyl groups) at the expense of water-insoluble ones, whereas the amount of compounds extractable with ethyl acetate remained at the same level.

**Size Exclusion Chromatography.** The degradation of polysaccharides under ammonoxidation conditions was studied for cellulose. Size exclusion chromatography—multilayer light scattering (SEC-MALLS) in combination with fluorescence labeling of carbonyl and carboxyl groups provides additional information on the extent and nature of alkaline degradation in presence of ammonia and oxygen. The total amount of carboxyls and carboxyls was measured according to the CCOA and FDAM labeling approaches, respectively, and the theoretical number of reducing end groups (REGs) was calculated from the number-average molecular weight (Mn) obtained through SEC-MALLS.

SEC-MALLS analysis of the studied cellulose (cotton linters, Mₚ = 153 kg/mol) prior to and after ammonoxidation confirmed that the increasing amounts of EtOAc-soluble and water-soluble compounds formed at higher temperature correlated with the obtained loss in molar mass of cellulose (Figure 3). However, at moderate ammonoxidation temperature of 70 °C, the chemical integrity of cotton linters is little compromised (Mₚ = 139 kg/mol). The amount of carbonyls and the difference between total carboxyls and REGs (equates to the content of along-chain carbonyls) remains unaffected, indicating only a very minor extent of oxidative damage along the chain as well as little participation in reactions with ammonia. A significant

$$A_2 = (M_1 + M_2 - (2 \cdot M_0)) \left( \frac{t_2 - t_3}{t_2 - t_3} \right) \frac{t_3}{2}$$

$$A_3 = (M_2 + M_3 - (2 \cdot M_0)) \left( \frac{t_1 - t_2}{t_1 - t_3} \right) \frac{t_3}{2}$$

$$A = A_1 + A_2 + A_3$$

$$\text{inhibition} [\%] = \left( \frac{A_B - A_1}{A_B} \right) \cdot 100$$

**RESULTS AND DISCUSSION**

Chromatograms of per-trimethylsilylated ethyl acetate extracts of the reaction mixtures obtained after ammonoxidation (100 °C, 0.2 MPa O₂, 3 h) of D-glucose (A) and D-xylose (B). For peak assignment see Table 1.

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**Figure 2.** Chromatograms of per-trimethylsilylated ethyl acetate extracts of the reaction mixtures obtained after ammonoxidation (100 °C, 0.2 MPa O₂, 3 h) of D-glucose (A) and D-xylose (B). For peak assignment see Table 1.
The ethyl acetate extracts were analyzed by GC/MS after per-trimethylsilylation, which is considered to be one of the most versatile derivatization methods. However, the number of carbonyls increased only slightly. At this temperature, alkaline peeling/stopping commence and fast alkali-induced chain scission (beta-elimination) play a major role. Along-chain carbonyls are immediately consumed by the latter processes, so that their content can be assumed to be nearly zero. Thus, the amount of carbonyls measured is approximately equal to the amount of REGs available for labeling. This amount is smaller than the theoretical amount derived from Mn, as many functionalities of the ethyl acetate extracts of ammoxidized D-glucose and D-xylose, even though some of the constituents are known to be formed in Maillard-type reactions,25−28 were not detected. As such compounds are sensitive to oxidative degradation of $M_w$ was observed with increasing temperature (100 °C) and oxygen pressure, as expected. At 140 °C, cellulose was severely degraded ($M_w$ = 43 kg/mol). However, the number of carbonyls increased only slightly. At this temperature, alkaline peeling/stopping commence and fast alkali-induced chain scission (beta-elimination) play a major role. Along-chain carbonyls are immediately consumed by the latter processes, so that their content can be assumed to be nearly zero. Thus, the amount of carbonyls measured is approximately equal to the amount of REGs available for labeling. This amount is smaller than the theoretical amount derived from Mn, as many reducing ends are converted by peeling/stopping-type processes and thus cannot be labeled. In addition, carbonyls and carboxyls of cellulose engage in reactions with ammonia as demonstrated by increasing nitrogen content.

Thereby, fluorescent aromatic structures are formed which is indicated by a weak fluorescence signal of the unlabeled cellulose in SEC-MALLS. The formation of numerous poly(hydroxalkyl) pyrazine or -imidazole derivatives from monomeric sugars suggests that similar structures can be formed at the reducing ends of cellulose or by reaction of other carbonyl groups along the cellulose chains (Figure 4).

**Derivatization and GC/MS Analysis of the EtOAc Extracts.** The ethyl acetate extracts were analyzed by GC/MS after per-trimethylsilylation, which is considered to be one of the most versatile derivatization methods. However, 1H-imidazoles, which turned out to be major ammoxidation products, are somewhat resistant to the initially employed silylation reagent N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA). In an attempt to optimize the yield of silylated reaction products, a test mixture consisting of 4-methyl-1H-imidazole, glycolic acid, glycine, urea, D-glucose, D-gluconic acid, glucosamine-hydrochloride, and 4-(D-arabino tetrahydroxybutyl)-1H-imidazole was silylated with a mixture of trimethylchlorosilane (TMS-Cl) and/or catalytic amounts of N,N-dimethyl-4-aminopyridine (DMAP) in BSTFA. While urea, carboxy- and hydroxy-functionalities were confirmed to give comparable yields with or without catalysts, the use of TMS-Cl alone increased significantly the yields of per-silylated glucosamine and glycine, and DMAP was especially effective in increasing the yields of 1H-imidazoles with free NH functionalities. A mixture of BSTFA containing 10% of TMCS and 1.5 g/L of DMAP performed best. Higher concentrations of any of the reagents did not further increase yields, but rather promoted side reactions, such as dehydration of silylated urea to N,N′-bis(trimethylsilyl) carbodiimide.

Ethyl acetate is considered to be a solvent that is well-suited for extracting compounds of different polarity from complex mixtures as its polarity index ($P_I$ 4.3) is almost exactly between those of water ($P_I$ 9.0) and the nonpolar alkanes ($P_I$ 0.1). The extracts obtained from the ammoxidation mixtures contained a large variety of products, which is shown in the chromatograms of the ethyl acetate extracts of ammoxidized (100 °C, 0.2 MPa O<sub>2</sub>, 3 h) D-glucose and D-xylose (Figure 2). The comparison of the gas chromatograms revealed many similarities in the product pattern of the EtOAc extracts of ammoxidized D-glucose and D-xylose, even though some of the constituents are present in significantly different amounts. Derivatives of 1H-imidazole, pyrazine, and pyridine that carry short side-chains such as methyl, hydroxymethyl, hydroxyl, or 1,2-dihydroxyethyl groups constitute the major fraction of extractives, with 4-methyl-1H-imidazole (5) and pyrazine-2-methanol (7) being the dominating compounds. Apart from N-heterocyclic compounds, a variety of rather hydrophilic compounds, such as glycol amide, urea and sugars, were also detected, albeit in smaller amounts. Pyrrole, furan and oxazole derivatives, which are known to be formed in Maillard-type reactions,25−28 were not detected. As such compounds are sensitive to oxidative processes and thus cannot be labeled. In addition, carbonyls increased only slightly. At this temperature, alkaline peeling/stopping commence and fast alkali-induced chain scission (beta-elimination) play a major role. Along-chain carbonyls are immediately consumed by the latter processes, so that their content can be assumed to be nearly zero. Thus, the amount of carbonyls measured is approximately equal to the amount of REGs available for labeling. This amount is smaller than the theoretical amount derived from Mn, as many reducing ends are converted by peeling/stopping-type processes and thus cannot be labeled. In addition, carbonyls and carboxyls of cellulose engage in reactions with ammonia as demonstrated by increasing nitrogen content.

Thereby, fluorescent aromatic structures are formed which is indicated by a weak fluorescence signal of the unlabeled cellulose in SEC-MALLS. The formation of numerous poly(hydroxalkyl) pyrazine or -imidazole derivatives from monomeric sugars suggests that similar structures can be formed at the reducing ends of cellulose or by reaction of other carbonyl groups along the cellulose chains (Figure 4).

**Figure 3.** $M_w$ (left) and carbonyl/carboxyl groups (right) of cotton linter cellulose after ammoxidation at different temperature and oxygen pressure.

**Figure 4.** Proposed formation of N-heterocyclic moieties covalently attached to the reducing ends of cellulose: pyrazines from α-amino carbonyl compounds (left) and 1H-imidazoles from aldehydes (right).
polymerization reactions\textsuperscript{29,30} their incorporation into the polymeric melanoidin fraction under oxidative conditions is assumed.

Major differences in the peak pattern were found only for those constituents in which carbon skeleton and stereo-configuration of the parent sugar molecule are partially preserved, such as the tetrahydroxybutyl- (24, 25) and trihydroxybutyl pyrazines (29), which each were only found in the glucose and xylose extracts, respectively, or the different dihydroxyethyl-5-methyl-pyrazine isomers (21).

The amounts of major N-heterocyclic compounds present in the ethyl acetate extracts, which are the most critical constituents with respect to ecotoxicity differ considerably for mono- and polysaccharides (Table 3). Whereas the total amounts of N-heterocyclic compounds reached 5 mg/g for cellulose and 16 mg/g for xylan, they were considerably higher for the monosaccharides (≥100 mg/g of educt).

Ammoxidation temperature has a great impact on the amount of N-heterocyclic compounds formed. While for the studied polysaccharides the fraction of extractable nitrogenous products increased with temperature, there was a maximum of yield at 100 °C reaction temperature for the monosaccharides (D-glucose: 158 mg/g of educt, D-xylose: 122 mg/g of educt). Interestingly, the total amount of N-heterocyclic compounds decreased again for the monosaccharides when the temperature was increased from 100 to 140 °C, and additional compounds were formed. Furthermore, larger differences were found between the yields of individual N-heterocyclic compounds at higher temperature (100, 140 °C). 4-Methyl-1H-imidazole contributed the main share to the product spectrum from monosaccharides at all studied temperature levels. However, from the polysaccharides it was formed at high (140 °C) temperature only. 1H-Imidazole was formed in higher yields from xylose and xylan as compared to glucose and cellulose at all temperatures. The yields of 1H-imidazoles are highest at 100 °C. Below 100 °C, pyrazine derivatives were generally formed in minute amounts only. At higher temperatures, comparatively large amounts of pyrazines are formed from glucose, whereas xylose afforded only about one-third of the amount of glucose, and polysaccharides produced them only in traces.

Nitrogenous Constituents of the Crude Reaction Mixture. Besides the afore-discussed nitrogenous constituents of the ethyl acetate extracts, a fraction of more hydrophilic nitrogenous compounds were detected in the crude reaction mixtures. These are N-heterocyclic pyrazine- and 1H-imidazole derivatives carrying hydrophilic side chains, such as deoxyfructosazine and 4-(arabino-tetrahydroxybutyl)-1H-imidazole, aminosugars, such as glucose amine, fructose amine and di(glucopyranosyl)-amine. Surprisingly, some hydroxymides (glycol- and lactamide) and amino acids (glycine, alanine, serine and 2-amino malonic acid) were also found. For glucose ammoxidized at 100 °C (0.2 MPa O\textsubscript{2}, 3 h), more than 60 water-soluble nitrogenous compounds were unambiguously confirmed to be present in the crude reaction mixture.\textsuperscript{18}

Formation of N-Heterocyclic Compounds under Ammoxidative Conditions: Mechanistic and Kinetic Considerations. 1H-imidazoles. 1H-Imidazoles are well-known products of the reaction of sugars with ammonia.\textsuperscript{10,26,31,32} However, no mass spectra of trimethylsilylated substituted 1H-imidazoles could be found in the literature. 4(5)-Methyl-, 4(5)-(hydroxymethyl)-, and 4(5)-(tetrahydroxybutyl)-1H-imidazole were therefore identified by comparison with authentic, trimethylsilylated samples. Further 1H-imidazole derivatives known to be formed under these conditions\textsuperscript{33–35} were tentatively assigned by the fragmentation pattern of their trimethylsilyl derivatives (Table 4). Pertrimethylsilylated polyhydroxyalkyl-1H-imidazoles can be easily identified by their abundant M· ion along with the characteristic m/z 241 fragment ion which is formed by scission of the alkyl chain in beta position to the imidazole ring.

### Table 3. Amounts of Major N-Heterocyclic Compounds (mg/g of Educt) in the Ethyl Acetate Extracts of the Crude Products Obtained at Different Ammoxidation Temperature (0.2 MPa O\textsubscript{2}, 3 h)

| Tempeature | Glucose | Cellulose | Xylose | Xylan |
|------------|---------|-----------|--------|-------|
| 70 °C      | 0       | 0         | 0      | 0     |
| 100 °C     | 1.2     | 0         | 0      | 0     |
| 140 °C     | 6.1     | 67.4      | 65.0   | 65.0  |

### Table 4. Relative Amount of 1H-Imidazole and Its Most Abundant Derivatives in the Crude Products Obtained from Ammoxidation of D-Glucose at 70 °C and 100 °C (0.2 MPa O\textsubscript{2}, 3 h)\textsuperscript{a}

| Rt (min) | compound | relative percentage (%) |
|----------|----------|-------------------------|
| 12.32    | 1H-imidazole | 1.58 4.51 |
| 14.21    | 4-methyl-1H-imidazole | 0.00 43.07 |
| 20.47    | 4-(hydroxymethyl)-1H-imidazole | 67.42 46.45 |
| 28.38    | 4-(dihydroxyethyl)-1H-imidazole | 1.58 5.64 |
| 34.93    | 4-(2,3,4-trihydroxybutyl)-1H-imidazole | 6.09 44.87 |
| 35.41    | 4-(arabino-tetrahydroxybutyl)-1H-imidazole | 834.99 74.41 |
| 35.80    | 2-acetyl-4-(tetrahydroxybutyl)-1H-imidazole | 0.45 7.22 |

\textsuperscript{a}Values were calculated as ratio of the peak areas of the analytes relative to the internal standard phenylglucoside.
N-Silylated, 4-substituted 1H-imidazoles give rather broad peaks with the chosen weakly polar phase, which is useful to identify them within the chromatogram, but increases the detection limit. However, there is evidence from the mass spectra that the special peak shape is not only due to the particular retention behavior on the stationary phase but also due to the existence and virtual coelution of isomer pairs formed by N-trimethylsilylation of 4-substituted 1H-imidazole derivatives at either the N-1 or the N-3 position. Table 4 shows 4-(arabino-tetrahydroxybutyl)-1H-imidazole to be by far the most abundant imidazole derivative formed from D-glucose at 70 °C ammoxidation temperature, followed by 4-(hydroxy-methyl)-1H-imidazole. At 100 °C, the total yield of 1H-imidazole derivatives decreased to less than one-third, and the spectrum of products changed considerably. While the amount of tetrahydroxybutyl-1H-imidazole dropped to less than 10%, the content of other 1H-imidazole derivatives such as 4-methyl-1H-imidazole, 4-(2,3,4-trihydroxybutyl)-1H-imidazole, or 2-acetyl-4-(tetrahydroxybutyl)-1H-imidazole increased significantly.

The obtained results provide strong evidence that the formation of 1H-imidazoles involves condensation of an α-dicarbonyl compound, two molecules of ammonia and a highly reactive aldehyde such as formaldehyde or methyl glyoxal (pathway A, Figure 5; Radziszewski reaction). Other mechanisms proposed such as condensation of α-amino hemiaminals with carboxylic acids (pathway B),^26 reaction of α-dicarbonyl compounds with ammonia and an amide (pathway C)^31 or condensation of a glycosyl amine and an amino sugar to a dihydroimidazole with two polyhydroxyalkyl side chains, followed by heat-induced scission of the side-chains (pathway D)^10 are not likely either due to the alkaline pH which renders carboxylic acids less reactive, and/or the absence of 2-substituted 1H-imidazoles (R2 substituent in Figure 5) in the product mixtures.

Pyrazines. The TMS derivatives of poly(hydroxyalkyl) pyrazines afford readily discernible M⁺ and characteristic fragment ions, most importantly those produced by cleavage of the C–C bond in α–β position to the pyrazine ring. By thoroughly screening the chromatograms for these ions, many combinations of polyhydroxalkyl and 1-deoxy-polyhydroxalkyl side chains in the position 2 and 5 (or 2 and 6) of the pyrazine ring were identified. Two peaks with very similar mass spectra were found for all disubstituted pyrazines and assigned
to the respective 2,5- and 2,6-disubstituted isomers. For
fructosazine and 2-(tetrahydroxybutyl)-pyrazine, additional
isomers were found which were assigned to diastereomers
with a different configuration of the side chain.

Pyrazines are formed by condensation of two molecules of α-
amino carbonyl compounds, e.g. amino sugars, initially to a
dihydropyrazine and subsequent aromatization to pyrazines.
This aromatization step proceeds either through oxidation
(pathway A, Figure 6), or elimination of a hydroxy group from
the side-chain (pathway B, Figure 6).

The greatest share of the obtained pyrazine derivatives
contained a methyl or hydroxymethyl substituent (i.e., C1
substituent in Figure 6), which, according to the above
mechanisms, should originate from a C3 fragment. Therefore,
we conclude that the main route of glucose degradation
comprises its conversion to fructose or 1-amino-1-deoxy-
fructose, followed by retro-aldol scission into two C3
fragments. The minor importance of other scissions is reflected
by the frequency of the occurrence of pyrazine substituent
chain lengths which decreased in the order C1 > C0 > C2 > C4
> C3 for all studied temperatures.

Formation of 2-(tetrahydroxybutyl)-pyrazine derivatives
carrying a (poly)hydroxyalkyl substituent of different chain
length (n = 0–4) at 5 or 6 position of the heterocyclic ring
occurred already at 70 °C ammoxidation temperature, however
at a very slow rate (Figure 7). Much higher amounts were
obtained at 100 °C. The rate of formation was proportional to
the amount of sugars and amino sugars present in the reaction
mixture. Prolonged reaction times, and in particular further
elevation of the temperature (140 °C), promoted follow-up
reactions and hence decreased the concentrations of the 2,5(6)-
bis(polyhydroxyalkyl) pyrazines. The formation of 3-methyl-
2,5-bis(polyhydroxyalkyl) pyrazines as found previously could
not be confirmed to occur during ammoxidation. Besides 2,5-
and 2,6- polyhydroxyalkyl pyrazines, pyrazin-2-ols were also
found in the crude reaction products. For their formation see
Figure 6, pathway C.

To the best of our knowledge, this is the first time that this
compound class is actually identified in reaction mixtures of
sugars and ammonia, although their presence has been
hypothesized. 2-Hydroxypyrazine itself was identified by
comparison with an authentic sample, while peak 78 (Figure 1)
was tentatively assigned to 2-hydroxy-5(6)-tetrahydroxybu-

Figure 7. Ammoxidation of D-glucose at 70 °C, 100 °C, and 140 °C: Kinetics of the formation of 2-(tetrahydroxybutyl)-pyrazine derivatives with
different chain lengths of the substituents in 5(6)-position (isobar 0.2 MPa O2). Derivatives with same chain length are grouped together. Legend:
solid black squares, C0; solid black circles, C1; solid gray triangles, C2; open white circles, C3; open white squares, C4.

Figure 8. Target substances for the conducted ecotoxicity tests according to OECD 201.
tyl pyrazine, based on the mass fragmentation pattern. MS data suggested the presence of other (polyhydroxalkyl)pyrazinols, however, the respective peaks were too small or too overlapped for unambiguous assignments.

**Pyridinols.** Pyridinols have long been known as reaction products of glucose and ammonia. They constitute only a very small fraction of the crude products, but their formation is highly undesirable due to their considerable ecotoxicity, as observed in the conducted OECD 201 screening tests. 3-Hydroxyppyrrolidine is the most abundant pyridinol being present in the ammoxidation products of all studied mono- and polysaccharides, with its yield strongly increasing with reaction temperature (Table 3). Furfural is usually considered to be very small fraction of the crude products, but their formation is observed in the conducted OECD 201 screening tests. 3-Hydroxyppyrrolidine is the most abundant pyridinol being present in the ammoxidation products of all studied mono- and polysaccharides, with its yield strongly increasing with reaction temperature (Table 3). Furfural is usually considered to be very small fraction of the crude products, but their formation is

**Phytotoxicity Testing.** Unicellular algae, such as the freshwater alga *Pseudokirchneriella subcapitata* (OECD 201 test) respond with growth inhibition to a large variety of nonspecific toxic substances and are thus widely used as reliable bioindicators for a first phytotoxicity (ecotoxicity) screening of water-soluble chemicals or environmental samples.

Besides the crude reaction mixtures and the ethyl acetate extracts, phytotoxicity screening was focused on a set of target substances that was compiled based on the analytical results of this study and literature data (Figure 8). Low-molecular, moderately lipophilic N-heterocyclic compounds were in the main focus of the study as many of them are known to exhibit phytotoxicity (ecotoxicity) screening of water-soluble chemicals or environmental samples.

All phytotoxicologically active compounds share a medium lipophilicity and basic functionalities with medium basicity of pK_B = 6.5−9.0. In principle, this enables them to cross biological membranes easily, and to accumulate in more acidic cell compartments, such as chloroplasts. The less basic (pyrazine) and the more hydrophilic (4-tetrahydroxybutyl-1H-imidazole) do not show any toxic response.

The ethyl acetate extracts of reaction mixtures obtained from ammoxidation of glucose and xylose at 100 and 140 °C, respectively, were shown to have EC_{50} values similar to that of their main nitrogenous constituent, 4-methyl-1H-imidazole (Table 5). The xylose extract seems to be slightly more bioactive than the glucose extract. The shallow slope of the sigmoidal dose/response curve, which is especially pronounced for the xylose sample, is likely caused by the multitude of different components of the extract (Figure 10). The extract of the glucose ammoxidation at 140 °C is significantly less active.

### Table 5. EC_{50} Values of Selected N-Heterocyclic Compounds As Obtained from the Phytotoxicity Screening According to OECD 201

| N-heterocyclic compound                          | EC_{50} value (mg/L) | ethyl acetate extract of ammonoxided samples | EC_{50} value (mg/L) |
|-------------------------------------------------|----------------------|---------------------------------------------|----------------------|
| pyridine                                        | 0.9                  | D-xylose (100 °C)                           | 45                   |
| 3-hydroxy-6-methyl-pyridine                    | 1.2                  | D-glucose (100 °C)                          | 62                   |
| 4-(hydroxymethyl)-1H-imidazole                 | 18                   |                                             | 160                  |
| 2-methyl-1H-imidazole                          | 28                   |                                             |                      |
| 3-hydroxy pyridine                             | 44                   |                                             |                      |
| 4-methyl-1H-imidazole                          | 66                   |                                             |                      |
| 1H-imidazole                                   | 500                  |                                             |                      |

### Figure 9. Dose–response relationships for different N-heterocyclic compounds as obtained from freshwater algae test according to OECD 201.
than the 100 °C extract, which coincides with the lower percentage of the more toxic imidazoles and higher percentage of less toxic pyrazines formed at higher reaction temperatures. For all ammoxidation batches conducted with the polysaccharides as starting materials, and for all crude products obtained from the monosaccharide at 70 °C reaction temperature, the amount of organically extractable compounds was too low for meaningful phytotoxicity screening.

In contrast to the organic phases, the crude ammoxidation mixtures were demonstrated to exhibit low phytotoxicity, which approximately corresponds to their content of 4-methyl-1H-imidazole. The crude reaction mixtures of both cellulose and xylan, as well as those of glucose and xylose subjected to imidazole. The crude reaction mixtures of both cellulose and xylan, as well as those of glucose and xylose subjected to ammoxidation at 70 °C did not show any inhibition.

The phytotoxicity tests of the ammoxidized samples and their extracts demonstrated that acute phytotoxicity must only be expected from lignocellulosic materials with a high monosaccharide content ammoxidized at temperatures >70 °C. By appropriately choosing reaction temperature and/or a pretreatment procedure capable of largely reducing the monosaccharide content, lignocellulosic biomass can be converted into nitrogen-rich (soil improving) materials without formation of phytotoxic substances.

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