Quantification of Fructans in Bioethanol Stillage Based On a Simplified Analytical Method

Andreas Zimmermann (andreas.zimmermann@tuhh.de)
Hamburg University of Technology: Technische Universitat Hamburg https://orcid.org/0000-0003-2876-8778

Martin Kaltschmitt
Hamburg University of Technology: Technische Universitat Hamburg

Research Article

Keywords: Fructan, Stillage, HPLC, Fructan analysis, Fructan quantification, Fructan hydrolysis

Posted Date: May 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-507266/v1

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

Zimmermann, A.; Kaltschmitt, M.; Institute of Environmental Technology and Energy Economics,

Hamburg University of Technology, Germany

Corresponding author: Zimmermann, A.; andreas.zimmermann@tuhh.de

Abstract

Bioethanol stillage, the main by-product of industrial bioethanol production, is a potential substrate for fructans. However, the determination and quantification of fructans in such complex sample matrices is still a challenge for the corresponding analytics to be overcome in order to allow for the identification and utilisation of such unused fructan sources. Especially a possible utilisation or rather the corresponding process development requires appropriate analytics first.

Thus, this paper aims to illuminate the basics of fructan quantification in stillage and the corresponding challenges particularly arising with widely used HPLC-RID systems. On this basis, a new approach for fructan quantification is presented based on such HPLC-RID systems allowing for a reliable and especially simple fructan determination in bioethanol stillage for comparably high sample throughput. The developed method performs fructan quantification by determination of fructose and glucose equivalents after a targeted acidic hydrolysis adapted to the respective sample matrix. By means of two different stationary phases, the problem of limited resolution in case of HPLC-RID is overcome and thus measurement errors are reduced. The approach towards the adapted analytical method can be transferred easily to comparable complex sample matrices.

Keywords

Fructan, Stillage, HPLC, Fructan analysis, Fructan quantification, Fructan hydrolysis

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 reserve. Typically larger quantities can be found in plants such as chicory (20 to 25 $\%_{FM}$ (fresh mass)) or artichoke (15 to 20 $\%_{FM}$) [8].

Furthermore, grasses and thus cereals such as wheat (1 to 2 $\%_{DM}$ (dry matter)) and rye (2 to 6 $\%_{DM}$) contain fructans in noticeable amounts [10, 36].

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 glyosidic bond, chain-length). For example, short-chain fructans with a degree of polymerisation (DP) smaller than 10 are usually called fructooligosaccharides (FOS). Fructans can also be differentiated with regard to their type of glyosidic 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 [34].

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 [27]. In general, fructans of the GF_n-type and F_nGF_m-type are non-reducing saccharides as their anomeric hydroxyl groups are interconnected. Accordingly, a ring opening with subsequent oxidation towards 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 non reducing fructans [16, 24, 32].

As fructans are non-digestible for humans while simultaneously enhancing intestinal bacterial growth being beneficial for the host's health, fructans are part of prebiotic dietary fibre. Additionally, their functional properties allow for a usage as a fat-replacer, texturizer or low caloric sweetener [33]. Consequently, fructans are an attractive ingredient for food products with increasing importance due to a continuously growing prebiotic market [31].

Besides plants, complex sample matrices like residues from industrial biomass processing (e.g., chicory roots [39]) 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 [9]. If obtained from a 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 [10]. 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 hydrolysed by either enzymes or acids and the released glucose and primarily fructose are quantified. Nevertheless, quantitative fructan analysis is still a major challenge. This is especially true for unknown samples additionally containing other polysaccharides. In case of stillage, its complex composition impedes the determination of specific carbohydrate compounds and analytical methods for comparable sample matrices are rare and laborious [28]. However, if such potential fructan sources are to be used, simple analytical methods for high sample throughput are required not only for identification of fructan sources but especially for a respective utilisation 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 HPLC-RID system is presented. Finally, the developed analytical method is applied to a specific bioethanol stillage in order to show the applicability and the results to be expected.

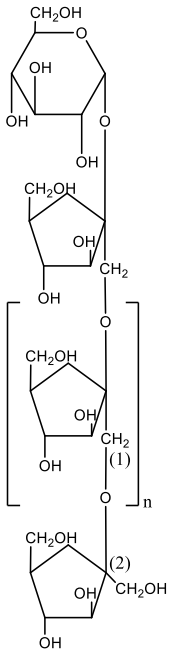

Figure 1: Inulin-type fructan with β-2,1-bonds.

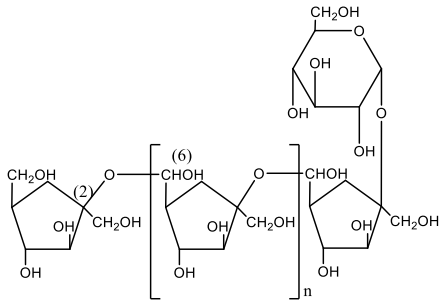

Figure 2: Phlein-type fructan with β-2,6-bonds.

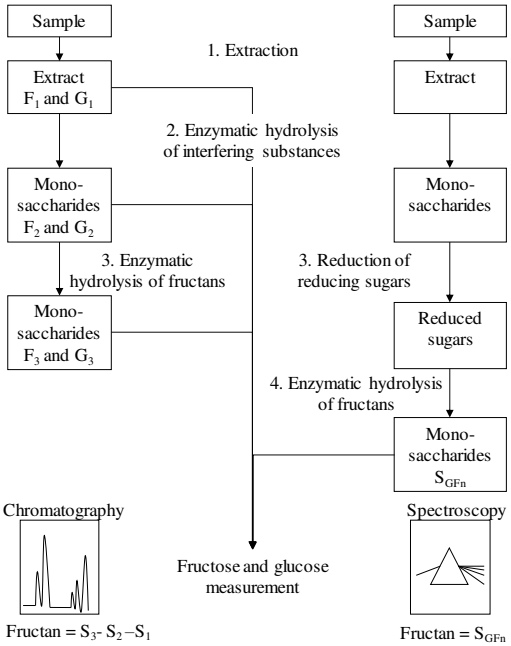

Figure 4: Schematic diagram of AOAC method 997.08 (l.) and 999.03 (r.); Saccharides S (glucose and fructose).

1.1 Bioethanol stillage

Stillage is the main by-product of bioethanol production (Figure 3). In 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) [9]. 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 of stillage dry matter were produced in 2016 potentially containing fructans [15].

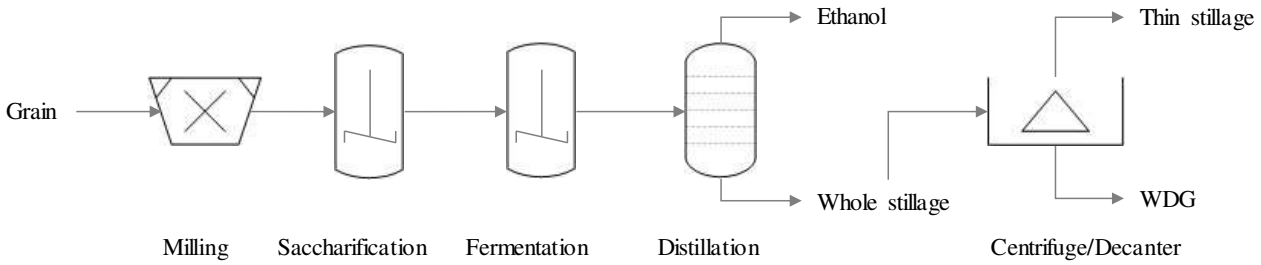

Figure 3: Schematic flow sheet of a conventional bioethanol production; Wet distillers grain (WDG)

1.2 Common analytical methods for fructan quantification

The main problem of quantitative fructan analysis is the lack of available standards, especially in 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 [7]. In general, state of the art fructan analysis always includes the following steps which also apply to the described methods in the following.

A. Extraction of fructans from the sample matrix and determination of free glucose and fructose in the extract.
B. (Selective) hydrolysis of the fructans by means of enzymes or acids and determination of the released glucose and fructose.
C. 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 (also referred to as AACC 32-32.01) and method 997.08 (also referred to as AACC 32-31.01), are both applicable for fructan determination. Commonly these are used for foods and differ, inter alia, in the method of monosaccharide analysis [7, 21, 22].

AOAC method 997.08

Method 997.08 (Figure 4, left) is based on HPLC analysis requiring three chromatographic runs in order 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. Thereof, fructose and glucose released from fructans can be determined. The total fructan amount $S_{fructan}$ is (re)calculated according to equation (1.1). By means of the anhydrous factor k (equation (1.2)), the water uptake per monomer (during hydrolysis) is taken into account. This factor requires the fructans' average degree of polymerisation (DP_{av}) being estimated by the ratio of fructose units per terminal glucose unit (equation (1.3)); i.e. 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 molar mass of a hexose molecule) can be assumed for the anhydrous factor, for example, in case of F_n-type fructans [5, 17]. 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 non-reducing 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 for glucose-containing polymers as well.

AOAC method 999.03

In contrast, method 999.03 is more suitable for samples with high contents of fructose, glucose and/or respective interfering polysaccharides, since disturbing carbohydrates are chemically reduced prior to 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) [22]. Based on this, the total fructan content ($S_{fructan}$) is recalculated via the sum of released fructose and glucose molecules (S_{GFn}) 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 distinct 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 prior to 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) [30]. On the other hand, the presence of galactooligosaccharides (GOS) such as raffinose-derived stachyose may result in an fructan overestimation as they contain fructose and glucose. As these saccharides are non-reducing, GOS are not oxidized in step 3 but partly hydrolysed in step 4 (Figure 4, right). Thus, the use of galactosidase in step 2 may be additionally necessary in order to avoid a respective fructan overestimation [21].

Conclusion

Despite being established, the outlined enzymatic approaches are expensive and especially laborious. As a consequence, these enzymatic methods are considered poorly suitable for bioethanol stillage and a large number of samples as required for instance in process development.

$$S_{fructan} = k\,(F_3 - F_2 - F_1 + G_3 - G_2 - G_1) \qquad 1.1$$

$$k = \frac{180 + 162(DP_{av} - 1)}{180 DP_{av}} \qquad 1.2$$

$$DP_{av} = \left(\frac{F_3 - F_2 - F_1}{G_3 - G_2 - G_1}\right) + 1 \qquad 1.3$$

1.3 Challenges in fructan quantification

Apart from the outlined method-specific difficulties (Chapter 1.2), fructan quantification is generally faced with two main challenges. On the one hand, fructans need to be hydrolysed 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.

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 interferences and thus an overestimation of the fructan content results.

For this reason, the methods outlined above (Chapter 1.2) use specific fructanase enzymes for a 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 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 [4, 20].

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 [29], hydrolysis time [25], and used acids (mostly hydrochloric acid [17, 36] and sulphuric acid [25]). The principle for fructan quantification is always analogous and the aim is to hydrolyse the fructans and to recover the resulting glucose and fructose as completely as possible. However, for acidic hydrolysis a conflict of objectives remains. On the one hand, a complete hydrolysis

should be achieved, and one the other hand, a complete determination of the released glucose and fructose equivalents is envisaged. The latter is impeded by 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 is considered thoughtless [14]. 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, a mild acidic hydrolysis can be relatively selective with regard to fructans and may not hydrolyse starch, cellulose or hemicellulose, if adapted correspondingly to the sample matrix as the latter ones are more stable against temperature and acids [36].

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, liquid chromatography is the method of choice using either refractive index detection (RID) [37], evaporative light scattering detection (ELSD) [1, 11], mass spectrometry (MS) [3], or pulsed amperometric detectors (PAD) [17, 36]. 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 [1]. As a result, particularly in the case of RID, other monosaccharides like xylose and arabinose may strongly interfere, coelute and/or overlay in the resulting chromatogram impeding the determination of fructose and glucose [37].

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.

Conclusion

So far, fructan analysis usually deals with the analysis of comparatively fructan-rich plant materials. However, when it comes to sample matrices with low fructan contents hardly 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 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). An enzymatic approach was considered to be unsuitable in this context, since being costly and time-consuming. For these reasons an approach with acidic fructan hydrolysis has been chosen using HPLC-RID.

2 Materials and methods

2.1 Materials

Chemicals and calibration standards are obtained from Merck (Germany) 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. Sulphuric acid (96 %, H_2SO_4) for hydrolysis and for the preparation of the mobile phase are also purchased from Carl Roth. Calcium carbonate ($CaCO_3$) for neutralisation is obtained from Merck (Germany).

The liquid fraction of stillage after decantation (thin stillage, Figure 3) from a bioethanol production plant processing cereals 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 4,500 min^{-1} for 30 min in order 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 equation (2.1) and (2.2) [2].

$$\log_{10}(R_0) = \log_{10}\left(t \cdot \exp\left(\frac{\vartheta(t) - 100}{14.75}\right)\right) \qquad (2.1)$$

$$\log_{10}(R_0'') = \log_{10}(R_0) + |pH - 7| \qquad (2.2)$$

in which R_0 is the combined severity factor comprising the hydrolysis temperature ϑ in °C and the reaction time t in min; R_0" is the severity factor additionally including the pH and thus the acid concentration.

2.3 Fructan analysis

The developed analytical method for the quantification of fructans in bioethanol stillage is the result of the respective method development presented below (Chapter 3.1 and 3.2).

Fructan hydrolysis is done with H_2SO_4 and subsequent neutralisation with $CaCO_3$. Therefore, a reaction cell (16 mm, pressure tight with screw cap) containing 10 mL of the prepared stillage supernatant is incubated in a thermoreactor (Spectroquant TR420, Merck, Germany) at 100 °C for 80 min with a final H_2SO_4 concentration in the sample of 0.25 wt%. In order 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 neutralisation reaction may slowly continue. For this reason and in order to minimize foaming, $CaCO_3$ should be added slowly. The neutralized sample is centrifuged for 20 min with 4,500 min^{-1} in order to remove the formed calcium sulphate ($CaSO_4$) and the excess $CaCO_3$. Subsequently, the supernatant is centrifuged again with 14,000 min^{-1} at 4 °C for 30 min prior to analysis in order to guarantee a particle-free sample for HPLC analysis. Similarly, a respective non-hydrolysed stillage sample is centrifuged with 14,000 min^{-1} for 30 min before HPLC analysis for determining the free monosaccharides prior to hydrolysis.

An Agilent 1260 Infinity II LC system with a refractive index detector (RID) is used for carbohydrate separation by means of both an Agilent Hi-Plex H column (7.7 x 300 mm, 8 µm) and a Hi-Plex Pb column (7.7 x 300 mm, 8 µm) each with the respective guard column. In case of the Pb column an additional deashing guard column (Bio-Rad) was used 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 (RID) operating at 55 °C. The injection volume was ≥ 10 µL. The fructan content was calculated based on dry matter of the stillage analogous to equation (1.1) to (1.3) via the fructose and glucose equivalents released after fructan hydrolysis (subtracted from fructose and glucose before hydrolysis).

The HPLC conditions can be summarized as follows:

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

3 Results and discussion

In accordance with the outlined challenges (Chapter 1.3), 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.

3.1 Optimisation of the HPLC-RID analysis

An appropriate separation and thus reliable determination of the hexose equivalents is mandatory for fructan analysis. In order to allow for the identification of potentially interfering substances during analysis, the composition of the used stillage was determined initially (Table 1). Based on this an appropriate HPLC-RID setup with the accompanying HPLC method was searched.

Approach

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. Here, either cationic exchange resins (mainly hydrogen, lead or sodium form) or amino phases are typically applied [6]. These potential columns for fructan analysis together with their main drawbacks are listed in Table 2.

For the column screening experiments, mixtures of glucose, fructose, xylose and arabinose were analysed. The last-mentioned pentoses are part of hemicellulose, one of the main potentially interfering polysaccharides in bioethanol stillage (Table 1). Moreover, stillage samples and fructan standards were analysed using the mentioned columns and assessed with regard to monosaccharide resolution. For the column assessment, particularly the resolution of glucose and fructose and their overlays with interferences were compared (data not shown).

Table 1 Composition of the liquid supernatant of the used thin stillage from bioethanol production with about 8 $\%_{DM}$ (dry matter).

$\%_{DM}$	Carbohydrates							
	Mono-saccharides[1)]	Hemicellulose[2a)]	Glucan[2b)]	Proteins[3)]	Lipids[4)]	Ash[5)]	Glycerol[1)]	Residual[6)]
	14	17	≥ 14	24	< 0.2	11	10	< 10

[1)] HPLC mainly glucose, arabinose and xylose
[2)] HPLC after hydrolysis with 2 wt% H_2SO_4, 1 h, 120 °C;
[2a)] released xylose and arabinose
[2b)] released glucose mainly from cellulose and starch
[3)] Kjeldahl: Factor 6.25 DIN EN ISO 20483
[4)] Soxleth: DIN EN ISO 11085
[5)] Gravimetery: 550 °C DIN EN ISO 18122
[6)] Including organic acids, e.g. formic and acetic acid

Results

Based on the experimental column screening, the following assessment of the commonly used stationary phases for fructan analysis with an HPLC-RID can be summarized as shown in Table 2. Columns with calcium [29] and silver [35] form resins have also been considered but a priori assessed as inappropriate for stillage. In case of the former this is due to the coelution of monosaccharides and in case of the latter this is due to high sensitivity against impurities.

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 (especially coelutions with fructose). However, based on Table 2 an hydrogen form column (H) was considered to be most appropriate for frequently carried out fructan analyses due to its durability in sample matrices like stillage. Additionally, a 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. In case of the Pb column, the problem of ion interferences (from salts) can be overcome by means of respective deashing cartridges prior to the main column.

Consequently, both columns need to be used in succession to determine fructose and glucose reliable 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 (e.g., xylose and arabinose) and alcohol (e.g., glycerol) tending to coelution and overlay on the respective columns.

As a result of these considerations and experimental column screening, the optimized HPLC method has been obtained as outlined in Chapter 2.3 using a Pb as well as a H column.

3.2 Optimisation of the acidic fructan hydrolysis

Acidic fructan hydrolysis is performed in order to release the glucose and fructose equivalents for the subsequent determination by the developed HPLC method. The aim is to hydrolyse the fructans as completely as possible while avoiding consecutive reactions (mainly dehydration to HMF). Usually hydrochloric acid (HCl) [37], trichloroacetic acid (TFA) [16] or H_2SO_4 [26] is used with or without subsequent neutralisation, depending, inter alia, on the hexose equivalent analysis method (e.g., in case of acid sensitive HPLC columns). If performed, neutralisation is commonly done by addition of sodium carbonate (Na_2CO_3) or $CaCO_3$, sodium hydroxide (NaOH) or acid removal in case of TFA [16, 26, 37].

Due to toxicity and the laborious removal via evaporation, TFA was a priori considered to be inappropriate for routine analysis. However, a neutralisation step was basically considered to be reasonable as ions show interfering signals in the resulting chromatogram and may continuously damage the Pb column. Hence, the ion proportion in the sample should be

reduced as much as possible prior to HPLC analysis. Consequently, H_2SO_4 with subsequent $CaCO_3$ treatment was considered to be most appropriate, because during neutralisation poorly soluble $CaSO_4$ is formed, visibly precipitates and thus reduces the ion load within the sample. In contrast, HCl generally forms well soluble salts resulting in a higher ion load within the sample consequently requiring a more frequent exchange of the deashing cartridges.

Table 2 Overview of stationary phases (functional groups) typically used for fructan quantification via HPLC-RID, example applications and main drawbacks for stillage analysis; Fructooligosaccharides (FOS).

Sodium Na^+
Application example: Fermentation broth from fructan production [12]. **Main drawback for fructan analysis in stillage:** Sensitivity, e.g. to salts and acids; coelution of fructose and xylose. **Conclusion:** The sodium 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). Moreover, these resins are sensitive against acids and salts making sample preparation laborious.
Amino NH_2
Application example: Fermentation broth from FOS production [26]. **Main drawback for fructan analysis in stillage:** Insolubility of high-molecular substances (e.g. polysaccharides) in the mobile phase (acetonitrile/water); coelution of fructose and arabinose. **Conclusion:** The widely used amino phase 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.
Hydrogen H^+
Application example: Fermentation broth from FOS production [13]. **Main drawback for fructan analysis in stillage:** Coelution of fructose and xylose. **Conclusion:** The hydrogen form column showed coelution of xylose and fructose as well, but 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. Column degradation can be overcome by using diluted sulphuric acid as an eluent guarantying continuous stationary phase regeneration.
Lead Pb^{2+}
Application example: Sensitivity, e.g. to ions and proteins. **Main drawback for fructan analysis in stillage:** (Hydrolysed) commercial fructans and grasses [25, 37]. **Conclusion:** The lead form column (Pb) showed the comparatively best resolution for the respective monosaccharides (glucose, fructose, xylose, arabinose) allowing for a 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. Moreover, the coelution of glycerol (Table 1) and fructose could be observed while analysing stillage.

Approach

Response Surface Methodology was used for hydrolysis optimisation with H_2SO_4 and $CaCO_3$. Hydrolysis temperature, time and acid concentration were varied with regard to a maximisation of the recovery of glucose and fructose equivalents

and thus fructan recovery; i.e. the maximum detectable fructan content was wanted. Simultaneously, the formation of HMF from hexose was considered for optimisation as well; i.e. HMF formation should be concurrently minimized. A Box-Behnken design (Table 3) was employed for these experiments using the commercially available statistical software Design-Expert (Stat-Ease). In case of the fructan content, a quadratic model was fitted to the experimental data, while in case of the HMF formation a Two Factor Interaction (2FI) model was used.

Following the outlined procedure above (Chapter 2.3), 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 3). The design space boundaries were selected based on preliminary screening tests (data not shown).

Table 3 Box-Behnken design for fructan hydrolysis optimisation: factors and values for three different levels.

	Level		
Variable	**-1**	**0**	**+1**
Temperature in °C	90	100	110
Time in min	40	60	80
Acid concentration in wt%	0.1	0.5	0.9

Results

Figure 5 shows surface plots 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 5, left) shows a maximum due to an increasing degradation of fructans to its monomers with increasing temperature and acid concentration and thus increasing severity factor (equation (2.1)). For comparably higher severity factors 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 5, right) increases steadily with increasing severity factor due to an increasing release of hexoses and an increasing reaction rate with higher temperature and/or acid concentration.

By means of numerical optimisation, the optimal hydrolysis conditions can be found based on the regression models. The aim is to achieve a fructan hydrolysis as complete as possible while completely detecting the released monosaccharides. The target figures for the optimisation are rated with respect to their importance as follows. The maximisation of the fructan content is rated high and the minimisation of the HMF formation is rated medium. A further boundary condition is the minimisation of the acid concentration (low importance) as ions in the sample may impede HPLC analysis as outlined above. Furthermore, the neutralisation procedure is simplified by using less $CaCO_3$ being also a practical benefit. The hydrolysis temperature and time are not limited for this optimisation and thus may lie in the range of the design space. The numerical solution for this optimisation is given in Table 4.

Using these numerically determined conditions, the hydrolysis was conducted using the stillage with the aim of 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 in order to successfully confirm the numerical result on an experimental basis (supplementary material). 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. Thus, the analytical method described in Chapter 2.3 results.

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

	Temperature in °C	**Time in min**	**Acid concentration in wt%**
Numerical Optimisation	103	80	0.27
Experimental Optimisation	**100**	**80**	**0.25**

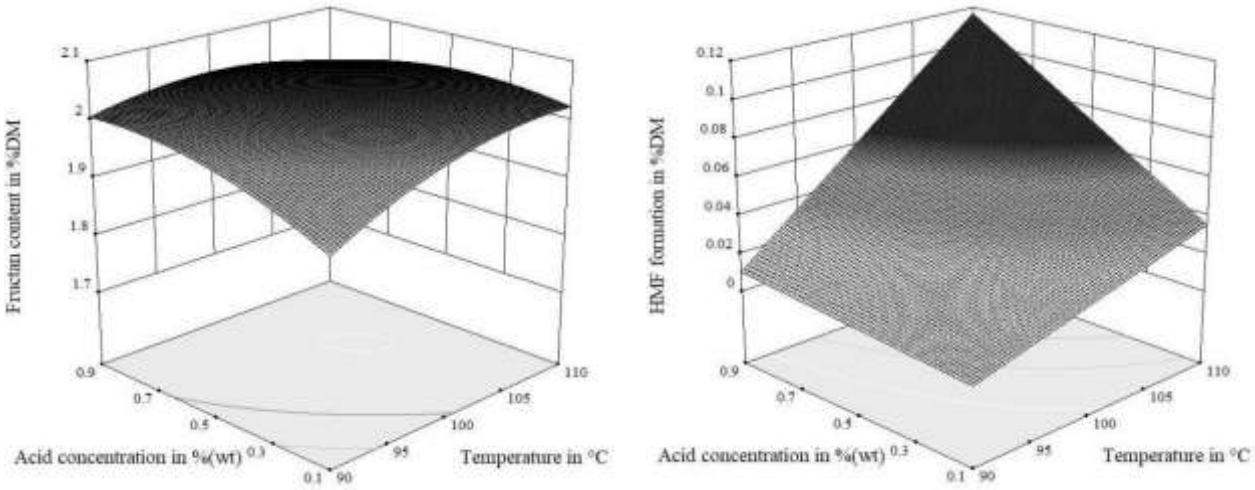

Figure 5: Response surface plots for the fructan content (l.) and HMF formation (r.) both based on stillage dry matter depending on temperature in °C and acid concentration in wt% (H_2SO_4) for a hydrolysis time of 80 min and a subsequent neutralisation with $CaCO_3$.

3.3 Method suitability and application

Potential errors and drawbacks of the developed analytical method are discussed below for both the HPLC analysis and the fructan hydrolysis procedure.

HPLC-RID method

A comparable HPLC method has been used for plant and feed material determining monosaccharides by means of a Pb column with good reproducibility [37]. Another study used a combination of H and Pb column for quantifying carbohydrates after hydrolysis of plant material analogous to this paper; i.e. a H column has been used 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 hydrolyse other polysaccharides than fructans [1]. Nevertheless, these studies corroborate the suitability of the developed HPLC method for the quantification of fructose and glucose equivalents from fructans in stillage.

Fructan hydrolysis method

The developed hydrolysis method using H2SO4 and CaCO3 (Table 4) was conducted using commercial FOS from chicory and inulin from dahlia in order to determine the fructan recovery. The recovery was determined to be greater than 92 ± 0.2 % for both fructan types. The discrepancy can be explained by potential measurement inaccuracies and/or incomplete hydrolysis. In order to exclude the latter, the Pb column for the detection of potential non-hydrolysed oligomeric or polymeric carbohydrates. Using this HPLC setup did not significantly detect any respective saccharides with DP > 2; i.e. under the optimized conditions, a more or less total fructan hydrolysis can be assumed (supplementary material). 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).

As shown in Table 1, glucose containing oligo- and/or polysaccharides (glucans) are present in the stillage, probably starch residues and cellulose. In this regard, it has been 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 (supplementary material). Due to its greater stability (in comparison to starch), cellulose degradation can be neglected as well [38]. Even though these compounds are hardly hydrolysed under the optimized conditions, however, high proportions of especially starch may falsify the fructan measurement. In such cases as prior amylase treatment might become necessary in order to degrade starch prior to fructan hydrolysis.

The presence of sucrose is one of the most important error sources for fructan quantification as sucrose is easily acidly hydrolysed and released fructose and glucose may falsify the results. In 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 prior to hydrolysis in the initial stillage (data not shown). However, if sucrose is present, its amount has to be determined by HPLC prior to hydrolysis and respectively subtracted.

Fructose and glucose degradation may also falsify the measured fructan content. However, fructose and glucose standards showed 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 case of glucose due to the reduced stability of fructose [25]. In this context, a sugar recovery standard containing the monosaccharides of interest may be used analogous to the sample

in order to correct for respective losses. Therefore, the amount of formed HMF is multiplied by the correction factor 180/126 in order to obtain the amount of respective initial hexose equivalents.

Hexoses like galactose or mannose, potentially interfering the HPLC analysis, could not be detected under the found hydrolysis conditions. In conclusion, such hexoses are not significantly released from polysaccharides within stillage. In contrast, potentially interfering pentoses like xylose are slightly released (from hemicellulose) under the found hydrolysis conditions. As the xylose may coelute with fructose on the H column, the auxiliary use of the Pb column is required. In comparison, arabinose is released significantly under these conditions from the stillage, but does not cause any problems during quantification with the used HPLC setup, neither on the H column nor on the Pb column (supplementary material).

A further potential source of error is the neutralisation step. Potential losses of HMF (if considered) during the neutralisation with $CaCO_3$ (as the produced CO_2 may entrain HMF) may result in an underestimation of the hexose equivalents and thus fructans. This also applies for a potential co-precipitation of monosaccharides by the formed $CaSCO_4$. However, this co-precipitation could not be detected in a respective precipitation trial with a solution containing glucose, fructose, arabinose and xylose standards (data not shown).

In comparison to the developed hydrolysis method, an equivalent acidic hydrolysis method can be found in literature [25]. 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). Based on the severity factor, the presented optimized hydrolysis conditions were compared additionally to other literature on acidic fructan hydrolysis [17–19, 23, 36]. The conditions within this paper or rather the severity factor is consistent with the average calculated severity factor of 7.6 ($n = 6$, supplementary material). These circumstances corroborate the developed hydrolysis procedure.

Reference analytics

As no literature values on fructans in stillage have been reported so far, a reference analysis was conducted for the assessment of the developed overall fructan analysis procedure. Therefore, the widely-used Fructan Assay Kit (Megazyme) was used following the outlined AOAC method 999.03 using enzymatic hydrolysis and a spectroscopic measurement. The following values for the fructan content in the stillage sample were obtained using the developed method in quintuplicate and the Megazyme procedure in triplicate.

Acidic hydrolysis and HPLC: 2.4 ± 0.1 $\%_{DM}$ ($n = 5$) Megazyme Fructan Kit: 2.5 ± 0.1 $\%_{DM}$ ($n = 3$)

Both method repetitions show similar standard deviations but a slight deviation of the measured value. As the recovery of the developed method has shown to slightly underestimate the actual fructan content (> 92 %), this method comparison is assed to be reasonable. This comparison substantiates the suitability of the entire developed method.

Apart from that, the acidic hydrolysis with subsequent HPLC analysis offers the advantage of determining fructose and glucose separately allowing for an estimation of DP_{av} via fructose units per glucose unit (equation (1.3)). Moreover, the developed method is comparably convenient, easy to establish for existing HPLC systems and suited for large sample volumes in comparison to the laborious AOAC method.

Application of the developed analytical method

The developed method has been used for the analysis of another batch of cereal-based raw thin stillage and its corresponding fractions after centrifugation in laboratory scale (Table 5). Here, the stillage was diluted to a dry matter content of 8 % prior to centrifugation in order to reduce its viscosity.

As expected, the fructan proportion in the stillage's dry matter increases in the liquid supernatant, as the soluble fructans accumulate in this aqueous phase. Simultaneously, the fructan proportion in the sediment decreases, while insolubles (in particular insoluble proteins) enrich [15]. For this reason, the supernatant is assessed to be the appropriate stillage fraction for a potential fructan separation and may be 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 utilisation so far. Against the background of an increasing demand for prebiotics like fructans not only for human but also animal nutrition, such low value residues could come into the focus [39]. Here, the established analytical method may be used for the investigation of respective fructan separation processes. As the stillage's fructans originate from cereals, a branched structure can be assumed [36]. However, a respective characterisation of the molecular structure is of interest in addition to the conducted quantification.

The established method may be used directly or as a starting point for method adaption analysing liquid media similar to bioethanol stillage containing dissolved fructans of cereal origin (Table 1), for example, stillage from drinking ethanol production and fermentation broths based on cereals.

Table 5 Fructan content in cereal-based thin stillage, its supernatant and the corresponding sediment after aqueous dilution and subsequent centrifugation

Stillage Fraction	Dry matter (DM) in %	Fructan content in $\%_{DM}$
Thin stillage; raw	17	1.0
Thin stillage, supernatant; diluted, 4,500 min^{-1}, 30 min	8	2.2
Thin stillage, sediment; diluted, 4,500 min^{-1}, 30 min	20	0.5

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; i.e. fructose and glucose monomers are determined before and after a targeted acidic fructan hydrolysis. Based on this, the released hexoses and thus the fructan amount is 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% sulphuric 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 a 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 analysed cereal-based bioethanol stillage comprises traceable fructan contents of about 2.0 to 2.5 $\%_{DM}$ in the liquid supernatant. As the dry matter content of the analysed 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 a corresponding process development and thus can help to expedite higher-value utilisation of bioethanol stillage.

Acknowledgement

We would like to thank the editorial board and the anonymous reviewers for their valuable assistance.

5 Declarations

Funding

This research has been funded by the German Federal Office for Agriculture and Food.

Data availability

The authors declare that the data and materials are transparent.

Ethics approval and consent to participate

This article followed the ethical standard of the institute. All authors consented to participation in this research.

Authors' contributions and consent for publication

Conceptualisation and writing of the original draft: A.Z., M.K.; Methodology and investigation: A.Z. All authors consented to publish this research in this journal.

Conflict of interest

The authors declare that they have no conflict of interest.

6 References

1. 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. doi:10.1007/s00253-016-8049-8.
2. Andersen, L. F., Parsin, S., Lüdtke, O., & Kaltschmitt, M. (2020). Biogas production from straw—the challenge feedstock pretreatment. *Biomass Conversion and Biorefinery*. doi:10.1007/s13399-020-00740-y.
3. 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. doi:10.1016/j.carbpol.2013.09.081.
4. Benkeblia, N. (2013). Fructooligosaccharides and fructans analysis in plants and food crops. *Journal of chromatography. A,* 1313, 54–61. doi:10.1016/j.chroma.2013.08.013.
5. 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). New York, NY: Springer New York.
6. Dos Santos Lima, M., Nunes, P. C., Lourdes Araújo Silva, B. de, da Silva Padilha, C. V., do Bonfim, T. H. F., Stamford, T. L. M., et al. (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. doi:10.1007/s13197-019-03936-x.
7. Hoebregs, H. (1997). Fructans in foods and food products, ion-exchange chromatographic method: collaborative study. *Journal of AOAC International, (*80), 1029–1036.
8. 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. doi:10.1080/10942912.2012.680221.
9. Kaltschmitt, M., Hartmann, H., & Hofbauer, H. (2016). *Energie aus Biomasse* . Berlin, Heidelberg: Springer Berlin Heidelberg.
10. Karppinen, S., Myllymäki, O., Forssell, P., & Poutanen, K. (2003). Fructan Content of Rye and Rye Products. *Cereal Chemistry Journal,* 80(2), 168–171. doi:10.1094/CCHEM.2003.80.2.168.
11. 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. doi:10.1007/s00706-011-0485-7.
12. Ko, H., Bae, J.-H., Sung, B. H., Kim, M.-J., Kim, C.-H., Oh, B.-R., et al. (2019). Efficient production of levan using a recombinant yeast Saccharomyces cerevisiae hypersecreting a bacterial levansucrase. *Journal of industrial microbiology & biotechnology,* 46(11), 1611–1620. doi:10.1007/s10295-019-02206-1.

13. Ko, H., Bae, J.-H., Sung, B. H., Kim, M.-J., Park, H.-J., & Sohn, J.-H. (2019). Microbial production of medium chain fructooligosaccharides by recombinant yeast secreting bacterial inulosucrase. *Enzyme and microbial technology,* 130, 109364. doi:10.1016/j.enzmictec.2019.109364.
14. Kuster, B. F. M. (1990). 5-Hydroxymethylfurfural (HMF). A Review Focussing on its Manufacture. *Starch - Stärke,* 42(8), 314–321. doi:10.1002/star.19900420808.
15. Lamp, A., Kaltschmitt, M., & Lüdtke, O. (2020). Protein recovery from bioethanol stillage by liquid hot water treatment. *The Journal of Supercritical Fluids,* 155, 104624. doi:10.1016/j.supflu.2019.104624.
16. Lichtenthaler, F. W. (2000). Carbohydrates: Occurrence, Structures and Chemistry. In *Ullmann's Encyclopedia of Industrial Chemistry* (1-4, p. 372). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA.
17. 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. doi:10.1016/j.ab.2011.08.004.
18. 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. doi:10.1021/jf501198k.
19. 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. doi:10.1111/gfs.12315.
20. 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.
21. 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. doi:10.5740/jaoacint.18-0330.
22. 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.
23. 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. doi:10.1021/jf800158p.
24. Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry,* 31(3), 426–428. doi:10.1021/ac60147a030.
25. 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. doi:10.1007/s12010-009-8596-x.
26. Nobre, C., Teixeira, J. A., & Rodrigues, L. R. (2012). Fructo-oligosaccharides purification from a fermentative broth using an activated charcoal column. *New biotechnology,* 29(3), 395–401. doi:10.1016/j.nbt.2011.11.006.
27. Pavis, N., Chatterton, N. J., Harrison, P. A., Baumgartner, S., Praznik, W., Boucaud, J., et al. (2001). Structure of fructans in roots and leaf tissues of Lolium perenne. *New Phytologist,* 150(1), 83–95. doi:10.1046/j.1469-8137.2001.00069.x.
28. 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. doi:10.1007/s11157-017-9421-6.
29. 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. doi:10.1016/j.tifs.2005.05.003.
30. 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.
31. Sukumaran, N. P., & Gopi, S. (2021). Overview of biopolymers. In *Biopolymers and their Industrial Applications* (pp. 1–19): Elsevier.
32. Tomasik, P. (Ed.) (2004). *Chemical and functional properties of food saccharides* (Chemical and functional properties of food components series). Boca Raton: CRC Press.
33. (2016). *Ullmann's food and feed, 3 volume set* : Wiley-VCH.

34. van den Ende, W., Michiels, A., Roover, J. de, & van Laere, A. (2002). Fructan biosynthetic and breakdown enzymes in dicots evolved from different invertases. Expression of fructan genes throughout chicory development. *TheScientificWorldJournal,* 2, 1281–1295. doi:10.1100/tsw.2002.288.
35. 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. doi:10.1016/j.foodchem.2014.10.119.
36. Verspreet, J., Pollet, A., Cuyvers, S., Vergauwen, R., van den Ende, W., Delcour, J. A., et al. (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. doi:10.1021/jf204774n.
37. 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. doi:10.3390/fermentation3030036.
38. 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. doi:10.1021/acs.energyfuels.6b01390.
39. Zimmermann, A., Visscher, C., & Kaltschmitt, M. (2021). Plant-based fructans for increased animal welfare: provision processes and remaining challenges. *Biomass Conversion and Biorefinery*. doi:10.1007/s13399-021-01473-2.

Figures

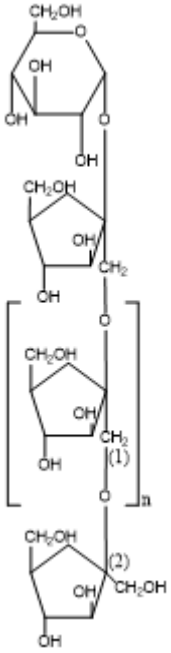

Figure 1

Inulin-type fructan with β-2,1-bonds.

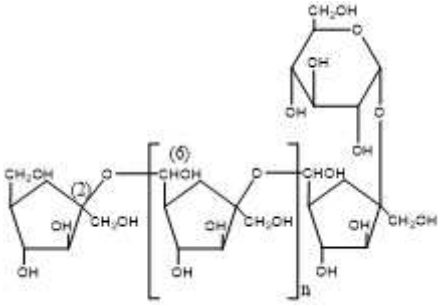

Figure 2

Phlein-type fructan with β-2,6-bonds.

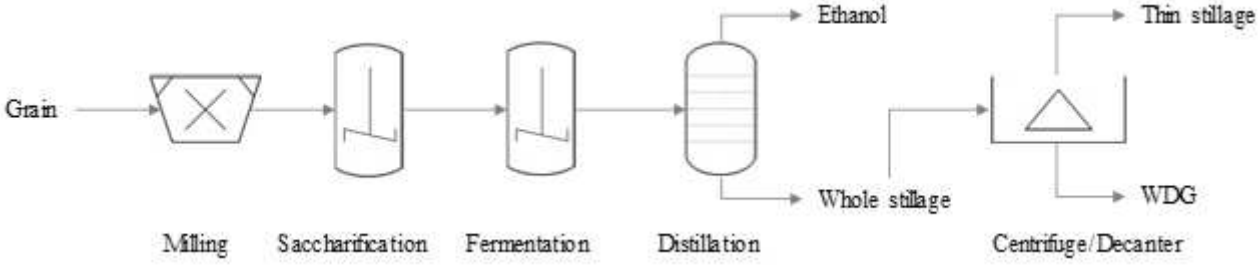

Figure 3

Schematic flow sheet of a conventional bioethanol production; Wet distillers grain (WDG)

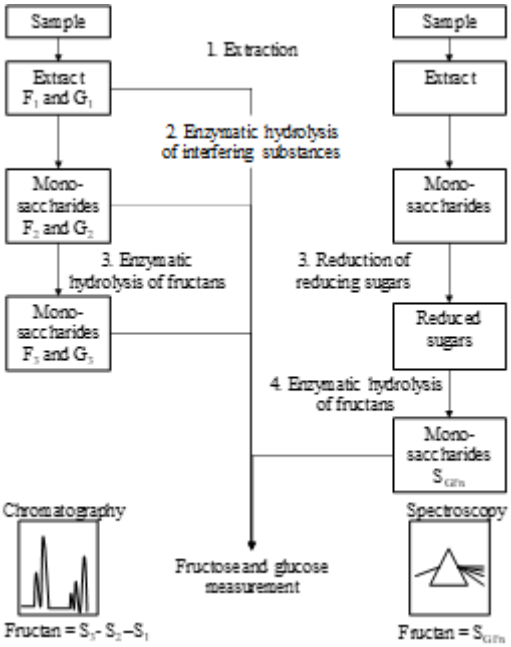

Figure 4

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

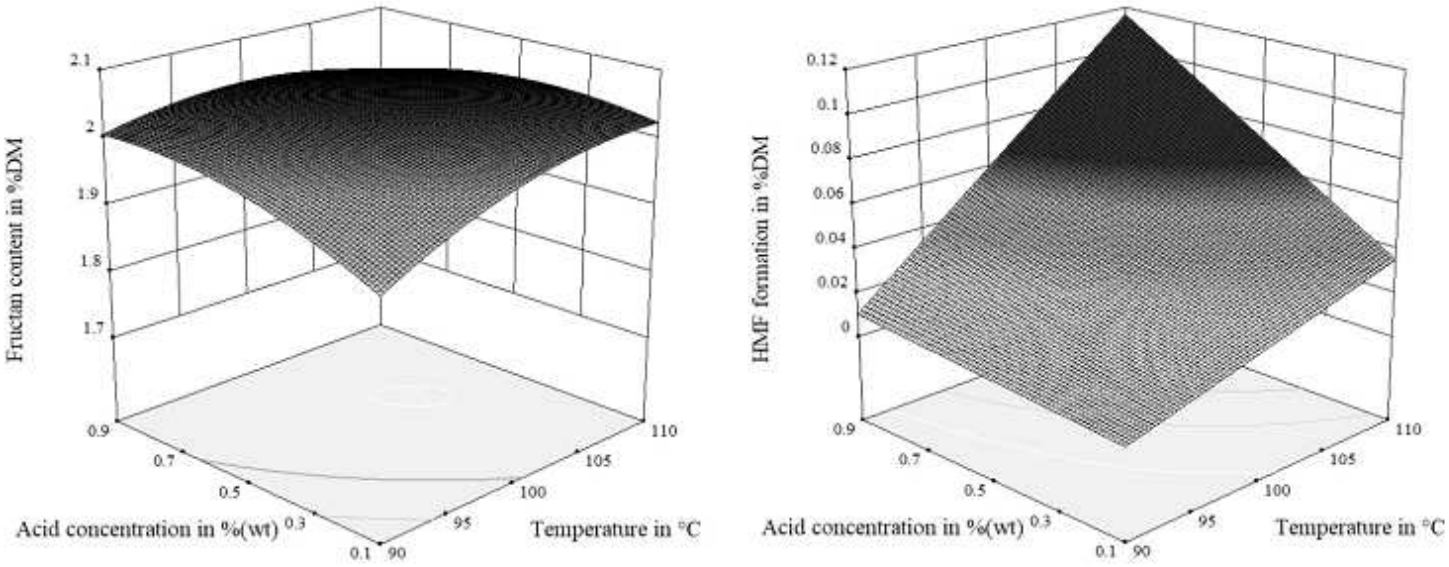

Figure 5

Response surface plots for the fructan content (l.) and HMF formation (r.) both based on stillage dry matter depending on temperature in °C and acid concentration in wt% (H2SO4) for a hydrolysis time of 80 min and a subsequent neutralisation with CaCO3.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ResearchFructananalyticsSupplementarymaterial.docx