Talanta 85 (2011) 2014–2026

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Composition of structural carbohydrates in biomass: Precision of a liquid chromatography method using a neutral detergent extraction and a charged aerosol detector

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ARTICLE INFO

Article history:
Received 13 April 2011
Received in revised form 4 July 2011
Accepted 9 July 2011
Available online 19 July 2011

Keywords:
Precision
Analytical sulfuric acid hydrolysis
Charged aerosol detector
Lignocellulosic biomass
Cellulose
Hemicellulose

ABSTRACT

We adapted and optimized a method to quantify the cellulose, hemicellulose, xylan, arabinan, mannan, galactan contents in lignocellulosic biomass. This method is based on a neutral detergent extraction (NDE) of the interfering biomass components, followed by a sulfuric acid hydrolysis (SAH) of the structural polysaccharides, and a liquid chromatography with charged aerosol detection (LC-CAD) to analyze the released monosaccharides. The first step of this NDE-SAH-LC-CAD method aims at removing all compounds that interfere with the subsequent sulphuric acid hydrolysis or with the subsequent chromatographic quantification of the cellulosic and hemicellulosic monosaccharides. This step includes starch hydrolysis with an analytical thermostable α-amylase followed by an extraction of soluble compounds by a Van Soest neutral detergent solution (NDE). The aim of this paper was to assess the precision of this method when choosing fiber sorghum (*Sorghum bicolor* (L.) Moench), tall fescue (*Festuca arundinacea* Schreb.) and fiber hemp (*Cannabis sativa* L.) as representative lignocellulosic biomass. The cellulose content of fiber sorghum, tall fescue and fiber hemp determined by the NDE-SAH-LC-CAD method were 28.7 ± 1.0, 29.7 ± 1.0 and 43.6 ± 1.2 g/100 g dry matter, respectively, and their hemicellulose content were 18.6 ± 0.5, 16.5 ± 0.5 and 14.5 ± 0.2 g/100 g dry matter, respectively. Cellulose, mannan and galactan contents were higher in fiber hemp (dicotyledon) as compared to tall fescue and fiber sorghum (monocotyledons). The xylan, arabinan and total hemicellulose contents were higher in tall fescue and fiber sorghum as compared to fiber hemp. The precision of the NDE-SAH-LC-CAD method was better for polysaccharide concentration levels above 1 g/100 g dry matter. Galactan analysis offered a lower precision, due to a lower CAD response intensity to galactose as compared to the other monosaccharides. The dispersions of the results (expanded uncertainty) of the NDE-SAH-LC-CAD method were smaller as compared to the Van Soest (VS) method. In addition, the NDE-SAH-LC-CAD method was able to provide additional information on the composition of the hemicellulose (xylan, arabinan, mannan and galactan content) that is not provided by the Van Soest method. The NDE-SAH-LC-CAD method offers also the advantage of a better specificity for hemicellulose and cellulose, as compared to the NREL and Uppsala methods.

Abbreviations: ADF, acid detergent fiber residue; ADL, acid detergent lignin; CAD, charged aerosol detector; DM, dry matter; ELSD, evaporating light scattering detectors; GC, gas chromatography; LC, liquid chromatography; NDE, neutral detergent extraction; NDF, neutral detergent fiber residue; NREL, National Renewable Energy Laboratory; PAD, pulsed amperometric detectors; RI, Refractive index detectors; RSD, relative standard deviation; RSDi, intermediate precision RSD; RSDr, repeatability RSD; SAH, sulfuric acid hydrolysis; SD, standard deviation; SDi, intermediate precision SD; SDr, repeatability SD; VS, Van Soest; WM, wet matter.

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doi:10.1016/j.talanta.2011.07.044

1. Introduction

The most abundant compounds in lignocellulosic biomass such as lignocellulosic crops, agricultural residues or wood are two types of structural polysaccharides: cellulose and hemicellulose. Cellulose is a linear homogeneous polysaccharide made of β-1,4-linked D-glucose units, which is mainly in the form of crystalline microfibrils [1]. It represents 25–40% DM of lignocellulosic biomass [reviewed by 2]. Hemicellulose is ramified heterogeneous polysaccharides mainly made of linked D-xylose, L-arabinose, D-mannose and D-galactose units [1]. They represent 10–30% DM of lignocellulosic biomass [reviewed by 2]. Lignocellulosic biomass also contains other minor compounds such as lignin (phenolic polymer), pectins (structural polysaccharide), proteins and inorganic compounds [1]. Cellulose and hemicellulose represent the largest pool in nature of organic carbon coming from the photosynthetically collected and stored solar energy [3]. They represent therefore a huge amount of renewable resource for a sustainable bio-based economy. They can be used in lignocellulosic feedstock based biorefineries for the production of biofuels and chemicals as an alternative to products issued from fossil oil refineries [4]. In order to optimize the production of value added products in biorefineries, it is necessary to refine our knowledge of the composition of lignocellulosic feedstocks, especially the amount of cellulose and hemicellulose and the monosaccharide composition of the hemicellulose fraction [3].

Cellulose and hemicellulose are insoluble in water [5,6]. Hemicellulose can be hydrolyzed into their constitutive monosaccharides by diluted sulphuric acid, usually 4% (w/w) at 121 °C, but cellulose needs to be first solubilized by concentrated sulphuric acid, usually 72% (w/w) at 30 °C, in order to be subsequently hydrolyzed by the diluted sulphuric acid hydrolysis step [5,6]. The history of the various developed SAH methods has been reviewed by [7]. Prior to the determination of the cellulosic and hemicellulosic content of biomass, it is necessary to remove all compounds that can interfere with the sulphuric acid hydrolysis: nitrogen compounds, inorganic compounds, chlorophyll, waxes and other minor compounds [5,8]. Non-structural carbohydrates and pectins must also be removed before the sulphuric acid hydrolysis as their monosaccharidic constituents could lead to overestimation of the monosaccharides derived from cellulose (which equates to glucan) and hemicellulose [9,10]. Cellulosic and hemicellulosic monosaccharides obtained after the sulfuric acid hydrolysis are usually separated by gas chromatography (GC) or liquid chromatography (LC) [11]. The need for chemical derivatization of the monosaccharides to volatile compounds is the major drawback of GC [11]. Over the last 20 years, refractive index detectors (RI) [6], pulsed amperometric detectors (PAD) [11] or evaporating light scattering detectors (ELSD) [12] have usually been used after LC separation to quantify monosaccharides [11,7]. The charged aerosol detector (CAD) is an innovative type of detector to quantify monosaccharides with LC [12]. The CAD first nebulizes the LC column eluent with nitrogen; the LC solvent evaporates form the droplets and leaves the non-volatile analytes as aerosol particles. The latter are charged by a positively charged nitrogen stream that has passed through a high-voltage platinum corona wire. This positive charge is then measured by a highly sensitive electrometer [12]. The CAD is a nebulization detector where no optimization of the settings is necessary [12]. The signal of a detector based on a nebulization step like the CAD depends on the total amount of the analyte. The CAD signal increases with the power of the injected analyte mass. This relationship can be linearized by a log transformation to have a linear calibration [13,14]. The advantages of the CAD are its better sensitivity compared to an ELSD [14,15] and a RI [14,15], its reproducibility and dynamic range as compared to an ELSD [14], its compatibility with gradient elution [16] and with crude, non derivatized monosaccharides [16] and its ease of use [16]. The drawbacks of CAD are its incompatibility with eluents that are not volatile, its unability to quantify volatile compounds and the destruction of the sample in the detector [16].

The Van Soest (VS) method is a routine gravimetric method used to predict the animal feed quality of forage crops based on the cell wall characteristics. The VS method determines the lignocellulosic structural compounds (cellulose, hemicellulose and lignin) of biomass by the sequential extraction and separation of three cell wall fractions [17,18]: the neutral detergent fiber residue (NDF), the acid detergent fiber residue (ADF) and the acid detergent lignin (ADL) [19]. The NDF is considered as the cell wall fraction of biomass [19]. The VS method determines cellulose as ADF-ADL and hemicellulose as NDF-ADF [19].

We developed a new and innovating method to analyze the structural carbohydrates, cellulose and hemicellulose, in lignocellulosic biomass. This new method is based on the VS method [17], the Uppsala [5], and the National Renewable Energy Laboratory (NREL) methods [6]. In our method, we use a modified Van Soest neutral detergent extraction to remove all compounds interfering with the subsequent sulphuric acid hydrolysis (nitrogen compounds, inorganic compounds, chlorophyll, waxes and other minor compounds) [5,8] or with the subsequent chromatographic quantification of the cellulosic and hemicellulosic monosaccharides (non-structural carbohydrates and pectins that can lead to overestimation of the monosaccharides derived from cellulose and hemicellulose) [9,10,19]. The structural carbohydrates are then submitted to analytical sulfuric acid hydrolysis and the released monosaccharides are analyzed by LC-CAD.

ISO 17025 [20] introduces new definitions to specify the variabilities of analytical results. According to ISO 17025 [20], the random variability of an analytical method is assessed by the *repeatability* (same analytical method, same operator, same instrument and same day) and *the intermediate precision* (same analytical method but different operators, different instruments and/or different days) [20,21]. The repeatability and intermediate precision are calculated as standard deviation (SD) or relative standard deviation (RSD). The *uncertainty* of an analytical method represents an interval around the mean of the results where the unknown true value can be found with a confidence level of 68%, i.e. the standard deviation [20,21]. This uncertainty depends on the repeatability, the intermediate precision and the variability of the mean's bias [20,21]. The *expanded uncertainty* (Ux) represents an interval around the results where the unknown true value can be observed with a confidence level of 95% [20,21]. The expanded uncertainty corresponds to the uncertainty multiplied by 2 (coverage factor) [20,21].

The aim of the present paper is to determine the precision (relative standard deviation of repeatability and intermediate precision tests, respectively, RSDr and RSDi) and the expanded uncertainty [20] of our NDE-SAH-LC-CAD method and to compare it to the expanded uncertainty of the Van Soest (VS) method. The latter was established by interlaboratory studies of the Bureau InterProfessionnel d'Etudes Analytiques (BIPEA) [22].

The goal of this paper is also to build the precision profile of our NDE-SAH-LC-CAD method according to the accuracy profile concept [20] and the guidelines of the French Society of Pharmaceutical Sciences and Techniques (SFSTP) [21,23–31]. The precision profile is a decision tool that enables to interpret and compare adequately results obtained with routine analyses using the same method. The *β expectation at 95% tolerance limits* of the precision profile corresponds to the interval wherein at least 95% of the results of the analytical method are expected to fall. The *acceptance limits* of the precision profile are arbitrarily fixed values for each compound and concentration levels based on previous studies. The acceptance limit is the maximum accepted variability limit for the method. Therefore, they are expected to fall outside the β expectation at

95% tolerance limits [21,24–26,32]. The precision profile concept was already used for pharmaceutical [33–38] and food products [23,39]. The *precision* (random variability) of an analytical method can be combined with the *trueness* (systematic variability; bias) of the method to define the accuracy profile. However, the trueness of our NDE-SAH-LC-CAD could not be measured because there are no pure oligosaccharides available for this kind of analysis [40]. The NIST materials certified for the NREL and Uppsala methods cannot be used as reference for our method, as their non-structural carbohydrate and pectin contents have not been standardized. The same issue appeared with a similar approach of Theander et al. [5]. As a result, we were able to determine the precision of the method but not the trueness. This paper presents the first use of the innovating precision profile approach for a LC-CAD based analytical method in the field of lignocellulosic biomass.

2. Materials and methods

2.1. Chemicals and biomass material

All chemicals were of analytical grade or equivalent and were purchased from VWR (Heverlee, Belgium) and Chem-Lab (Zeldelgem, Belgium). The samples of fiber sorghum (*Sorghum bicolor* (L.) Moench), tall fescue (*Festuca arundinacea* Schreb.), fiber hemp (*Cannabis sativa* L.), miscanthus (*Miscanthus* × *giganteus* J.M. Greef & Deuter ex Hodk. & Renvoize) and jerusalem artichoke (*Helianthus tuberosus* L.; aerial part) came from lignocellulosic crop trials performed in 2009 at Libramont (Altitude of 522 m; 49°55′N, 05°22′E; Belgium) and harvested in October 2009, except miscanthus which was harvested in March 2010. For each biomass sample, a plot of 10 m^2 of the whole above ground biomass was harvested and chopped. Two representative subsamples of 750 g of each biomass were then directly dried after the harvest.

The biomass samples were dried at 60 °C for 72 h in a Memmert UFP800 oven (VWR, Heverlee, Belgium). The DM content (drying at 60 °C) for fiber sorghum, tall fescue, fiber hemp, miscanthus and jerusalem artichoke were respectively: 0.202, 0.147, 0.391, 0.834 and 0.336 g DM/g WM. After drying, they were first milled with a 4 mm screen hammer mill (BOA, Waterleau, Herent, Belgium) followed by a second milling step with a 1 mm screen cyclone mill (Cyclotec, FOSS Benelux N.V., Bruxelles, Belgium). The samples were stored in airtight bags at room temperature and protected from light in a dark box.

2.2. Structural polysaccharides analysis by the Van Soest method

The neutral detergent fiber residue (NDF) of the VS method was determined as described by [17]. The acid detergent fiber residue (ADF) and acid detergent lignin (ADL) of the VS method were determined as described by [18]. The cellulose content was calculated as ADF-ADL, with ADF the weight of the acid detergent fiber residue and ADL the weight of the acid detergent lignin residue. The hemicellulose content was calculated as NDF-ADF with NDF the weight of the neutral detergent fiber residue [17,18].

2.3. Structural polysaccharides analysis by the NDE-SAH-LC-CAD method

2.3.1. Neutral detergent extraction (NDE)

2 g of dried and milled biomass were weighted in a filtering crucible and were extracted on a reflux apparatus (Fibertec, FOSS Benelux N.V., Bruxelles, Belgium) for 15 min at 90 °C with 100 ml of a 0.1 mM phosphate buffer at pH 7 containing 1000 U of an analytical thermostable α-amylase (Megazyme, Ireland). After the first extraction the sample crucible was vacuum filtered. The solid retentate was then extracted with 100 ml of Van Soest neutral detergent for 1 h at 100 °C [17]. The sample was vacuum filtered, rinsed with deionized water and the solid retentate was dried at 40 °C for 72 h. The dried retentate was ground in a water-cooled laboratory grinder (A10, IKA, Staufen, Germany).

2.3.2. Sulfuric acid hydrolysis (SAH)

200 mg of the dried and ground retentate was weighted in a 100 ml pressure tube (Simax 100 ml reagent bottle, part 2070; Kavalier, Prague, Czech Republic) and was dispersed in 3 ml of 72% (w/w) H_2SO_4 containing 0.1% (w/v) of phenol. After flushing the tube headspace with nitrogen, the tube was stoppered with a screw cap and incubated in a water bath for 1 h at 30 °C. Then the H_2SO_4 was diluted to 4% (w/w) by adding 84 ml of deionized water. After flushing the tube headspace with nitrogen, the tube was stoppered with a screw cap and incubated in a forced air convection oven for 2 h at 121 °C. Phenol and nitrogen flushing were used against oxidation.

The pressure tube was cooled to room temperature before removing the cap. The hydrolysis solution was filtered with a filtering crucible of pore size from 40 to 100 μm. The pressure tube and filtering crucible were rinsed with deionized water. The filtrate was collected in a 100 ml flask.

10 ml of the hydrolysis filtrate were transferred into a 50 ml centrifugation tube and neutralized to pH 7 with solid calcium carbonate. The tube was mixed and centrifuged for 5 min at 6000 × *g*. 1.0 ml of the supernatant was transferred into a 1.5 ml centrifugation tube containing 0.5 ml of acetonitrile to precipitate the residual calcium sulfate. The tube was mixed and centrifuged for 5 min at 6000 g. The supernatant fraction was filtered through a 0.2 μm filter (Filter Service S.A., Eupen, Belgium) into a LC vial (Filter Service S.A., Eupen, Belgium) and its monosaccharidic content was quantified by LC-CAD.

To prevent any under-estimation of the monosaccharide concentration due to acidic degradation, a sugar recovery standard mixture (SRSM) of monosaccharides was used [11,41]: a mixture of 60.0 mg/ml of D-glucose, 32.5 mg/ml of D-xylose, 2.5 mg/ml of L-arabinose, 3.1 mg/ml of D-mannose and 3.1 mg/ml of D-galactose was prepared into a 10 ml flask containing 72% (w/w) H_2SO_4 with 0.1% (w/v) of phenol. After flushing the tube headspace with nitrogen, the tube was stoppered with a screw cap and incubated in a water bath for 1 h at 30 °C. The flask was cooled down to room temperature. 3 ml were transferred into a 100 ml pressure tube. The concentrated H_2SO_4 was diluted to 4% (w/w) by adding 84 ml of deionized water. The SRSM tube was then handled and diluted as described above for the retentate samples. The recovery factor RF was determined as the ratio of the amounts of monosaccharides detected by the LC-CAD relative to the corresponding amounts introduced in the SRSM.

Blank sample for SAH biomass were prepared as described above but without biomass.

2.3.3. LC-CAD analysis

The chromatographic run was carried out by injecting 35 μl of the prepared solutions into an Alliance 2695 Separation module LC (Waters S.A., Zellik, Belgium) using a Carbo Sep CHO-682 Pb analytical LC column (300 mm × 7.8 mm I.D.; 7 μm particle size) (Interchrom, Montluçon, France) with a Carbo Sep CHO-682 Pb LC precolumn (20 mm × 4.0 mm I.D.; 7 μm particle size) (Interchrom, Montluçon, France). The samples were eluted with deionized water at 80 °C for 30 min at 0.4 ml/min.

The charged aerosol detection was performed by an ESA Corona CAD detector (ESA Biosciences, Chelmsford, MA, USA). The CAD was set at a 50 pA maximum current and gas pressure of 246 kPa. Equipment control, data acquisition and integration were performed with Empower Pro 2.0 software (Waters S.A., Zellik, Belgium).

The resolution (R) between peaks was assessed by [32]:

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2} \quad (1)$$

where t is the peak retention time in seconds with $t_2 > t_1$ and W is the baseline peak width in seconds.

2.3.4. Calculation of the cellulose and hemicellulose content

The calculation of the cellulose, xylan, arabinan, mannan and galactan contents of the sample were calculated as the total mass of, respectively, D-glucose, D-xylose, L-arabinose, D-mannose and D-galactose content determined by the NDE-SAH-LC-CAD method (with correction for the H_2O release upon polymerisation). The content (g/100 g DM) of an individual neutral polysaccharide (NP = cellulose, xylan arabinan, mannan and galactan) in a sample given as anhydrous-sugar was calculated the following way:

$$\mathrm{NP} = \frac{\mathrm{MF} \times \mathrm{CF} \times \mathrm{ND} \times 100}{\mathrm{RF} \times \mathrm{MS} \times \mathrm{DM}} (\mathrm{g}/100\mathrm{g\,DM}) \quad (2)$$

where MF = mass of a given monosaccharide in the 100 ml flask after the sulfuric acid hydrolysis (in g), CF = mass conversion factor of the considered monosaccharide to a polysaccharide residue (0.90 for D-glucose, D-mannose and D-galactose; 0.88 for D-xylose and L-arabinose), ND = ratio of the dry mass of the retentate after the neutral detergent extraction relative to the dry mass of the sample before this extraction (in g DM/g DM), MS = mass of the dried retentate used for the sulfuric acid hydrolysis (in g of WM), RF = sugar recovery factor of the considered monosaccharide, DM = dry matter content determined at 103 °C for 4 h of the sample used for the sulfuric acid hydrolysis (in g DM/g WM).

Hemicellulose of the NDE-SAH-LC-CAD method was calculated as the sum of the xylan, arabinan, mannan and galactan content. Cellulose + hemicellulose of the NDE-SAH-LC-CAD method was calculated as the sum of the cellulose and hemicellulose contents.

2.4. Calibration and quantification of LC-CAD results

Five stock solutions were prepared in deionized water/acetonitrile (2/1, v/v). Each solution contained a mixture of D-glucose, D-xylose, L-arabinose, D-mannose and D-galactose. A new calibration curve was build with 5 concentration levels analyzed twice for each series (day) of hydrolysis and each monosaccharide. The CAD response follows the law: area = a * (mass injected)^b. To be accurate, the calibration curve of each monosaccharide was build based on a simple linear regression model: log(area) = b * log(mass injected) + log(a) [13,14].

2.5. Optimization of the sulfuric acid hydrolysis

The responses of the cellulose (considered as glucan), xylan, arabinan, mannan, galactan, hemicellulose and cellulose + hemicellulose contents as a function of the type of biomass (tall fescue, fiber sorghum, fiber hemp, miscanthus and jerusalem artichoke) were optimized for SAH using a Box-Behnken experiments design approach. A second-order polynomial quadratic equation was fitted to assess the effect of each independent variable to the response resulting from the Box-Behnken experimental design. The second-order polynomial quadratic equation is made of an intercept, a linear, a quadratic and an interaction component. The selected factors and levels used for the Box-Behnken design are shown in Table 1. The considered factors and ranges were based on preliminary experiments. The design of experiments was solved using JMP version 7.0.1 (SAS Institute, Cary, NC, USA). The significance of the factors was analyzed by an analysis of the variance (ANOVA) using JMP.

Table 1
Experimental factors and levels used for the Box-Behnken design.

Level of the factors	−1	0	1
Temperature (°C) of the first H_2SO_4 step	20	30	40
Incubation time (min) of the first H_2SO_4 step	15	45	75
H_2SO_4 concentration (N) of the second H_2SO_4 step	0.80	0.90	1.00
Incubation time (min) of the second H_2SO_4 step	30	90	150

Table 2
Acceptance limits as arbitrarily fixed in this study on the basis of previous works [5,21,24–26,32]. X refers to the concentration level of cellulose, hemicellulose, xylan, arabinan, mannan, galactan and cellulose + hemicellulose.

Concentration level = X	Acceptance limit (λ) (%)
X < 1 g/100 g DM	±20
1 g/100 g DM ≤ X < 5 g/100 g DM	±15[a]
5 g/100 g DM ≤ X	±10

[a] For galactan λ = ±20% for this concentration range.

2.6. Statistical treatment of the results

2.6.1. Dispersion of the results

The procedure to determine the dispersion of the results for each compound can be summarized as follows [21,24–26,32]:

- *Step 1*: Select k concentration levels of the compound on which the dispersion study will be determined in order to have a large concentration range. In our case, there were no certified reference materials or pure oligosaccharides with a known composition available [40]. Then, we selected the following 3 biomass samples for their distinctive cellulose and hemicellulose concentration levels: fiber hemp (dicotyledon), fiber sorghum (annual monocotyledon), tall fescue (perennial monocotyledon).
- *Step 2*: Define the experimental design for the determination of the dispersion study profile. It was characterized by the number of series (p = 5), by the number of replicates (n = 3) per series and concentration level and the number of concentration levels of the dispersion study (k = 3).
- *Step 3*: Calculate for each k concentration level the mean content ($\bar{x}_k$), the repeatability SD (SDr), repeatability RSD (RSDr), the intermediate precision SD (SDi), the intermediate precision RSD (RSDi), the expansion factor of the variability of the mean (k_s; Eq. (9)), the absolute uncertainty (ux), the absolute expanded uncertainty (with a coverage factor according to ISO 17025 [20]; k_e = 2) (Ux), the relative uncertainty, the relative expanded uncertainty (with a coverage factor according to ISO 17025 [20]; k_e = 2) [20,21,24,26].

$$\mathrm{MSM} = \frac{1}{p-1}\sum_{j=1}^{p} n_i(\bar{x}_j - \bar{x}_k)^2$$

is the ANOVA mean square of the model of the series (3)

$$\mathrm{MSE} = \frac{1}{pn-p}\sum_{j=1}^{p}\sum_{i=1}^{n}(x_{ij} - \bar{x}_j)^2$$

is the ANOVA mean square error of the series (4)

If MSE < MSM, then

$\hat{\sigma}_W^2 = \mathrm{MSE}$ is the variance within series (intra-series) (5)

$\hat{\sigma}_B^2 = \frac{\mathrm{MSM} - \mathrm{MSE}}{n}$ is the variance between series (inter-series) (6)

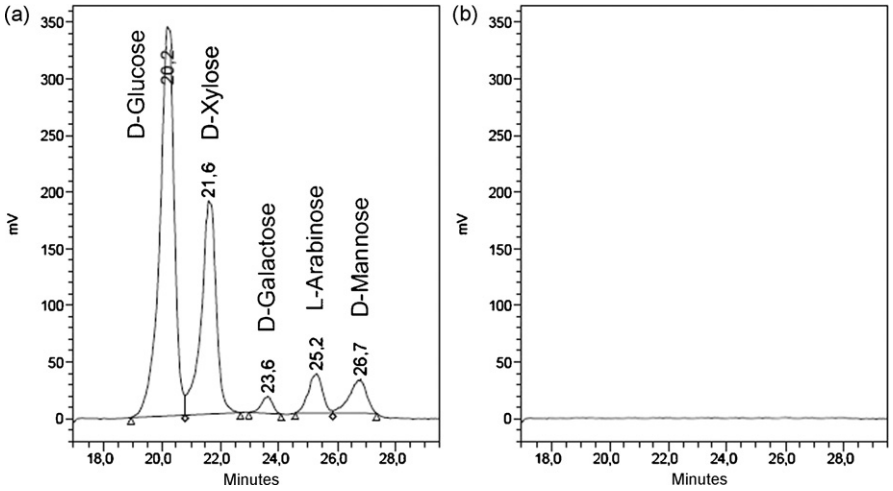

Fig. 1. (a) Chromatogram of D-glucose (24.4 μg), D-xylose (12.3 μg), D-galactose (2.13 μg), L-arabinose (2.20 μg) and D-mannose (2.03 μg) standards analyzed by LC-CAD. Numbers on top of each peak indicate the peak elution time. (b) Chromatogram of SAH biomass blank samples.

Otherwise

$$\hat{\sigma}_W^2 = \frac{1}{pn-1}\sum_{j=1}^{p}\sum_{i=1}^{n}(x_{ij}-\bar{x}_k)^2 \quad (7)$$

$$\hat{\sigma}_B^2 = 0 \quad (8)$$

$$\mathrm{SDr} = \sqrt{\hat{\sigma}_W^2} \quad (9)$$

$$\mathrm{SDi} = \sqrt{\hat{\sigma}_W^2 + \hat{\sigma}_B^2} \quad (10)$$

$$k_s = \sqrt{1+\frac{1}{pnB^2}}(9) \quad \text{with } B = \sqrt{\frac{A+1}{nA+1}}(10) \quad \text{and } A = \frac{\hat{\sigma}_B^2}{\hat{\sigma}_W^2} \quad (11)$$

$$\mathrm{ux} = k_s * \mathrm{SDi} \quad (11)$$

$$\mathrm{Ux} = ke * \mathrm{ux} \quad (12)$$

- *Step 4*: Interpretation and conclusion on the dispersion of the method.

2.6.2. Precision profile

The procedure to determine the precision profile for each compound can be summarized as follows [21,24–26,32]:

- *Step 1*: Arbitrarily define the acceptance limits (noted ±λ) of each compound and concentration levels based on previous studies [5,21,24–26,32]. We fixed arbitrarily the acceptance limits shown in Table 2.
- *Step 2*: Same step as step 1 of the method dispersion study described above.
- *Step 3*: Same step as step 2 of the method dispersion study described above.
- *Step 4*: Calculate for each *k* concentration level the mean content, the absolute β expectation at 95% tolerance limits (TL), the absolute acceptance limits, the relative β expectation at 95% tolerance limits, the relative acceptance limits [21,24,26].

$$\mathrm{TL} = \pm\bar{x}_k * k_s * Qt * \mathrm{SDi} \quad (13)$$

$Qt\left(\nu; \frac{1+\beta}{2}\right)$ is the β quantile of Student *t* distribution with ν degrees of freedom (14)

$$\nu = \frac{(A+1)^2}{(A+(1/n)^2/(p-1)+(1-(1/n))/pn} \quad (15)$$

- *Step 5*: Build the precision profile for each *k* concentration level with the observed values, the β expectation at 95% tolerance limits and the acceptance limits.
- *Step 6*: Same step as step 4 of the method dispersion study described above.

2.6.3. Uncertainty of the VS method

For the results obtained with the VS method, the reference values of the maximal tolerated NDF, ADF and ADL uncertainty (ux) values were based on the interlaboratory studies of the Bureau InterProfessionnel d'Etudes Analytiques (BIPEA) [22]. The uncertainty (ux) values for NDF, ADF and ADL are 2.0 g/100 g DM, 1.5 g/100 g DM and 0.75 g/100 g DM respectively. The uncertainty for the cellulose content determined by the VS method was calculated as follows [20]:

$$\mathrm{ux(cellulose)} = \sqrt{\mathrm{ux(ADF)}^2 + \mathrm{ux(ADL)}^2} \quad (16)$$

The uncertainty for the hemicellulose content determined by the VS method was calculated as follows [20]:

$$\mathrm{ux(hemicelluloses)} = \sqrt{\mathrm{ux(NDF)}^2 + \mathrm{ux(ADF)}^2} \quad (17)$$

The coverage factor value used to calculate the expanded uncertainty based on the uncertainty of the value is equal to 2 [20,21].

3. Results and discussion

3.1. LC-CAD chromatograms and calibration curve

Fig. 1a illustrates a typical LC-CAD chromatogram obtained with a mixture of D-glucose, D-xylose, D-galactose, L-arabinose and D-mannose standards. The peaks of Fig. 1a show some fronting. This could be explained by the special nature of the stationary phase of the used HPLC column [42]. The selectivity of the chromatographic conditions depends on the resolution between two peaks. The lowest resolution was observed between L-arabinose and D-mannose, with $R = 1.51$. This resolution is acceptable as it is below 1.50 [32]. The selectivity of the chromatographic conditions also depends on interfering peaks. With SAH biomass blank samples, no interfering peaks coming the chemicals used by the

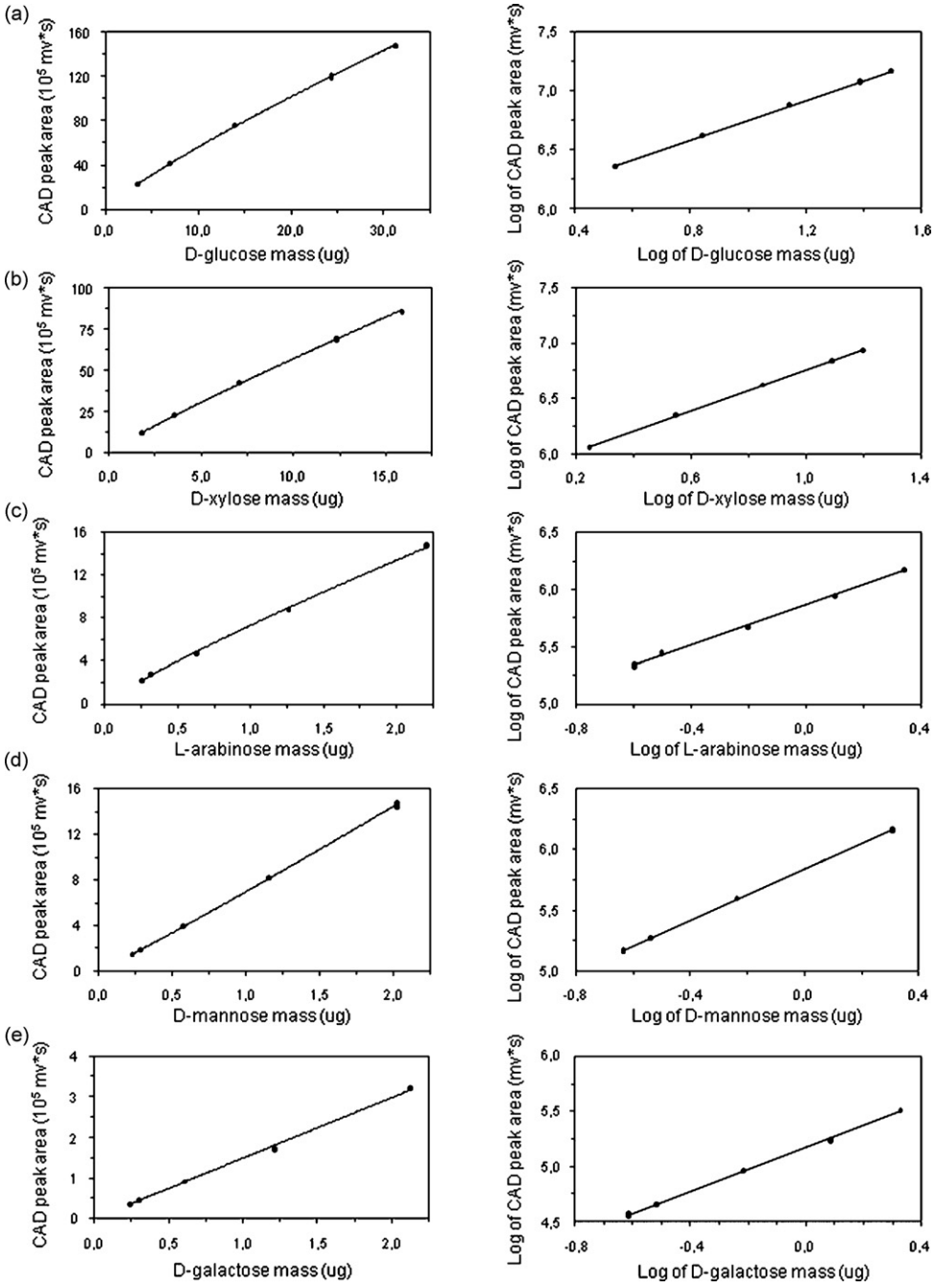

Fig. 2. LC-CAD non linearized (left) and log linearized (right) calibration lines of (a) D-glucose, (b) D-xylose, (c) L-arabinose, (d) D-mannose and (e) D-galactose.

NDE-SAH-LC-CAD method were observed at the retention times corresponding to D-glucose, D-xylose, L-arabinose, D-mannose and D-galactose (Fig. 1b). In addition, according to the peak retention chart for the Carbo Sep CHO-682 Pb analytical LC column [43] and the components that can be present in the SAH solution of the NDE-SAH-LC-CAD method, no peaks is expected to coelute with the peaks of D-glucose, D-xylose, L-arabinose, D-mannose and D-galactose. We thus concluded that no peak interferes with the peaks of D-glucose, D-xylose, L-arabinose, D-mannose and D-galactose.

Fig. 2 illustrates a typical LC-CAD non linearized and log linearized calibration curve obtained for D-glucose, D-xylose, L-arabinose, D-mannose and D-galactose). The equation terms (log(a) and b) that fit the calibration lines of D-glucose, D-xylose, L-arabinose, D-mannose and D-galactose of the LC-CAD are presented in Table 3. Log(a) represents the response intensity and b represents the response shape [15]. The main difference between these terms for the different analyzed monosaccharides is observed for the log(a) term (response intensity) of D-galactose which is lower compared to the log(a) term of D-glucose, D-xylose, L-arabinose and D-mannose. This difference could come from the nebulization step of the CAD [44].

3.2. Optimization of the sulfuric acid hydrolysis

The optimal SAH conditions were identified using a Box-Behnken experimental design. As presented in Table 1, the response of the cellulose, hemicellulose, xylan, arabinan, mannan, galactan and cellulose + hemicellulose contents for 5 different biomass (tall fescue, fiber sorghum, fiber hemp, miscanthus and jerusalem artichoke) were tested for the following parameters: the incubation

Table 3
Equation terms (log(*a*): response intensity; *b*: response shape) that fit the calibration lines of D-glucose, D-xylose, L-arabinose, D-mannose and D-galactose, respectively, based on 5 independent calibration for each compound.

	D-Glucose	D-Xylose	L-Arabinose	D-Mannose	D-Galactose
log(*a*)	5.872 ± 0.018	5.825 ± 0.020	5.828 ± 0.036	5.824 ± 0.015	5.209 ± 0.041
b	0.859 ± 0.009	0.913 ± 0.006	0.875 ± 0.048	1.014 ± 0.048	1.020 ± 0.009

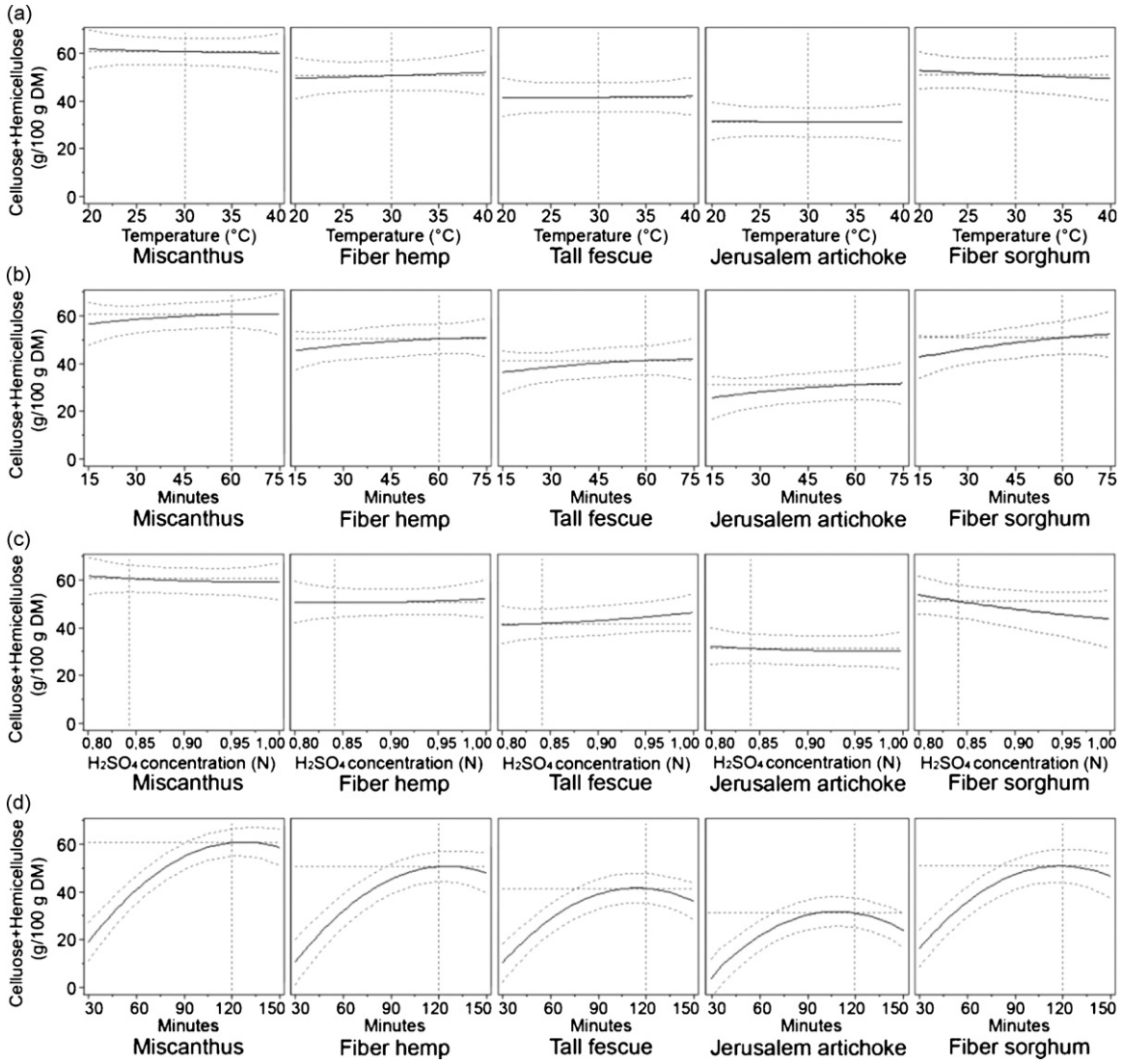

Fig. 3. Cellulose + hemicellulose content response for the different lignocellulosic biomass tested, as a function of (a) the incubation temperature (°C) of the first H_2SO_4 step (b) the incubation time (min) of the first H_2SO_4 step (c) the H_2SO_4 concentration (N) of the second H_2SO_4 step (d) the incubation time (min) of the second H_2SO_4 step. The dotted lines correspond to the 95% confidence interval.

temperature of the first H_2SO_4 step, the incubation time of the first H_2SO_4 step, the concentration of the second H_2SO_4 step and the incubation time of the second H_2SO_4 step. The ANOVA analysis determined that the incubation time of the second H_2SO_4 step was by far the most significant factor (*P*-value < 0.0001) of the sulfuric acid hydrolysis (excluding the type of biomass factor) for the cellulose + hemicellulose content whereas the incubation time and temperature of the first H_2SO_4 step and the H_2SO_4 concentration of the second H_2SO_4 step had *P*-value of 0.714, 0.013 and 0.819 respectively. Fig. 3 illustrates the influence of these 4 factors on the response of the cellulose + hemicellulose content for the 5 different lignocellulosic biomass tested, with the confidence interval (dotted line).

The optimal conditions for the SAH were similar for all compounds and all biomass. Therefore, we decided to continue the study with only 3 of the 5 biomass, i.e. one of the dicotyledons (fiber hemp), one of the perennial monocotyledons (tall fescue) and the annual monocotyledon (fiber sorghum), because of their distinctive botanical characteristics.

3.3. Comparison of structural carbohydrates determined by the NDE-SAH-LC-CAD and VS methods

The composition of the 3 selected biomass (tall fescue, fiber sorghum, fiber hemp) was determined in more details by both the NDE-SAH-LC-CAD and the VS methods. The mean contents of cellulose, hemicellulose and cellulose + hemicellulose are shown in Table 4, as well as the xylan, arabinan, mannan and galactan that can only be determined by the NDE-SAH-LC-CAD method. Both methods show similar significantly higher hemicellulose and

Table 4
Mean values of the results of the NDE-SAH-LC-CAD method for the analyzed polysaccharides and comparison with the mean values results of the VS method for the same biomass samples.

Compound		Fiber sorghum	Tall fescue	Fiber hemp
Mean content by VS method				
Cellulose	g/100 g DM	33.4 ± 1.7	33.5 ± 1.7	56.3 ± 1.7
Hemicellulose	g/100 g DM	23.8 ± 2.5	22.9 ± 2.5	11.0 ± 2.5
Cellulose + hemicellulose	g/100 g DM	57.2 ± 2.1	56.4 ± 2.1	67.3 ± 2.1
Mean content by NDE-SAH-LC-CAD method				
Cellulose	g/100 g DM	28.7 ± 1.0	29.7 ± 1.0	43.6 ± 1.2
Hemicellulose	g/100 g DM	18.6 ± 0.5	16.5 ± 0.5	14.5 ± 0.2
Xylan	g/100 g DM	15.8 ± 0.6	13.0 ± 0.4	9.3 ± 0.2
Arabinan	g/100 g DM	2.03 ± 0.06	2.40 ± 0.09	0.49 ± 0.04
Mannan	g/100 g DM	0.35 ± 0.02	0.34 ± 0.02	2.67 ± 0.12
Galactan	g/100 g DM	0.41 ± 0.03	0.73 ± 0.06	2.12 ± 0.13
Cellulose + hemicellulose	g/100 g DM	47.5 ± 1.3	46.3 ± 1.4	58.1 ± 1.3

Table 5
Dispersion values of the results of the NDE-SAH-LC-CAD method for the analyzed polysaccharides and comparison with the dispersion values results of the VS method for the same biomass samples.

Compound		Fiber sorghum	Tall fescue	Fiber hemp
Cellulose	VS method			
	Expanded uncertainty (Ux), g/100 g DM	3.4	3.4	3.4
	NDE-SAH-LC-CAD method			
	Repeatability RSD (RSDr), %	2.0	1.9	2.1
	Intermediate precision RSD (RSDi), %	3.1	3.1	2.5
	Uncertainty, %	3.3	3.3	2.6
	Expanded uncertainty (Ux), g/100 g DM	1.9	1.9	2.3
Hemicellulose	VS method			
	Expanded uncertainty (Ux), g/100 g DM	5.0	5.0	5.0
	NDE-SAH-LC-CAD method			
	Repeatability RSD (RSDr), %	1.8	2.7	1.6
	Intermediate precision RSD (RSDi), %	2.6	2.7	1.6
	Uncertainty, %	2.8	2.8	1.7
	Expanded uncertainty (Ux), g/100 g DM	1.0	0.9	0.5
Xylan	NDE-SAH-LC-CAD method			
	Repeatability RSD (RSDr), %	2.6	2.9	1.9
	Intermediate precision RSD (RSDi), %	3.5	2.9	2.1
	Uncertainty, %	3.7	3.0	2.2
	Expanded uncertainty (Ux), g/100 g DM	1.2	0.8	0.4
Arabinan	NDE-SAH-LC-CAD method:			
	Repeatability RSD (RSDr), %	1.7	2.4	2.9
	Intermediate precision RSD (RSDi), %	2.9	3.5	6.9
	Uncertainty (ux), %	3.1	3.7	7.5
	Expanded uncertainty (Ux), g/100 g DM	0.12	0.18	0.07
Mannan	NDE-SAH-LC-CAD method			
	Repeatability RSD (RSDr), %	2.8	2.8	2.9
	Intermediate precision RSD (RSDi), %	5.6	5.9	4.3
	Uncertainty, %	6.0	6.3	4.6
	Expanded uncertainty (Ux), g/100 g DM	0.04	0.04	0.25
Galactan	NDE-SAH-LC-CAD method			
	Repeatability RSD (RSDr), %	2.3	4.7	2.5
	Intermediate precision RSD (RSDi), %	6.4	7.4	5.5
	Uncertainty, %	6.9	8.0	5.9
	Expanded uncertainty (Ux), g/100 g DM	0.06	0.12	0.25
Cellulose + hemicellulose	VS method			
	Expanded uncertainty (Ux), g/100 g DM	4.3	4.3	4.3
	NDE-SAH-LC-CAD method			
	Repeatability RSD (RSDr), %	1.6	2.1	1.8
	Intermediate precision RSD (RSDi), %	2.5	2.8	2.2
	Uncertainty, %	2.7	2.9	2.3
	Expanded uncertainty (Ux), g/100 g DM	2.5	2.7	2.6

lower cellulose contents in the monocotyledons (fiber sorghum and tall fescue: about 17% hemicellulose and 29% cellulose by the NDE-SAH-LC-CAD method) as compared to the dicotyledon (fiber hemp: about 14% hemicellulose and 44% cellulose by the NDE-SAH-LC-CAD method). These differences are consistent with those previously reviewed by [2] and with the botanical differences between commeniloid monocotyldons and non commeniloid dicotyledons [1,2]. The cellulose and hemicellulose contents determined by the VS method are higher than those obtained by the NDE-SAH-LC-CAD method by 5–10 $g_{cellulose}/100\,g_{DM}$ and 5–6 $g_{hemicellulose}/100\,g_{DM}$, respectively (excepted for fiber hemp hemicellulose, that will be discussed below). These differences between the VS method and the SAH method are consistent with those previously observed by [45]. The NDE-SAH-LC-CAD method measures specifically the monosaccharide content of cellulose and hemicellulose, while the cellulosic and hemicellulosic contents

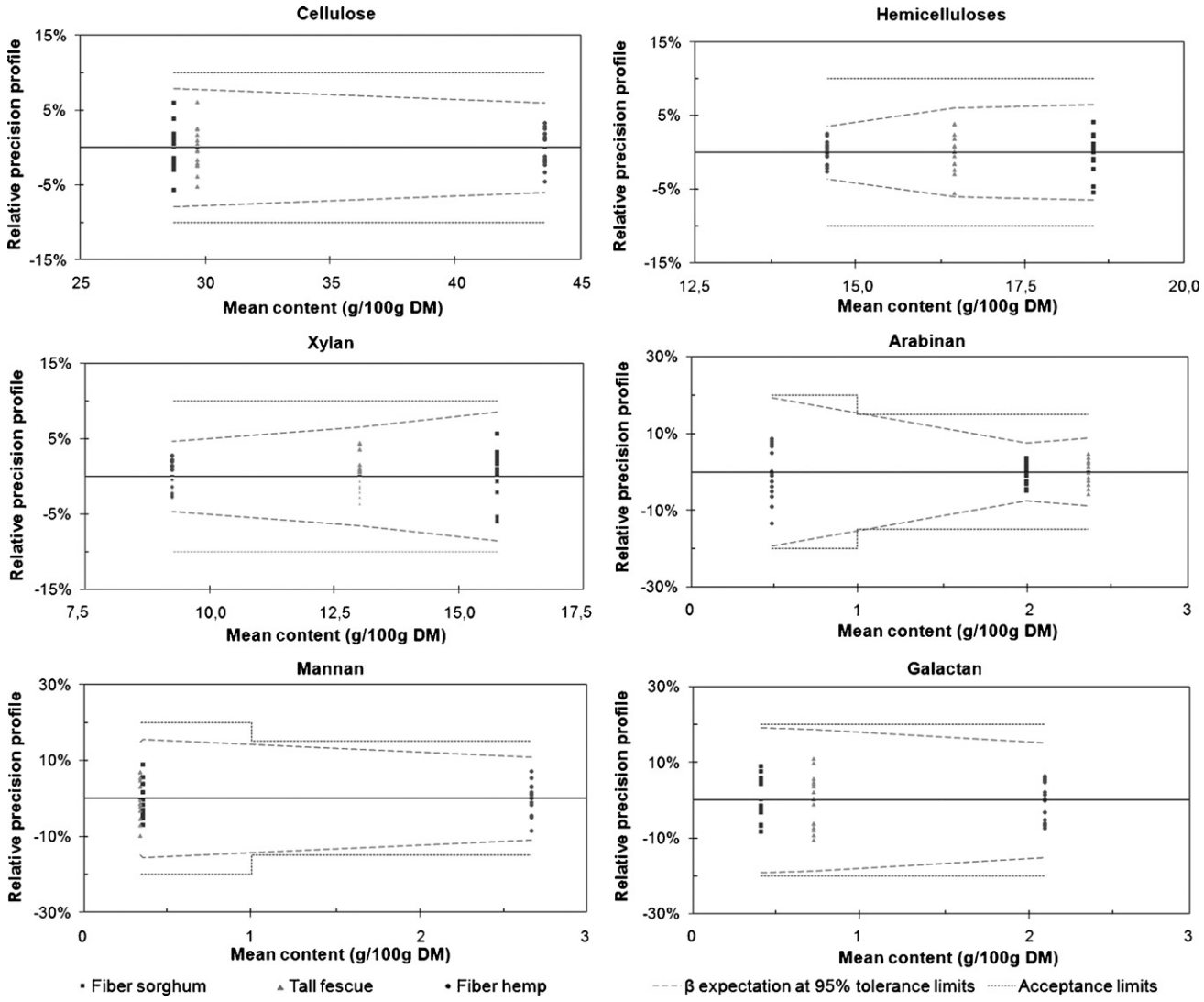

Fig. 4. Relative precision profile of the NDE-SAH-LC-CAD method for the analyzed polysaccharides. Each profile is based on 45 experimental results (15 analyses * 3 biomass).

determined by the VS method are overestimated because the NDF fraction also contains some proteins and the ADL does not contain the acid soluble lignin [10,46]. In the case of fiber hemp, the hemicellulosic content determined by the VS method was lower than with the NDE-SAH-LC-CAD. This can be explained by the presence of some pectins in the ADF fraction, as can be observed with pectin-rich dicotyledons [10,46,47].

The advantage of the NDE-SAH-LC-CAD chromatographic method, as compared to the VS gravimetric method, is that the NDE-SAH-LC-CAD method also provides the composition of the hemicellulose (xylan, arabinan, mannan and galactan). Table 4 shows that the characterization of this composition is useful as there is a significant difference in the hemicellulosic composition of fiber sorghum and tall fescue (monocotyledons, richer in xylan and arabinan) on one hand and fiber hemp (dicotyledon, richer in mannan and galactan) on the other hand. These differences can be explained by the botanical differences of the biomass, as explained above [1,2].

3.4. Comparison of the dispersion of the results of the NDE-SAH-LC-CAD and VS methods

As only the first extraction step is shared between the NDE-SAH-LC-CAD and the VS method and each step can be responsible for some variability, we decided to compare the precision of both methods. The precision of a method corresponds to the random variability within one series of measurements performed on different subsamples of the same sample. The precision of the NDE-SAH-LC-CAD method was assessed at the 3 different polysaccharides concentration levels offered by the 3 analyzed lignocellulosic biomass. For polysaccharide concentrations above 5 g/100 g DM (i.e. cellulose, hemicellulose, xylan and

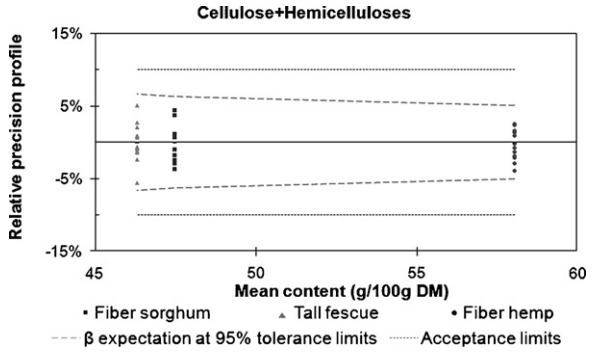

Fig. 5. Relative precision profile of the NDE-SAH-LC-CAD method for cellulose + hemicellulose, based on 45 experimental results (15 analyses * 3 biomass).

Table A1
Data of the dispersion results and precision profile of the NDE-SAH-LC-CAD method for cellulose.

Cellulose ($n = 3$, $p = 5$)	Fiber sorghum	Tall fescue	Fiber hemp
Mean content, g/100 g DM	28.7	29.7	43.6
Repeatability SD (SDr), g/100 g DM	0.6	0.8	0.6
Repeatability RSD (RSDr), %	2.0	1.9	2.1
Intermediate precision SD (SDi), g/100 g DM	0.9	0.9	1.1
Intermediate precision RSD (RSDi), %	3.1	3.1	2.5
Expansion factor of the variability of the mean, k_s	1.07	1.07	1.06
Absolute uncertainty (ux), g/100 g DM	1.0	1.0	1.2
Absolute expanded uncertainty (Ux), g/100 g DM	1.9	1.9	2.3
Relative uncertainty, %	3.3	3.3	2.6
Relative expanded uncertainty, %	6.7	6.5	5.3
Absolute β expectation at 95% tolerance limits, g/100 g DM	[26.5 to 31.0]	[27.4 to 32.0]	[40.9 to 46.2]
Absolute acceptance limits, g/100 g DM	[25.9 to 31.6]	[26.7 to 32.7]	[39.2 to 47.9]
Relative β expectation at 95% tolerance limits, %	[−7.9 to 7.9]	[−7.7 to 7.7]	[−6.0 to 6.0]
Relative acceptance limits, %	[−10 to 10]	[−10 to 10]	[−10 to 10]

Table A2
Data of the dispersion results and precision profile of the NDE-SAH-LC-CAD method for hemicellulose.

Hemicellulose ($n = 3$, $p = 5$)	Fiber sorghum	Tall fescue	Fiber hemp
Mean content, g/100 g DM	18.6	16.5	14.5
Repeatability SD (SDr), g/100 g DM	0.3	0.4	0.2
Repeatability RSD (RSDr), %	1.8	2.7	1.6
Intermediate precision SD (SDi), g/100 g DM	0.5	0.4	0.2
Intermediate precision RSD (RSDi), %	2.6	2.7	1.6
Expansion factor of the variability of the mean, k_s	1.07	1.03	1.03
Absolute uncertainty (ux), g/100 g DM	0.5	0.5	0.2
Absolute expanded uncertainty (Ux), g/100 g DM	1.0	0.9	0.5
Relative uncertainty, %	2.8	2.8	1.7
Relative expanded uncertainty, %	5.6	5.6	3.3
Absolute β expectation at 95% tolerance limits, g/100 g DM	[17.4 to 19.8]	[15.5 to 17.5]	[14.0 to 15.1]
Absolute acceptance limits, g/100 g DM	[16.8 to 20.5]	[14.8 to 18.1]	[13.1 to 16.0]
Relative β expectation at 95% tolerance limits, %	[−6.5 to 6.5]	[−6.0 to 6.0]	[−3.6 to 3.6]
Relative acceptance limits, %	[−10 to 10]	[−10 to 10]	[−10.0 to 10]

Table A3
Data of the dispersion results and precision profile of the NDE-SAH-LC-CAD method for xylan.

Xylan ($n = 3$, $p = 5$)	Fiber sorghum	Tall fescue	Fiber hemp
Mean content, g/100 g DM	15.8	13.0	9.3
Repeatability SD (SDr), g/100 g DM	0.4	0.4	0.2
Repeatability RSD (RSDr), %	2.6	2.9	1.9
Intermediate precision SD (SDi), g/100 g DM	0.6	0.4	0.2
Intermediate precision RSD (RSDi), %	3.5	2.9	2.1
Expansion factor of the variability of the mean, k_s	1.06	1.03	1.04
Absolute uncertainty (ux), g/100 g DM	0.6	0.4	0.2
Absolute expanded uncertainty (Ux), g/100 g DM	1.2	0.8	0.4
Relative uncertainty, %	3.7	3.0	2.2
Relative expanded uncertainty, %	7.5	6.1	4.3
Absolute β expectation at 95% tolerance limits, g/100 g DM	[14.4 to 17.1]	[12.2 to 13.9]	[8.8 to 9.7]
Absolute acceptance limits, g/100 g DM	[14.2 to 17.3]	[11.7 to 14.3]	[8.3 to 10.2]
Relative β expectation at 95% tolerance limits, %	[−8.6 to 8.6]	[−6.6 to 6.6]	[−4.7 to 4.7]
Relative acceptance limits, %	[−10 to 10]	[−10 to 10]	[−10 to 10]

Table A4
Data of the dispersion results and precision profile of the NDE-SAH-LC-CAD method for Arabinan.

Arabinan ($n = 3$, $p = 5$)	Fiber sorghum	Tall fescue	Fiber hemp
Mean content, g/100 g DM	2.03	2.40	0.49
Repeatability SD (SDr), g/100 g DM	0.04	0.06	0.01
Repeatability RSD (RSDr), %	1.7	2.4	2.9
Intermediate precision SD (SDi), g/100 g DM	0.06	0.08	0.03
Intermediate precision RSD (RSDi), %	2.9	3.5	6.9
Expansion factor of the variability of the mean, k_s	1.07	1.07	1.08
Absolute uncertainty (ux), g/100 g DM	0.06	0.09	0.04
Absolute expanded uncertainty (Ux), g/100 g DM	0.12	0.18	0.07
Relative uncertainty, %	3.1	3.7	7.5
Relative expanded uncertainty, %	6.1	7.4	15.0
Absolute β expectation at 95% tolerance limits, g/100 g DM	[1.87 to 2.18]	[2.19 to 2.61]	[0.39 to 0.58]
Absolute acceptance limits, g/100 g DM	[1.72 to 2.33]	[2.04 to 2.76]	[0.39 to 0.59]
Relative β expectation at 95% tolerance limits, %	[−7.5 to 7.5]	[−8.7 to 8.7]	[−19 to 19]
Relative acceptance limits, %	[−15 to 15]	[−15 to 15]	[−20 to 20]

Table A5
Data of the dispersion results and precision profile of the NDE-SAH-LC-CAD method for mannan.

Mannan ($n = 3$, $p = 5$)	Fiber sorghum	Tall fescue	Fiber hemp
Mean content, g/100 g DM	0.35	0.34	2.67
Repeatability SD (SDr), g/100 g DM	0.01	0.01	0.08
Repeatability RSD (RSDr), %	2.8	2.8	2.9
Intermediate precision SD (SDi), g/100 g DM	0.02	0.02	0.12
Intermediate precision RSD (RSDi), %	5.6	5.9	4.3
Expansion factor of the variability of the mean, k_s	1.08	1.07	1.07
Absolute uncertainty (ux), g/100 g DM	0.02	0.02	0.12
Absolute expanded uncertainty (Ux), g/100 g DM	0.04	0.04	0.25
Relative uncertainty, %	6.0	6.3	4.6
Relative expanded uncertainty, %	12.0	12.6	9.2
Absolute β expectation at 95% tolerance limits, g/100 g DM	[−0.30 to 0.41]	[−0.29 to 0.39]	[2.38 to 2.96]
Absolute acceptance limits, g/100 g DM	[−0.28 to 0.42]	[−0.27 to 0.40]	[2.27 to 3.07]
Relative β expectation at 95% tolerance limits, %	[−15 to 15]	[−15 to 15]	[−11 to 11]
Relative acceptance limits, %	[−20 to 20]	[−20 to 20]	[−15 to 15]

Table A6
Data of the dispersion results and precision profile of the NDE-SAH-LC-CAD method for galactan.

Galactan ($n = 3$, $p = 5$)	Fiber sorghum	Tall fescue	Fiber hemp
Mean content, g/100 g DM	0.41	0.73	2.12
Repeatability SD (SDr), g/100 g DM	0.01	0.03	0.05
Repeatability RSD (RSDr), %	2.3	4.7	2.5
Intermediate precision SD (SDi), g/100 g DM	0.03	0.05	0.12
Intermediate precision RSD (RSDi), %	6.4	7.4	5.5
Expansion factor of the variability of the mean, k_s	1.09	1.07	1.08
Absolute uncertainty (ux), g/100 g DM	0.03	0.06	0.13
Absolute expanded uncertainty (Ux), g/100 g DM	0.06	0.12	0.25
Relative uncertainty, %	6.9	8.0	5.9
Relative expanded uncertainty, %	13.9	16.0	11.9
Absolute β expectation at 95% tolerance limits, g/100 g DM	[0.33 to 0.48]	[0.59 to 0.86]	[1.80 to 2.45]
Absolute acceptance limits, g/100 g DM	[0.32 to 0.49]	[0.58 to 0.87]	[1.70 to 2.55]
Relative β expectation at 95% tolerance limits, %	[−19 to 19]	[−19 to 19]	[−15 to 15]
Relative acceptance limits, %	[−20 to 20]	[−20 to 20]	[−20 to 20]

Table A7
Data of the dispersion results and precision profile of the NDE-SAH-LC-CAD method for cellulose + hemicellulose.

Cellulose + hemicellulose ($n = 3$, $p = 5$)	Fiber sorghum	Tall fescue	Fiber hemp
Mean content, g/100 g DM	47.5	46.3	58.1
Repeatability SD (SDr), g/100 g DM	0.8	1.0	1.0
Repeatability RSD (RSDr), %	1.6	2.1	1.8
Intermediate precision SD (SDi), g/100 g DM	1.2	1.3	1.3
Intermediate precision RSD (RSDi), %	2.5	2.8	2.2
Expansion factor of the variability of the mean, k_s	1.07	1.06	1.06
Absolute uncertainty (ux), g/100 g DM	1.3	1.4	1.3
Absolute expanded uncertainty (Ux), g/100 g DM	2.5	2.7	2.6
Relative uncertainty, %	2.7	2.9	2.3
Relative expanded uncertainty, %	5.3	5.9	4.6
Absolute β expectation at 95% tolerance limits, g/100 g DM	[44.0 to 50.9]	[43.7 to 48.6]	[55.1 to 61.0]
Absolute acceptance limits, g/100 g DM	[42.7 to 52.2]	[41.6 to 50.9]	[52.3 to 63.9]
Relative β expectation at 95% tolerance limits, %	[−7.3 to 7.3]	[−5.5 to 5.5]	[−5.0 to 5.0]
Relative acceptance limits, %	[−10 to 10]	[−10 to 10]	[−10 to 10]

cellulose + hemicellulose), the highest values seen of the RSDr and the RSDi were respectively 2.9% and 3.5% (Table 5). In the concentrations range 1–5 g/100 g DM (i.e. arabinan, mannan and galactan), the highest values seen of the RSDr and the RSDi were respectively 2.9% and 4.3% (Table 5), excepted for galactan whose RSDi was equal to 5.5%. This higher RSDi value can be explained by the lower galactose response intensity (log(a)) (Table 3). For polysaccharide concentrations below 1 g/100 g DM (i.e. arabinan, mannan and galactan), the highest values seen of the RSDr and the RSDi were respectively 4.7% and 7.4% (Table 5). The RSDr and RSDi of the NDE-SAH-LC-CAD increase with decreasing polysaccharide concentrations, as also observed by the works of Horwitz [32,48]. The increase of the RSDi was higher than for the RSDr one. This can be explained by the fact that the intermediate precision variability (RSDi) depends on the variability within and between series whereas the variability of the repeatability (RSDr) only depends on the variability within series. The variability between series is usually higher than the variability within series. The RSDi of the cellulose, xylan, galactan and arabinan content of fiber sorghum, tall fescue and fiber hemp determined by our NDE-SAH-LC-CAD in this study are similar to the RSD of the cellulose, xylan, galactan and arabinan content of corn stover and sugarcane bagasse determined by the SAH method of Sluiter [6] in the study of Templeton [49].

The expanded uncertainty can be used to compare the dispersion of the NDE-SAH-LC-CAD method to the VS method [20,21]. Table 5 shows that the expanded uncertainties (Ux), i.e. the dispersion, for the cellulose and hemicellulose contents were lower for the NDE-SAH-LC-CAD method than for the VS method. This difference can probably be explained by the fact that each result of the VS method depend on two measurements (ADF-ADL and NDF-ADF) while the NDE-SAH-LC-CAD method measure directly the desired value.

The precision profiles of the NDE-SAH-LC-CAD method are illustrated in Figs. 4 and 5. The β expectation at 95% tolerance limits of each precision profile are comprised within the acceptance limits that we fixed arbitrarily to values based on previous studies (Table 2). These results mean that at least 95% of the results of NDE-SAH-LC-CAD method are expected to fall within the acceptance limits. For the quantification of the cellulose, hemicellulose, xylan, arabinan, mannan, galactan or cellulose + hemicellulose, the precision of the NDE-SAH-LC-CAD method was better at concentration levels equal or above 1 g/100 g DM than at concentrations levels below 1 g/100 g DM except for galactan. For polysaccharide concentrations above 5 g/100 g DM (for cellulose, hemicellulose, xylan and cellulose + hemicellulose), all the expanded uncertainty values seen were not higher than 2.6 g/100 g DM for the NDE-SAH-LC-CAD method (Table 5). In the polysaccharide concentration range 1–5 g/100 g DM (for arabinan, mannan and galactan), all the expanded uncertainty values seen were not higher than 0.25 g/100 g DM for the NDE-SAH-LC-CAD method (Table 5). For polysaccharide concentrations below 1 g/100 g DM (for arabinan, mannan and galactan), all the expanded uncertainty values seen were not higher than 0.12 g/100 g DM for the NDE-SAH-LC-CAD method.

For more exhaustive data about the dispersion study of the NDE-SAH-LC-CAD, the reader can refer to the Tables A1–A7 of the appendix that present all the data of the dispersion results and of the precision profile for each compound.

4. Conclusions

The NDE-SAH-LC-CAD method that we developed for the quantification of the cellulose and hemicellulose content of lignocellulosic biomass presents the advantage of providing information on the contents of xylan, arabinan, mannan and galactan, that are not available with the reference VS method. The cellulose and hemicellulose contents determined with the NDE-SAH-LC-CAD method are a bit lower than determined by the VS method. This can be explained by the presence of some other structural non-(hemi-)cellulosic compounds in the fractions that are considered as “cellulose” and “hemicellulose” in the VS method. The precision of this new NDE-SAH-LC-CAD method is better as compared to that of the VS method. The precision of the NDE-SAH-LC-CAD method increased for polysaccharides contents above 1 g/100 g DM, but to a lesser extent for galactan. The precision profiles show that at least 95% of the results of the NDE-SAH-LC-CAD method are expected to fall within the selected acceptance limits. A wide range of structural polysaccharides concentration levels was covered with the 3 selected lignocellulosic biomass; nevertheless, future work could complete this range with intermediate compositions and extend it with biomass such as gymnosperm wood that contains more cellulose, mannan and galactan [1], and beet pulp that contains less cellulose, and more arabinan and mannan [50].

Acknowledgements

This research was funded by the Walloon Agricultural Research Center (CRA-W) with the support of the Belgian Science Policy. The authors are grateful to A. Bridoux for her technical support.

Appendix A.

The Tables A1–A7 of the appendix present all the data of the dispersion results and of the precision profile of the NDE-SAH-LC-CAD method for each compound.

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