Received: 8 October 2021 | Revised: 12 December 2021 | Accepted: 23 January 2022

DOI: 10.1002/cche.10533

RESEARCH

Quantification of fructans in cereal-based bioethanol stillage based on a simplified analytical method

Andreas Zimmermann | **Martin Kaltschmitt**

Institute of Environmental Technology and Energy Economics, Hamburg University of Technology, Hamburg, Germany

Correspondence
Andreas Zimmermann, Institute of Environmental Technology and Energy Economics, Hamburg University of Technology, Eissendorfer Strasse 40, Hamburg 21073, Germany.
Email: andreas.zimmermann@tuhh.de

Funding information
Federal Ministry of Food and Agriculture (BMEL)

Abstract

Background and Objectives: Cereal-based bioethanol stillage, the main by-product of industrial bioethanol production, is a potential substrate for fructans. However, the quantification of fructans in such complex sample matrices is still a challenge for the corresponding analytics to be overcome to allow for the identification and utilization of such unused fructan sources. Especially a possible utilization or rather the corresponding process development requires appropriate analytics first. Thus, this paper aims to develop and apply a method for fructan quantification in bioethanol stillage using common a high-performance liquid chromatography–refractive index detector.

Findings: A new approach for fructan quantification is presented allowing for simple fructan determination in bioethanol stillage for comparably high sample throughput. The developed analytical method performs fructan quantification by means of fructose- and glucose-equivalent determination after targeted acidic hydrolysis adapted to the respective sample matrix. On the basis of this method, up to 2.5% of fructans were found in the dry matter of cereal-based bioethanol stillage.

Conclusion: Cereal-based bioethanol stillage is assessed to be a potential source of fructans. The presented analytical method can be the basis for respective process development and, thus, may contribute to a higher-value utilization of stillage.

Significance and Novelty: According to current knowledge, no determination of fructans in stillage or corresponding analytical methods have been published so far.

KEYWORDS

bioethanol stillage, fructan, fructan analysis, fructan hydrolysis, fructan quantification

1 | INTRODUCTION

The generic term fructans is used for carbohydrates almost exclusively consisting of fructose monomers with a linear or a branched molecular structure. Fructans are stored in leaves, bulbs, tubers, and roots as carbohydrate reserves. Typically larger quantities can be found in plants, such as chicory (20%–25%FM [fresh mass]) or artichoke (15%–20% FM) (Jovanovic-Malinovska et al., 2014). Furthermore, grasses and, thus, cereals, such as wheat (1%–2%DM [dry matter]) and rye (2%–6%DM), contain fructans in noticeable amounts (Karppinen et al., 2003; Verspreet et al., 2012).

Besides the term fructans, numerous names exist in literature with more or less the same meaning. Nevertheless, different terms may partly describe differences in the respective molecular structure (e.g., type of glycosidic bond, chain-length). For example, short-chain fructans with a degree of polymerization (DP) smaller than 10 are usually called fructooligosaccharides (FOS). Fructans can also be differentiated with regard to their type of glycosidic bond. The inuline-type fructans consist of β-2,1-glycosidic bonds (Figure 1), while in the phlein-type (or levan-type in the case of microbial origin) β-2,6-glycosidic bonds are present (Figure 2). Fructans, including both β-2,1- as well as β-2,6-glycosidic bonds, are called graminan- or just mixed-type. However, by far most literature addresses inulin-type fructans (van den Ende et al., 2002).

Due to their synthesis from sucrose, the fructan saccharide chain contains one terminal glucose molecule (G) linked by an α-1,2-glycosidic bond to a fructose unit (F). For this reason, GF_n is an appropriate and commonly used abbreviation for fructans containing a glucose unit originating from the precursor sucrose (GF) with a fructose chain (F_n) of varying length. Nonetheless, there are native F_nGF_m-type fructans as well, including one internal glucose unit (Pavis et al., 2001). In general, fructans of the GF_n-type and F_nGF_m-type are nonreducing saccharides as their anomeric hydroxyl groups are interconnected. Accordingly, a ring opening with subsequent oxidation toward the corresponding carboxylic acid is prevented. In case of the terminal glucose unit missing (e.g., due to partial hydrolysis), a fructan only consists of fructose molecules (F_n-type) and, thus, has a reducing end. This fact is important as carbohydrate analysis frequently makes use of this reducing character (e.g., colorimetric methods using 3,5-dinitrosalicylic acid) and hence does not include native nonreducing fructans (Lichtenthaler, 2000; Miller, 1959; Tomasik, 2004).

As fructans are nondigestible for humans while simultaneously enhancing intestinal bacterial growth being beneficial for the host's health, fructans are part of prebiotic dietary fiber. Additionally, their functional properties allow for usage as a fat-replacer, texturizer, or low caloric sweetener (Elvers, 2016). Consequently, fructans are an attractive ingredient for food products

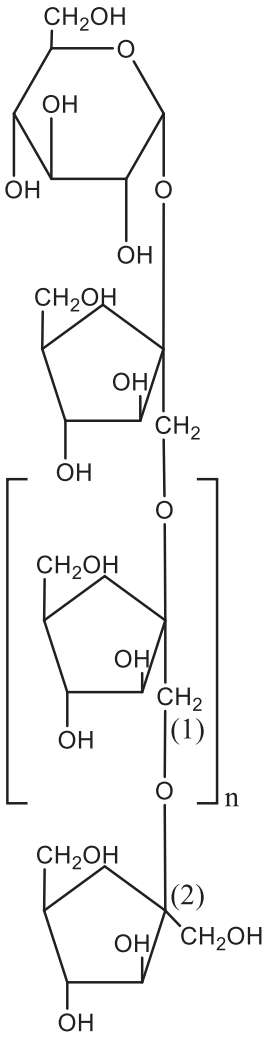


FIGURE 1 Inulin-type fructan with β-2,1-bonds

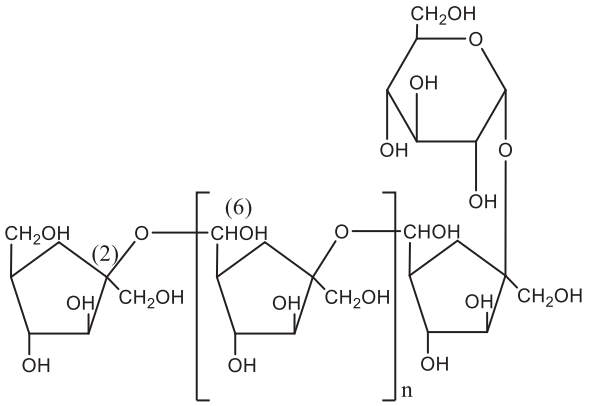


FIGURE 2 Phlein-type fructan with β-2,6-bonds

with increasing importance due to a continuously growing prebiotic market (Sukumaran & Gopi, 2021).

Besides plants, complex sample matrices like residues from industrial biomass processing (e.g., chicory roots; Zimmermann et al., 2021) can contain fructans. Thus, such residues can be promising resources for higher-value products like prebiotics for feed and food applications. In this context, bioethanol stillage is particularly of interest as a promising source of fructans. As the main by-product of bioethanol production, stillage is globally available in large quantities (Kaltschmitt et al., 2016). If obtained from cereal-based bioethanol production, significant amounts of fructans can be expected within the respective stillage as their content in the initial cereal is comparably high (Karppinen et al., 2003). According to current knowledge, no determination of fructans in stillage or respective analytical methods have been published so far.

In principle, fructan quantification is based on the determination of its monomeric hexose equivalents. This means, fructans are hydrolyzed by either enzymes or acids and the released glucose or primarily fructose molecules are quantified. Nevertheless, quantitative fructan analysis is still a major challenge. This is especially true for unknown samples additionally containing other polysaccharides. In the case of stillage, its complex composition impedes the determination of specific carbohydrate compounds and analytical methods for comparable sample matrices are rare and laborious (Reis et al., 2017). However, if such potential fructan sources are to be used, simple analytical methods for high sample throughput are required not only for the identification of fructan sources but especially for respective utilization and process development.

Against this background, this paper summarizes the state of knowledge in quantitative fructan analysis and discusses its main challenges. Based hereon, a new and simplified approach to fructan analysis adapted to bioethanol stillage using a common high-performance liquid chromatography–refractive index detector (HPLC-RID) system is presented. Finally, the developed analytical method is applied to a specific bioethanol stillage to show the applicability and the results to be expected.

1.1 | Bioethanol stillage

Stillage is the main by-product of bioethanol production (Figure 3). In the case of cereal-based ethanol production, the respective processing generally includes milling and saccharification of the raw cereal grains followed by alcoholic fermentation. Subsequently, raw ethanol (including water) is obtained by distillation. The aqueous residue from distillation called stillage is mainly used for low-value applications, such as biogas production or cheap animal feed. By means of solid-liquid separation (e.g., decanter) this (whole) stillage can be separated further into suspended solids and a liquid fraction (thin stillage) (Kaltschmitt et al., 2016). Especially the latter is expected to contain soluble saccharides such as fructans usually being highly water-soluble. Alone within the EU, about 4.7 Mt DM of stillage was produced in 2016 potentially containing fructans (Lamp et al., 2020).

1.2 | Common analytical methods for fructan quantification

The main problem of quantitative fructan analysis is the lack of available standards, especially in the case of fructans with DP > 5. Therefore, quantitative fructan analysis is almost exclusively performed by quantifying the comprised hexose equivalents (i.e., glucose and fructose) within a fructan molecule after hydrolysis (Hoebregs, 1997). In general, state-of-the-art fructan analysis always includes the following steps, which also apply to the described methods in the following.

1. Extraction of fructans from the sample matrix and determination of free glucose and fructose in the extract.

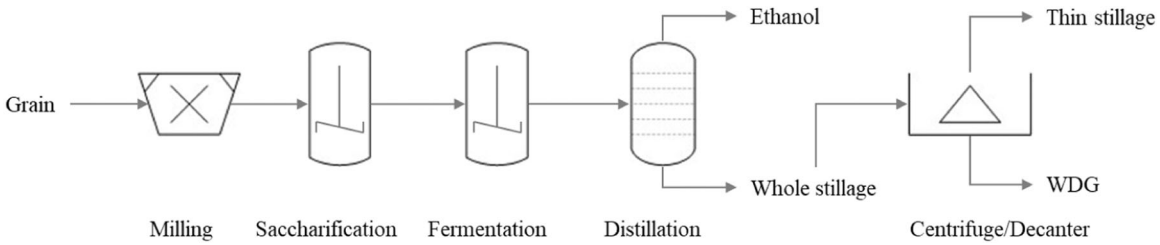


FIGURE 3 Schematic flow chart of a conventional bioethanol production. WDG, wet distillers grain

2. (Selective) hydrolysis of the fructans by means of enzymes or acids and determination of the released glucose and fructose.
3. Calculation of the original fructan amount by means of the released glucose and fructose equivalents.

The widely used and approved methods of the Association of Official Agricultural Chemists (AOAC), method 999.03 and method 997.08, are both applicable for fructan determination. Commonly these are used for foods and differ, inter alia, in the method of monosaccharide analysis (Hoebregs, 1997; McCleary et al., 2019, 2000).

1.2.1 | AOAC method 997.08 (AACC 32-31.01)

Method 997.08 (Figure 4, left) is based on HPLC analysis requiring three chromatographic runs to quantify the fructans. Fructose and glucose are the carbohydrates of interest and their respective amounts F_i and G_i are quantified in each step i. Hence fructose and glucose released from fructans can be determined. The total fructan amount S_{fructan} is (re)calculated according to Equation (1). By means of the anhydrous factor k (Equation 2), the water uptake per monomer (during hydrolysis) is taken into account. This factor requires

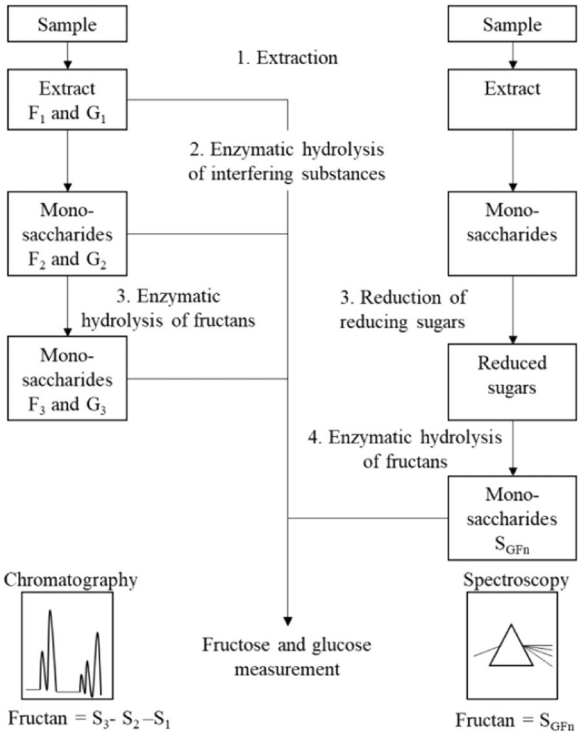


FIGURE 4 Schematic diagram of AOAC method 997.08 (l.) and 999.03 (r.); Saccharides S (glucose G and fructose F)

the fructans' average degree of polymerization (DP_{av}) to be estimated by the ratio of fructose units per terminal glucose unit (Equation 3); that is, the ratio of fructose and glucose released during step 3 (Figure 4, left). Alternatively, $k = 162/180 = 0.9$ (molar mass of the anhydrous hexose form divided by the molar mass of a hexose molecule) can be assumed for the anhydrous factor, for example, in the case of F_n-type fructans (Bosscher, 2009; Liu et al., 2011). The additional quantification of the initially present sucrose (step 1) allows for subtracting out the correspondingly released fructose and glucose and, thus, avoids a fructan overestimation.

Method 997.08 enables the determination of reducing and nonreducing fructans. It is well suited for relatively pure ingredients, as in such cases, step 2 (for interfering polymers) is not necessary. However, if fructose-containing polymers are not detected or only partly taken into consideration within step 2, an overestimation of fructose and, thus, of fructans in step 3 is the result. This applies to glucose-containing polymers as well.

$$S_{\text{fructan}} = k\,(F_3 - F_2 - F_1 + G_3 - G_2 - G_1), \quad (1)$$

$$k = \frac{180 + 162(DP_{av} - 1)}{180 DP_{av}}, \quad (2)$$

$$DP_{av} = \left(\frac{F_3 - F_2 - F_1}{G_3 - G_2 - G_1}\right) + 1, \quad (3)$$

in which F_i and G_i is the amount of fructose and glucose, respectively, where the index i is referred to in Figure 4.

1.2.2 | AOAC method 999.03 (AACC 32-32.01)

In contrast, method 999.03 is more suitable for samples with high contents of fructose, glucose, and/or respective interfering polysaccharides as disturbing carbohydrates are chemically reduced before the fructan hydrolysis (Figure 4, right). The measurement principle is here based on spectroscopy for reducing saccharides. After fructan hydrolysis, the released reducing saccharides (fructose and glucose) are determined by means of hydroxylbenzoic acid and a spectroscopic measurement with 410 nm (PAHBAH method) (McCleary et al., 2000). On this basis, the total fructan content is recalculated via the sum of released fructose and glucose molecules multiplied with the anhydrous factor $k = 0.9$.

Method 999.03 decreases the analysis expense significantly, as only one analysis step is necessary.

However, this procedure does not distinguish between fructose and glucose and, thus, no determination of DP_{av} is possible. Consequently, this approach may be combined with a chromatographic analysis, instead of the spectroscopic measurement, allowing a separate determination of glucose and fructose and, thus, DP_{av}. Nevertheless, the reduction step before fructan hydrolysis is not only extra effort but affects fructans of the F_n-type as well and might lead to an underestimation of the fructans as a result of partial recovery (up to about 20% error) (Steegmans et al., 2004). On the other hand, the presence of galactooligosaccharides (GOS) such as raffinose-derived stachyose may result in a fructan overestimation as they contain fructose and glucose as well. As these saccharides are nonreducing, GOS is not oxidized in step 3 but is partly hydrolyzed in step 4 (Figure 4, right). Thus, the use of galactosidase in step 2 may be additionally necessary to avoid a respective fructan overestimation (McCleary et al., 2019).

1.3 | Challenges in fructan quantification

Apart from the outlined method-specific difficulties above, fructan quantification is generally faced with two main challenges. On the one hand, fructans need to be hydrolyzed as specifically and completely as possible while not degrading the released fructose and glucose. On the other hand, these two hexose monomers need to be reliably determined (ideally separated from each other) next to interfering substances like other monosaccharides.

1.3.1 | Aimed fructan hydrolysis

The lack of analytical standards and, thus, the necessity for a hydrolytic step results in the need for a complete but simultaneously selective fructan hydrolysis. This is especially valid if other glucose- and fructose-containing saccharides (e.g., sucrose or starch) are present because of interferences and, thus, an overestimation of the fructan content results.

For this reason, the methods outlined above use specific fructanase enzymes for selective hydrolysis. Nonetheless, these procedures require numerous analytical steps, making them laborious and cost-intensive and, thus, large sample volumes are difficult to handle. As a result, there are lots of modifications described in the literature with the aim of being simpler. These modifications all differ with regard to sample preparation, hydrolysis procedure, and subsequent carbohydrate analytics, each with different assets and drawbacks (Benkeblia, 2013; Matros et al., 2018).

Besides enzymatic hydrolysis, mainly acidic hydrolysis for fructan quantification has been used. There are many approaches describing different hydrolytic conditions with variation in hydrolysis temperature (Sangeetha et al., 2005), hydrolysis time (Nguyen et al., 2009), and used acids (mostly hydrochloric acid; Liu et al., 2011; Verspreet et al., 2012) and sulfuric acid (Nguyen et al., 2009). The principle for fructan quantification is always analogous. However, for acidic hydrolysis, a conflict of objectives remains. On the one hand, complete hydrolysis should be achieved, and on the other, a complete determination of the released glucose and fructose equivalents is envisaged. The latter is impeded by acid-induced consecutive reactions. Especially the instability of fructose may cause problems leading to the formation of hydroxymethylfurfural (HMF). As other hexoses like galactose or mannose are basically able to form HMF as well, the fructan amounts are potentially falsified if HMF (of non-fructan origin) is considered in the calculation thoughtless (Kuster, 1990). Nonetheless, acidic hydrolysis has clear advantages like a simple implementation and no need for expensive plus partly rarely available enzymes. Thus, this approach is more suitable for routine analyses with large sample volumes. Additionally, mild acidic hydrolysis can be relatively selective with regard to fructans and may not hydrolyze starch, cellulose, or hemicellulose, if adapted correspondingly to the sample matrix as the latter ones are more stable against temperature and acids (Verspreet et al., 2012).

1.3.2 | Hexose equivalent analysis

Complete fructan determination may also be impeded by the subsequent monosaccharide analysis. After the hydrolytic step, the released fructose and glucose monomers need to be quantified. In the case of complex sample matrices like stillage comprising interfering substances, the differentiation and, thus, the correct quantification of fructose and glucose may cause problems.

Here, HPLC is the method of choice using either RID (Weiß & Alt, 2017), evaporative light scattering detection (Alassali et al., 2017; Kiss & Forgo, 2011), mass spectrometry (Apolinário et al., 2014), or pulsed amperometric detectors (Liu et al., 2011; Verspreet et al., 2012). RID is commonly used for carbohydrate and, thus, fructan analysis as it is a simple and robust measuring principle. However, RID has relatively high detection limits, offers no selective detection, and can only be operated in isocratic mode (Alassali et al., 2017). Thus, the resolution is

limited by the used column only and may not be improved by an eluent gradient. Depending on the used column, monosaccharides like fructose, glucose, xylose, and/or arabinose strongly interfere, coelute, and/or overlay in the resulting chromatogram impeding the determination of fructose and glucose (Weiß & Alt, 2017).

The outlined problems especially in terms of coelution are hardly described in literature on fructan analysis so far. However, within the scope of this paper coelution of monosaccharides has been found to be significantly impeding the analyses.

So far, fructan analysis usually deals with the analysis of comparatively fructan-rich plant materials. However, when it comes to sampling matrices with low fructan contents, hardly any literature exists and reliable values are rare (e.g., stillage). Even though HPLC-RID is commonly used, interferences by other substances are frequently neglected in the literature related to fructan analysis. Together with the outlined aspects above, several challenges have to be faced during fructan analysis of unknown samples like stillage, especially if interfering substances have to be expected. Consequently, the existing analytical procedures need to be modified and adapted to the respective sample matrix (here: stillage from bioethanol production) being the main objective of this paper. An enzymatic approach was considered to be poorly suitable in this context, since being costly and time-consuming and, thus, unsuitable for a large number of samples.

2 | MATERIALS AND METHODS

2.1 | Materials

Chemicals and calibration standards were obtained from Merck and Carl Roth: Glucose (ACS, anhydrous), fructose (≥99%), xylose (≥99%), arabinose (≥99%), sucrose (≥99.5%), hydroxymethylfurfural (97%), fructooligosaccharides from chicory (≥90%), and inulin from dahlia tubers. Sulfuric acid (96%, H_2SO_4) for hydrolysis and for the preparation of the mobile phase were also purchased from Carl Roth. Calcium carbonate ($CaCO_3$) for neutralization was obtained from Merck.

The liquid fraction of stillage after decantation (thin stillage, Figure 3) from a bioethanol production plant processing cereals (here: rye and triticale) is used. The received raw thin stillage was freeze-dried, fine grounded, and passed through a 1.0-mm sieve. These samples were stored tightly sealed at 4°C. For further use, the dried samples were mixed with distilled water and resolved at about 80°C for 15 min in a water bath. Subsequently, the samples were cooled to room temperature and centrifuged with 4500 min^{-1} for 30 min to remove the insoluble material. The resulting supernatant of this centrifuged thin stillage is used in the following.

2.2 | Severity factor

The severity factor is a conceptualized variable expressing the combination of time, temperature, and acid concentration (pH value), predicting conditions that result in similar hydrolysis yields. The severity factor might be used to compare conditions of a treatment (here: acidic hydrolysis) according to the below equations (Andersen et al., 2020):

$$\log_{10}(R_0) = \log_{10}\left(t \times \exp\left(\frac{\vartheta(t) - 100}{14.75}\right)\right), \quad (4)$$

$$\log_{10}(R_0'') = \log_{10}(R_0) + |\mathrm{pH} - 7|, \quad (5)$$

in which R_0 is the combined severity factor comprising the hydrolysis temperature ϑ in °C and the reaction time t (min); R_0'' is the severity factor additionally including the pH and, thus, the acid concentration.

2.3 | Final method

The developed analytical method (Figure 5) for the quantification of fructans in bioethanol stillage is the main result of this paper and the outcome of the method development described below.

For fructan hydrolysis, a reaction cell (16 mm, pressure-tight with screw cap) containing 10 ml of the

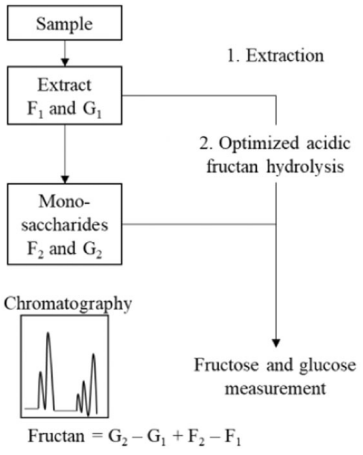


FIGURE 5 Schematic diagram of the developed method for fructan determination

prepared stillage supernatant is incubated in a thermoreactor (Spectroquant TR420; Merck) at 100°C for 80 min with a final H_2SO_4 concentration in the sample of 0.25 wt%. To keep the total volume and, thus, the concentration of the resulting acidic sample constant, 72% H_2SO_4 is used for acidification as volume changes can be neglected due to volume contraction. After incubation, the reaction cell is cooled in a water bath and neutralized with $CaCO_3$. The pH value should be adjusted to about 6 as $CaCO_3$ is poorly soluble and, thus, the neutralization reaction may slowly continue. For this reason and to minimize foaming, $CaCO_3$ should be added slowly. The neutralized sample is centrifuged for 20 min with 4500 min^{-1} to remove the formed calcium sulfate ($CaSO_4$) and the excess $CaCO_3$. Subsequently, the supernatant is centrifuged again with 14,000 min^{-1} at 4°C for 30 min before analysis to guarantee a particle-free sample for HPLC analysis. Similarly, a respective nonhydrolyzed stillage sample is centrifuged with 14,000 min^{-1} for 30 min before HPLC analysis for determining the free monosaccharides before hydrolysis.

An Agilent 1260 Infinity II LC system with an RID is used for carbohydrate separation by means of both an Agilent Hi-Plex H column (7.7 × 300 mm, 8 μm) and Hi-Plex Pb column (7.7 × 300 mm, 8 μm), each with the respective guard column. In the case of the Pb column, an additional deashing guard column (Bio-Rad) is required for ion removal. This means two chromatographic runs are performed in succession with their respective HPLC conditions. The detection was done by a refractive index detector operating at 55°C. The injection volume was ≥10 μl. The fructan content was calculated based on DM of the stillage analogous to Equations (1) to (3) via the fructose and glucose equivalents released after fructan hydrolysis (subtracted from free fructose and glucose before hydrolysis).

The HPLC conditions can be summarized as follows:

1. Agilent Hi-Plex H Column temperature 60°C, flow rate 0.5 ml/min 5 Mm H_2SO_4.
2. Agilent Hi-Plex Pb Column temperature 80°C, flow rate 0.6 ml/min H_2O.

2.4 | Method development

In accordance with the outlined challenges, the method development for fructan quantification in stillage is subdivided into the HPLC analysis method and the acidic hydrolysis procedure. Both steps are characterized by the objective of developing a reliable but time- and cost-saving method for fructan analysis in stillage using an HPLC-RID system.

2.4.1 | HPLC-RID method

To allow for the identification of potentially interfering substances during analysis, the composition of the used stillage was determined initially (Table 1). On this basis, an appropriate HPLC-RID setup with the accompanying HPLC method was searched.

Within the scope of method development, different column types have been experimentally checked and assessed with the aim of finding the most appropriate HPLC setup for stillage. For the column screening experiments, mixtures of glucose, fructose, xylose, and arabinose were analyzed. The last-mentioned pentoses are part of pentosans (from hemicellulose), one of the main potentially interfering polysaccharides in bioethanol stillage (Table 1). For the column assessment, particularly the resolution of glucose and fructose and their overlays with interferences were compared. Typically, either cationic exchange resins (mainly hydrogen, lead, or sodium form) or amino phases are applied (Dos Santos Lima et al., 2019) and, thus, are evaluated for fructan analysis in stillage (for more information on the column selection see Supporting Information). Columns with calcium (Sangeetha et al., 2005) and silver (Vega &

TABLE 1 Composition of the liquid supernatant of the used thin stillage from bioethanol production with about 8%DM (dry matter)

%DM	Carbohydrates							
	Monosaccharides[a]	Pentosans[b]	Glucan[c]	Proteins[d]	Lipids[e]	Ash[f]	Glycerol[a]	Others[g]
	14	17	≥14	24	<0.2	11	10	<10

[a]High-performance liquid chromatography (HPLC): mainly glucose, arabinose, and xylose.

[b]HPLC after hydrolysis with 4 wt% H_2SO_4, 1 h, 120°C: released xylose and arabinose (Sluiter et al., 2006).

[c]HPLC after hydrolysis with 4 wt% H_2SO_4, 1 h, 120°C: released glucose (Sluiter et al., 2006).

[d]Kjeldahl: Factor 6.25 DIN EN ISO 20483.

[e]Soxleth: DIN EN ISO 11085.

[f]Gravimetery: 550°C DIN EN ISO 18122.

[g]Including organic acids, for example, formic and acetic acid.

Zuniga-Hansen, 2015) phases have also been considered but a priori assessed as inappropriate for stillage. In the case of the former, this is due to the coelution of monosaccharides, and in the case of the latter, this is due to high sensitivity against impurities.

2.4.2 | Fructan hydrolysis method

Response Surface Methodology was used for hydrolysis optimization with H_2SO_4 and $CaCO_3$ (for more information on choosing H_2SO_4 and $CaCO_3$ for hydrolysis, see Supporting Information). Hydrolysis temperature x_1, time x_2, and acid concentration x_3 were varied with regard to a maximization of the recovery of glucose and fructose equivalents and, thus, fructan recovery; that is, the maximum detectable fructan content was wanted. Simultaneously, the formation of HMF from hexose was considered for optimization as well; that is HMF formation should be concurrently minimized. A Box-Behnken design (Table 2) was employed for these experiments using the commercially available statistical software Design-Expert (Stat-Ease). Following the outlined analytical procedure above, the hydrolysis temperatures were varied from 90 to 110°C for varying hydrolysis times from 40 to 80 min with different final H_2SO_4 concentrations in the sample ranging from 0.1 to 0.9 wt% (Table 2). The design space boundaries were selected based on preliminary screening tests (data not shown).

In the case of the fructan content Y_1, a quadratic model was fitted to the experimental data, while in the case of the HMF formation Y_2, a Two-Factor Interaction (2FI) model was used.

$$Y_1 = a_0 + \sum_{i=1}^{3} a_i x_i + \sum_{i=1}^{3} a_{ii} x_i^2 + \sum_{j=1}^{3} \sum_{i>1}^{3} a_{ji} x_j x_i, \quad (3.1)$$

$$Y_2 = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} \sum_{j>1}^{3} b_{ij} x_i x_j. \quad (3.2)$$

A statistical analysis of variance (ANOVA) based on the Box-Behnken design was performed using DesignExpert. The respective tables of ANOVA are attached in the Supporting Information for both models (Table S1.1 and S1.2).

By means of numerical optimization, the optimal hydrolysis conditions were searched using the regression models. The aim was to achieve fructan hydrolysis as completely as possible while detecting the released monosaccharides (and avoiding HMF formation). Thus, the target figures for the optimization were rated with respect to their importance as follows. The maximization of the fructan content was rated high and the minimization of the HMF formation was rated medium. A further boundary condition was the minimization of the acid concentration (low importance) as ions in the sample may impede HPLC analysis as outlined below. Furthermore, the neutralization procedure can be simplified by using less $CaCO_3$, which also has a practical benefit. The hydrolysis temperature and time are not limited for this optimization and, thus, may lie in the range of the design space.

TABLE 2 Box-Behnken design for fructan hydrolysis optimization: Factors and values for three different levels

		Level		
Variable	**Symbol**	**−1**	**0**	**+1**
Temperature (°C)	x_1	90	100	110
Time (min)	x_2	40	60	80
Acid concentration (wt%)	x_3	0.1	0.5	0.9

2.5 | Method suitability and usability

To assess the suitability of the method or rather the hydrolysis procedure, the developed analytical method is compared to existing literature using the combined severity factor according to Equations (4) and (5).

Furthermore, as no literature values on fructans in stillage have been reported so far, reference analyses were conducted for the assessment of the developed overall fructan analysis procedure. For this purpose, results obtained using the developed method are compared to those using the commercial Megazyme Fructan Assay Kit (K-Fruc) following the outlined AOAC method 999.03. Subsequently, potential sources of error are discussed and the method's recovery is assessed based on commercial fructans (FOS from chicory, inulin from dahlia) and other sugar standards.

Finally, the developed method was used for the analysis of a batch of cereal-based raw thin stillage with a view to a potential fructan separation. For this purpose, thin stillage and its corresponding fractions after centrifugation on laboratory scale (4500 min^{-1}, 30 min) were used. Here, the stillage was diluted to a DM content of 8% before centrifugation to reduce its viscosity.

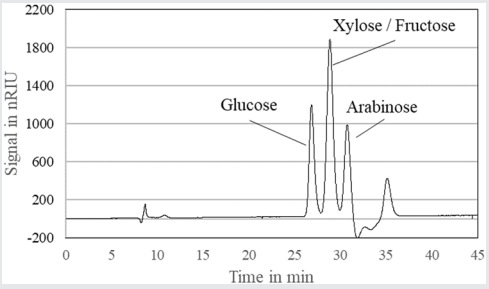
3 | RESULTS AND DISCUSSION

3.1 | Optimization of the HPLC-RID analysis

The following assessment of potential stationary phases for fructan analysis results from an experimentally carried out column screening. Table 3 gives an overview of

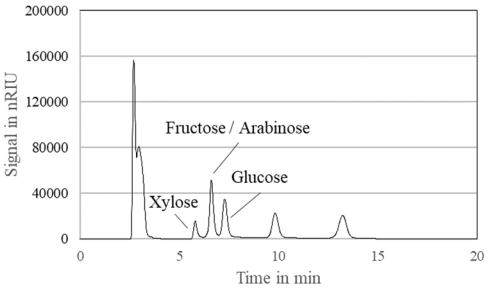
TABLE 3 Overview of stationary phases (a–d), typically used for fructan quantification via high-performance liquid chromatography with refractive index detector (HPLC-RID): Application examples, main drawbacks for stillage analysis, and representative chromatograms from the column screening experiments

(a) Sodium Na (Agilent, product number PL1170-6840)



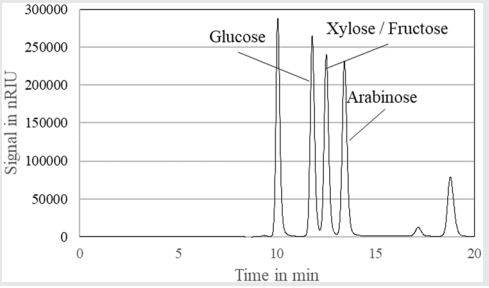
Application example: Fermentation broth from enzymatic fructan production (Ko et al., 2019). *Main drawbacks*: Sensitivity to salts and acids; coelution of fructose and xylose. *Conclusion*: The Na column was considered to be inappropriate for stillage due to the coelution of xylose and fructose and, thus, the impossibility of a reliable fructan determination in the presence of xylose-containing saccharides (e.g., hemicellulose).

(b) Amino NH_2 (Agilent, product number 840300-908)



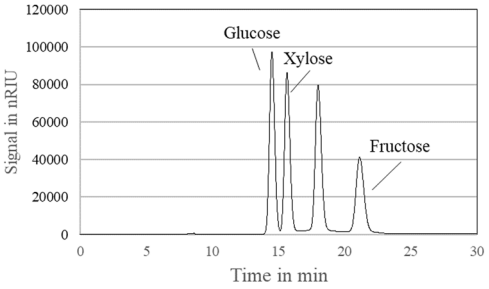
Application example: Fermentation broth from enzymatic fructooligosaccharides (FOS) production (Nobre et al., 2012). *Main drawbacks*: Insolubility of high-molecular substances (e.g., polysaccharides) in the mobile phase (aqueous acetonitrile); coelution of fructose and arabinose. *Conclusion*: The widely used NH_2 column was considered to be inappropriate for the given sample matrix due to the coelution of arabinose and fructose and, thus, the impossibility of a reliable fructan determination in the presence of arabinose-containing saccharides (e.g., hemicellulose). Moreover, the necessity of a high acetonitrile content in the mobile phase results in a poor solubility of the higher molecular substances (e.g., polysaccharides) continuously precipitating with the danger of capillary or column clogging within the HPLC system.

(c) Hydrogen H (Agilent, product number PL1170-6830)



Application example: Fermentation broth from enzymatic FOS production (Ko et al., 2019). *Main drawbacks*: Coelution of fructose and xylose. *Conclusion*: The H column shows coelution of xylose and fructose. This could be improved by the use of acetonitrile as a modifier in the eluent. However, this modification was found to be unsuitable for frequently carried out routine analyses due to poor solubility of high molecular substances and continuous degradation of the column.

(d) Lead Pb (Agilent, product number PL1170-6820)



Application example: (Hydrolyzed) commercial fructans and grasses (Nguyen et al., 2009; Weiß & Alt, 2017). *Main drawbacks*: Sensitivity, for example, to ions and proteins. *Conclusion*: The Pb column showed the comparatively best resolution for the respective monosaccharides (glucose, fructose, xylose, arabinose) allowing for separation from galactose, mannose, and other monosaccharides as well. However, ions within the sample, especially from the acidic hydrolysis procedure lead to overlays in the chromatogram impeding a reliable quantification.

the evaluated columns and their suitability for the sample matrix stillage. Respective selectivity factors are attached in Table S2.

Each of the assessed stationary phases has drawbacks with regard to fructan determination in stillage or rather the determination of the glucose and fructose equivalents, whereby, especially coelution causes problems. Some of these resins are additionally sensitive against acids and salts, making sample preparation laborious. This is also necessary as salts may interfere with the monosaccharides in the chromatogram (data not shown).

On the basis of Table 3, a hydrogen form column (H) was considered to be most appropriate for frequently carried out fructan analyses in sample matrices like

stillage due to its durability and the comparably good resolution (Table S2). However, an additional lead phase column (Pb) is required in parallel to reliable determine fructose in the presence of xylose. Simultaneously, the H column is required if the stillage contains glycerol (Table 1) as it coelutes with fructose on the Pb column. By means of respective deashing cartridges before the main column, the problem of ion interferences (from salts) can be overcome for the Pb column.

Consequently, both columns need to be used in succession to reliably determine fructose and glucose by capturing a picture of the stillage composition as complete as possible. This procedure allows for the reliable determination of fructose and glucose in the presence of interfering carbohydrates (mainly xylose) and alcohol (e.g., glycerol) tending to coelution and overlay on the respective columns. As a result of the experimental column screening and subsequent considerations, the optimized HPLC method has been obtained as outlined in Section 2.3 using a Pb as well as an H column.

Comparable HPLC methods can be found in the literature showing good reproducibility (Weiß & Alt, 2017). Alassali et al. (2017) used a combination of an hydrogen and a lead form column for quantifying carbohydrates after hydrolysis of plant material, analogous to this paper; that is, an H column for the determination of glucose, xylose, and arabinose and a Pb column for the quantification of fructose and glucose. However, the hydrolysis procedure was different with a view to hydrolyze other polysaccharides than fructans (Alassali et al., 2017).

3.2 | Optimization of the acidic fructan hydrolysis

Figure 6 shows the results of the acidic hydrolysis optimization via Response Surface Methodology. The surface plots are based on the measured fructan content in stillage and formed HMF during hydrolysis both exemplarily for a hydrolysis time of 80 min. As expected, the fructan content (Figure 6, left) shows a maximum due to increasing degradation of fructans to its monomers with increasing temperature and acid concentration and, thus, increasing severity factor (Equation 4). For comparably higher severity factors, the released fructose and partly glucose increasingly react to HMF and, thus, are not registered as fructans. This results in lower fructan amounts or rather contents for increasing severity. In contrast, the formation of HMF (Figure 6, right) increases steadily with an increasing severity factor due to an increasing release of hexoses and an increasing reaction rate with higher temperature and/or acid concentration.

The numerical solution for the optimization of the hydrolysis conditions is given in Table 4. Using these numerically determined conditions, the hydrolysis was conducted experimentally with the aim of

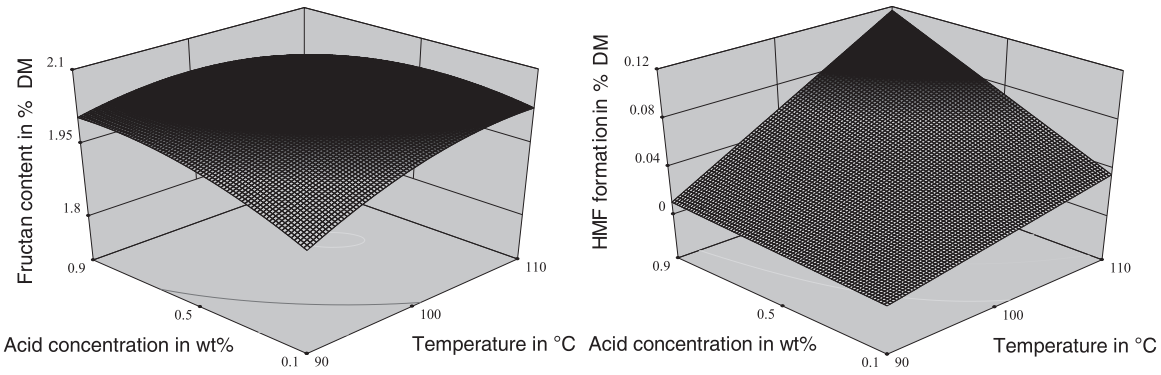


FIGURE 6 Response surface plots for the fructan content (left) and hydroxymethylfurfural (HMF) formation (right), both based on stillage dry matter (DM), depending on temperature (°C) and acid concentration (wt%) (H_2SO_4) for a hydrolysis time of 80 min and subsequent neutralization with $CaCO_3$

TABLE 4 Optimal conditions for fructan hydrolysis in stillage based on design of experiments using Box-Behnken design

	Temperature (°C)	Time (min)	Acid concentration (wt%)
Numerical optimization	103	80	0.27
Experimental optimization	100	80	0.25

reproducing the numerically determined optimum. Small adjustments have been made regarding temperature (set to 100°C) and acid concentration (set to 0.25 wt%). Simultaneously, the hydrolysis time was varied from 60 to 120 min in steps of 10 min to successfully confirm the numerical result on an experimental basis (Table S3).

As a result, the optimum hydrolysis conditions for fructans in stillage have been found to be 100°C, 80 min, 0.25 wt% H_2SO_4, as shown in Table 4. This results in the final analytical method described in Section 2.3.

3.3 | Method suitability

3.3.1 | Comparison of the severity factor

In comparison to the developed hydrolysis method, an equivalent acidic hydrolysis method can be found in the literature (Nguyen et al., 2009). The mentioned publication reports fructan hydrolysis conditions using 0.2 wt% H_2SO_4 at 121°C for 60 min corresponding to a severity factor of 8.0. These conditions are assessed to be comparable to the found conditions within this paper (severity factor 7.6). On the basis of the severity factor, the presented optimized hydrolysis conditions were compared additionally to other literature on acidic fructan hydrolysis (Liu & Rochfort, 2014; Liu et al., 2011; Maharjan et al., 2018; Michel-Cuello et al., 2008; Verspreet et al., 2012). The conditions within this paper or rather the severity factor are consistent with the average calculated severity factor of 7.6 ($n = 6$, Table S4) for the mentioned citations. This circumstance corroborates the developed hydrolysis procedure.

3.3.2 | Comparison to reference analytics

The following values for the fructan content in different stillage samples were obtained using the developed method and the Megazyme Fructan Assay Kit in comparison (Table 5). Both method repetitions show similar standard deviations but a slight deviation of the mean measured value substantiating the suitability of the entire developed method.

Apart from the good correlation between the two methods, the acidic hydrolysis with subsequent HPLC analysis offers the advantage of determining fructose and glucose separately. This allows for an estimation of DP_{av} via fructose units per glucose unit (Equation 3), while the commercial Fructan Assay Kit determines a sum parameter for both sugars. Moreover, in comparison to the laborious AOAC method, the developed method is convenient, easy to establish for existing HPLC systems, and suited for large sample volumes (Figures 4 and 5).

3.3.3 | Error analysis and discussion

The developed hydrolysis method using H_2SO_4 and $CaCO_3$ (Table 4) was additionally conducted using commercial fructans to determine the fructan recovery. The recovery of the method is determined to be greater than 92% ± 0.2% ($n = 2$). This discrepancy can be explained by potential measurement inaccuracies and/or incomplete hydrolysis. To exclude the latter, the Pb column was used for the detection of potential non-hydrolyzed oligomeric or polymeric fructans. Using this HPLC setup did not significantly detect any respective saccharides with DP > 2. This means, under the optimized conditions, a more or less total fructan hydrolysis can be assumed (Figure S2). Incomplete fructan hydrolysis could be additionally ruled out as even higher severity factors (compared to the optimized conditions) did not result in higher fructan contents or further degradation (data not shown).

Fructose and glucose degradation may also falsify the measured fructan content. However, fructose and glucose standards show hardly degradation to HMF under the optimized hydrolysis conditions. Fructose losses are found to be about 1.8% (related to the initial amount) and about 0.6% in the case of glucose due to the reduced stability of fructose (Nguyen et al., 2009). In this context, a sugar recovery standard containing the monosaccharides of interest can be used analogously to the sample to correct for respective losses. Therefore, the

TABLE 5 Comparison between commercial fructan assay kit and developed method for different stillage samples

Sample	Acidic hydrolysis and HPLC	Megazyme Fructan Kit
Rye-based stillage	2.4 ± 0.1%DM ($n = 5$)	2.5 ± 0.1%DM ($n = 3$)
Triticale-based stillage	0.3 ± 0.0%DM ($n = 2$)	0.2 ± 0.0%DM ($n = 2$)
Triticale-based stillage with inulin addition	0.8 ± 0.0%DM ($n = 2$)	0.8 ± 0.0%DM ($n = 2$)

Abbreviations: DM, dry matter; HPLC, high-performance liquid chromatography.

TABLE 6 Fructan content in cereal-based thin stillage, its supernatant, and the corresponding sediment after aqueous dilution and subsequent centrifugation with 4500 min^{-1} for 30 min

Stillage fraction	Dry matter (DM) in %	Fructan content in %DM
Thin stillage; raw	17	1.0
Thin stillage, supernatant; diluted	8	2.2
Thin stillage, sediment; diluted	20	0.5

amount of formed HMF is multiplied by the correction factor 180/126 to obtain the amount of respective initial hexose equivalents.

Another potential source of error is the unwanted cohydrolysis of other fructose- and glucose-containing oligo- and polysaccharides. As shown in Table 1, glucose-containing saccharides (glucans) are present in the stillage, probably starch residues and cellulose. In this regard, it could be shown that starch degradation is below the limit of detection under the optimized hydrolysis conditions and, thus, interferences with glucose from starch can be neglected (Figure S2). Due to its greater stability (in comparison to starch), cellulose degradation can be neglected as well (Whitfield et al., 2016). Even though these compounds are hardly hydrolyzed under optimized conditions, high proportions of starch may falsify the fructan measurement. In such cases as prior amylase treatment might become necessary to degrade starch before fructan hydrolysis. The presence of sucrose is usually one of the most important error sources for fructan quantification, as sucrose is easily acidly hydrolyzed and released fructose (and glucose) may falsify the results. In the case of stillage, sucrose was supposed to be fully degraded or converted during fermentation in the bioethanol process. This has been confirmed as no sucrose was detected before hydrolysis in the initial stillage (data not shown). However, if sucrose is present, its amount has to be determined by HPLC before hydrolysis and respectively subtracted.

A further potential source of error is the neutralization step. Potential coprecipitation of monosaccharides by the formed $CaSCO_4$ may result in an underestimation of the hexose equivalents and, thus, fructans. However, this coprecipitation could not be detected in a respective precipitation trial with a solution containing glucose, fructose, arabinose, and xylose standards (data not shown).

3.3.4 | Application of the developed analytical method

As shown in Table 5, the fructan content in stillage strongly depends on its origin (here: rye and triticale). Stillage based on rye, the fructan-richest cereal, shows significantly higher fructan contents in this comparison and, thus, is more interesting for a potential fructan separation. On this basis, Table 6 shows the results for another batch of cereal-based raw thin stillage and its corresponding fractions after centrifugation on a laboratory scale.

As expected, the fructan proportion in the stillage's DM increases in the liquid supernatant, as the soluble fructans accumulate in this aqueous phase. Simultaneously, the fructan proportion in the sediment decreases, while insoluble matter (in particular, insoluble proteins) enrich (Lamp et al., 2020). For this reason, the supernatant is assessed to be the appropriate stillage fraction for a potential fructan separation and maybe the starting point for further respective investigations. Even though the fructan content is relatively low, this material flow (bioethanol stillage) is considered to be a true residue without any other competitive utilization so far. Against the background of increasing demand for prebiotics like fructans not only for human but also animal nutrition, such low-value residues could come into the focus (Zimmermann et al., 2021). Here, the established analytical method may be used for the investigation of respective fructan separation processes.

The established method may be used directly or as a starting point for method adaption analyzing liquid media similar to bioethanol stillage containing dissolved fructans of cereal origin (e.g., stillage from drinking ethanol production).

4 | CONCLUSION

By means of a tailored analytical method, cereal-based bioethanol stillage has been identified as a potential and so far unused source of fructans. For this purpose, a simplified method for quantification of fructans in stillage was introduced allowing for a high sample throughput in comparison to time-consuming and more expensive enzymatic methods. The presented method is based on hexose equivalents analysis; that is, fructose and glucose monomers are determined before and after targeted acidic fructan hydrolysis. On this basis, the released hexoses and, thus, the fructan amount are calculated by means of an anhydrous factor, including the water uptake per monosaccharide unit.

As a result, adapted fructan hydrolysis in bioethanol stillage is conducted with 0.25 wt% sulfuric acid at 100°C

for 80 min. An HPLC-RID system for the determination of the released fructose and glucose monomers is used with both, a lead form and hydrogen form ion exchange column. This developed analytical approach has been successfully compared to literature and a widely used and well-established reference procedure confirming the method's suitability. The presented method can be used for quantification of fructans in ethanol stillages and is expected to be suitable for similar media containing cereal originating fructans and interfering substances like hemicellulose.

The exemplarily analyzed cereal-based bioethanol stillage comprises traceable fructan contents of up to 2.5%DM in the liquid supernatant depending on the stillage's origin. As the DM content of the analyzed stillages (here: about 8%) is simultaneously low as well, a potential separation of fructans makes high demands on the respective process technology. However, the developed analytical method can be the basis for corresponding process development and, thus, can help to expedite higher-value utilization of bioethanol stillage.

ACKNOWLEDGMENTS

The project is supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

Conceptualization and writing of the original draft: Andreas Zimmermann and Martin Kaltschmitt; *Methodology and investigation*: Andreas Zimmermann. All authors consented to publish this study in this journal.

ORCID

Andreas Zimmermann http://orcid.org/0000-0003-2876-8778

REFERENCES

Alassali, A., Cybulska, I., Galvan, A. R., & Thomsen, M. H. (2017). Wet fractionation of the succulent halophyte Salicornia sinus-persica, with the aim of low input (water saving) biorefining into bioethanol. *Applied Microbiology and Biotechnology*, *101*(4), 1769–1779. https://doi.org/10.1007/s00253-016-8049-8

Andersen, L. F., Parsin, S., Lüdtke, O., & Kaltschmitt, M. (2020). Biogas production from straw—The challenge feedstock pretreatment. *Biomass Conversion and Biorefinery*, *12*, 379–402. https://doi.org/10.1007/s13399-020-00740-y

Apolinário, A. C., Lima Damasceno, B. P. G., de Macêdo Beltrão, N. E., de Pessoa, A., Converti, A., & da Silva, J. A. (2014). Inulin-type fructans: A review on different aspects of biochemical and pharmaceutical technology. *Carbohydrate Polymers*, *101*, 368–378. https://doi.org/10.1016/j.carbpol.2013.09.081

Benkeblia, N. (2013). Fructooligosaccharides and fructans analysis in plants and food crops. *Journal of Chromatography. A*, *1313*, 54–61. https://doi.org/10.1016/j.chroma.2013.08.013

Bosscher, D. (2009). Fructan prebiotics derived from inulin. In D. Charalampopoulos, & R. A. Rastall (Eds.), *Prebiotics and probiotics science and technology* (Vol. 151, pp. 163–205). Springer New York. https://doi.org/10.1007/978-0-387-79058-9_6

Dos Santos Lima, M., Nunes, P. C., de Lourdes de Araújo Silva, B., da Silva Padilha, C. V., do Bonfim, T., Stamford, T., da Silva Vasconcelos, M. A., & de Souza Aquino, J. (2019). Determining 1-kestose, nystose and raffinose oligosaccharides in grape juices and wines using HPLC: Method validation and characterization of products from Northeast Brazil. *Journal of Food Science and Technology*, *56*(10), 4575–4584. https://doi.org/10.1007/s13197-019-03936-x

Elvers, B. (Ed.). (2016). *Ullmann's food and feed, 3 volume set*. Wiley-VCH.

Hoebregs, H. (1997). Fructans in foods and food products, ion-exchange chromatographic method: Collaborative study. *Journal of AOAC International*, *80*(80), 1029–1036.

Jovanovic-Malinovska, R., Kuzmanova, S., & Winkelhausen, E. (2014). Oligosaccharide profile in fruits and vegetables as sources of prebiotics and functional foods. *International Journal of Food Properties*, *17*(5), 949–965. https://doi.org/10.1080/10942912.2012.680221

Kaltschmitt, M., Hartmann, H., & Hofbauer, H. (2016). *Energie aus Biomasse*. Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-662-47438-9

Karppinen, S., Myllymäki, O., Forssell, P., & Poutanen, K. (2003). Fructan content of rye and rye products. *Cereal Chemistry Journal*, *80*(2), 168–171. https://doi.org/10.1094/CCHEM.2003.80.2.168

Kiss, A., & Forgo, P. (2011). Investigations on inulin-type oligosaccharides with regard to HPLC analysis and prospective food applicability. *Monatshefte Für Chemie—Chemical Monthly*, *142*(6), 547–553. https://doi.org/10.1007/s00706-011-0485-7

Ko, H., Bae, J.-H., Sung, B. H., Kim, M.-J., Kim, C.-H., Oh, B.-R., & Sohn, J.-H. (2019). Efficient production of levan using a recombinant yeast Saccharomyces cerevisiae hypersecreting a bacterial levansucrase. *Journal of Industrial Microbiology & Biotechnology*, *46*(11), 1611–1620. https://doi.org/10.1007/s10295-019-02206-1

Kuster, B. F. M. (1990). 5-Hydroxymethylfurfural (HMF). A review focussing on its manufacture. *Starch—Stärke*, *42*(8), 314–321. https://doi.org/10.1002/star.19900420808

Lamp, A., Kaltschmitt, M., & Lüdtke, O. (2020). Protein recovery from bioethanol stillage by liquid hot water treatment. *The Journal of Supercritical Fluids*, *155*, 104624. https://doi.org/10.1016/j.supflu.2019.104624

Lichtenthaler, F. W. (2000). Carbohydrates: Occurrence, structures and chemistry, *Ullmann's Encyclopedia of Industrial Chemistry* (p. 372). Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/14356007.a05_079.pub2

Liu, Z., Mouradov, A., Smith, K. F., & Spangenberg, G. (2011). An improved method for quantitative analysis of total fructans in

plant tissues. *Analytical Biochemistry*, *418*(2), 253–259. https://doi.org/10.1016/j.ab.2011.08.004

Liu, Z., & Rochfort, S. (2014). A simple method for simultaneous quantification of total arabinoxylans and fructans in wheat flour. *Journal of Agricultural and Food Chemistry*, *62*(33), 8319–8324. https://doi.org/10.1021/jf501198k

Maharjan, P., Jacobs, J. L., Deighton, M. H., & Panozzo, J. F. (2018). A high-throughput method using ultra-performance liquid chromatography to determine water-soluble carbohydrate concentrations in pasture plants. *Grass and Forage Science*, *73*(2), 562–571. https://doi.org/10.1111/gfs.12315

Matros, A., Peukert, M., Lahnstein, J., Seiffert, U., & Burton, R. (2018). Determination of fructans in plants: Current analytical means for extraction, detection, and quantification. In J. A. Roberts (Ed.), *Annual plant reviews online* (Vol. 13, pp. 117–156). Wiley. https://doi.org/10.1002/9781119312994.apr0672

McCleary, B. V., Charmier, L. M. J., McKie, V. A., McLoughlin, C., & Rogowski, A. (2019). Determination of fructan (Inulin, FOS, Levan, and Branched Fructan) in animal food (animal feed, pet food, and ingredients): Single-laboratory validation, first action 2018.07. *Journal of AOAC International*, *102*(3), 883–892. https://doi.org/10.5740/jaoacint.18-0330

McCleary, B. V., Murphy, A. A., & Mugford, D. C. (2000). Measurement of total fructan in foods by enzymatic/spectrophotometric method: Collaborative study. *Journal of AOAC International*. *83*(2), 356–364.

Michel-Cuello, C., Juárez-Flores, B. I., Aguirre-Rivera, J. R., & Pinos-Rodríguez, J. M. (2008). Quantitative characterization of nonstructural carbohydrates of mezcal Agave (Agave salmiana Otto ex Salm-Dick). *Journal of Agricultural and Food Chemistry*, *56*(14), 5753–5757. https://doi.org/10.1021/jf800158p

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*(3), 426–428. https://doi.org/10.1021/ac60147a030

Nguyen, S. K., Sophonputtanaphoca, S., Kim, E., & Penner, M. H. (2009). Hydrolytic methods for the quantification of fructose equivalents in herbaceous biomass. *Applied Biochemistry and Biotechnology*, *158*(2), 352–361. https://doi.org/10.1007/s12010-009-8596-x

Nobre, C., Teixeira, J. A., & Rodrigues, L. R. (2012). Fructo-oligosaccharides purification from a fermentative broth using an activated charcoal column. *New Biotechnology*, *29*, 395–401. https://doi.org/10.1016/j.nbt.2011.11.006

Pavis, N., Chatterton, N. J., Harrison, P. A., Baumgartner, S., Praznik, W., Boucaud, J., & Prud'homme, M. P. (2001). Structure of fructans in roots and leaf tissues of Lolium perenne. *New Phytologist*, *150*(1), 83–95. https://doi.org/10.1046/j.1469-8137.2001.00069.x

Reis, C. E. R., Rajendran, A., & Hu, B. (2017). New technologies in value addition to the thin stillage from corn-to-ethanol process. *Reviews in Environmental Science and Bio/Technology*, *16*(1), 175–206. https://doi.org/10.1007/s11157-017-9421-6

Sangeetha, P. T., Ramesh, M. N., & Prapulla, S. G. (2005). Recent trends in the microbial production, analysis and application of Fructooligosaccharides. *Trends in Food Science & Technology*, *16*(10), 442–457. https://doi.org/10.1016/j.tifs.2005.05.003

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J. & Templeton, D. (2006). *Determination of sugars, byproducts, and degradation products in liquid fraction process samples: Laboratory Analytical Procedure (LAP)* (Technical Report NREL/TP-510-42623).

Steegmans, M., Iliaens, S., & Hoebregs, H. (2004). Enzymatic, spectrophotometric determination of glucose, fructose, sucrose, and inulin/oligofructose in foods. *Journal of AOAC International*, *87*(5), 1200–1206.

Sukumaran, N. P., & Gopi, S. (2021). Overview of biopolymers, *Biopolymers and their industrial applications* (pp. 1–19). Elsevier. https://doi.org/10.1016/B978-0-12-819240-5.00001-8

Tomasik, P. (Ed.). (2004). *Chemical and functional properties of food components series*. CRC Press. http://search.ebscohost.com/login.aspx?direct=true%26scope=site%26db=nlebk%26db=nlabk%26AN=110759

van den Ende, W., Michiels, A., de Roover, J., & van Laere, A. (2002). Fructan biosynthetic and breakdown enzymes in dicots evolved from different invertases. Expression of fructan genes throughout chicory development. *The Scientific World Journal*, *2*, 1281–1295. https://doi.org/10.1100/tsw.2002.288

Vega, R., & Zuniga-Hansen, M. E. (2015). The effect of processing conditions on the stability of fructooligosaccharides in acidic food products. *Food Chemistry*, *173*, 784–789. https://doi.org/10.1016/j.foodchem.2014.10.119

Verspreet, J., Pollet, A., Cuyvers, S., Vergauwen, R., van den Ende, W., Delcour, J. A., & Courtin, C. M. (2012). A simple and accurate method for determining wheat grain fructan content and average degree of polymerization. *Journal of Agricultural and Food Chemistry*, *60*(9), 2102–2107. https://doi.org/10.1021/jf204774n

Weiß, K., & Alt, M. (2017). Determination of single sugars, including inulin, in plants and feed materials by high-performance liquid chromatography and refraction index detection. *Fermentation*, *3*(3), 36. https://doi.org/10.3390/fermentation3030036

Whitfield, M. B., Chinn, M. S., & Veal, M. W. (2016). Improvement of acid hydrolysis procedures for the composition analysis of herbaceous biomass. *Energy & Fuels*, *30*(10), 8260–8269. https://doi.org/10.1021/acs.energyfuels.6b01390

Zimmermann, A., Visscher, C., & Kaltschmitt, M. (2021). Plant-based fructans for increased animal welfare: Provision processes and remaining challenges. *Biomass Conversion and Biorefinery*. Advance online publication. https://doi.org/10.1007/s13399-021-01473-2

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Zimmermann, A., & Kaltschmitt, M. (2022). Quantification of fructans in cereal-based bioethanol stillage based on a simplified analytical method. *Cereal Chemistry*, 1–14. https://doi.org/10.1002/cche.10533