Optimization and Validation of a HPAEC-PAD Method for the Quantification of FODMAPs in Cereals and Cereal-based Products

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ABSTRACT: This study presents an analytical method for the quantification of fermentable oligo-, di-, monosaccharides and polyols (FODMAPs) in cereals and cereal-based products, considering diverse ingredients, such as different cereals in addition to wheat, pulses or pseudocereals. All carbohydrates have been separated, identified and quantified with a high-performance anion exchange chromatographic system coupled with pulsed amperometric detection (HPAEC-PAD). The total fructan content and the average degree of polymerization (DP_{av}) have been determined after enzymatic hydrolysis to the monomers glucose and fructose, based on the principle of the official method for fructan quantification in food products, AOAC 997.08. Extraction, separation and detection as well as fructan determination methods are based on several other studies and were modified in order to minimize interferences in the analysis. The method has been validated with regard to the limits of detection and quantification, linearity, repeatability and accuracy as well as the DP_{av} of the fructans.

KEYWORDS: FODMAPs, IBS, cereals, HPAEC-PAD, fructans, enzymatic hydrolysis, quantification, degree of polymerization, galactooligosaccharides

INTRODUCTION

Fermentable oligo-, di-, monosaccharides and polyols (FODMAPs) are carbohydrates often associated with symptoms of irritable bowel syndrome (IBS). They comprise galactooligosaccharides, fructans and fructooligosaccharides (FOS), lactose, fructose in excess of glucose and polyols. *GOS* (often in not FODMAP related literature referred to as raffinose family oligosaccharides, RFO) are α-galactose derivates (1 → 6 linked) from sucrose (α-glucose 1 → 2 linked to ß-fructose) and found especially in pulses, but also in different grains such as wheat, barley or rye.[1,2] *Fructans* are composed of fructose-chains, containing one glucose residue. Depending on the fructan-source different types are classified. Inulin-type and levan-type fructans are linear β (2 → 1) or β (2 → 6) fructosyl-fructose chains with one final glucose residue. The branched group, called graminan-type fructans, contains both types of linkages (β (2 → 1) or β (2 → 6)) and is commonly found in cereals. As humans do not possess the enzymes α-galactosidase or fructanases, fructans as well as GOS are not digested in the small intestine, delivered to the large intestine and fermented by colonic bacteria, inducing gas formation.[3] Also the β (1 →4) linked galactosyl-glucose disaccharide, *lactose*, is not tolerated by some individuals, due to the lack of the enzyme lactase. Lactose is the major FODMAP in dairy products. If the monosaccharide *fructose* appears *in excess of glucose*, it is not absorbed sufficiently. This may be relevant in fermented cereal-products, depending on the fermentation-conditions.[4,5] At the last, *polyols*, which are reduced forms of sugars in their chemical structure, also called sugar alcohols are as well poorly absorbed and may also be found in fermented cereal-products.[6]

Studies have shown that a reduction in the intake of FODMAPs (the low FODMAP diet) is an efficacious therapeutic approach to reduce IBS-symptoms.[7,8] Hence, current research increasingly focusses on the development of functional food products with lowered FODMAP contents; whereas a standardized analytical tool for the determination of FODMAPs in diverse food matrices is required[6,9] This study aimed to develop an analytical method for the quantification of FODMAPs in cereals and cereal-based products, considering the composition

of the products may be very diverse, in particular due to beneficial techno-functional or nutritional properties of specific novel ingredients.

The successfully applied analytical methodology, described by Muir et al. (2007) comprises the liquid chromatographic separation coupled with evaporative light scattering detection (HPLC-ELSD) for the quantification of the smaller FODMAPs, combined with the photometric determination of glucose and fructose after enzymatic hydrolysis of fructans, using an enzyme assay (HK-FRUC from Megazyme), a modified version of the original AOAC method 999.03 (K-FRUC from Megazyme).[10,11] However, these two photometric methods do not deliver information about the average degree of polymerization (DP_{av}) of fructans. Knowledge about chain length is important as putative beneficial properties or induction of IBS-symptoms may relate to the DP of fructan molecules.[12] Muir et al. (2007) proposed a modification of the HK-FRUC assay, which enables an estimation of the DP_{av}.[10] Yet, the photometric determination has a low analytical sensitivity and thus high detection limits, leading to unreliable results if fructan levels are below 1 % on dry weight basis. Furthermore, the combination of different analytical methods may be laborious for many samples. Also, both fructan assays must be amended with the additional GOS correction step (incubation of the sample extracts with α-galactosidase) as fructose released from GOS by the enzyme inulinase, included in the assay, leads to an overestimation of the total fructan content, which increases the complication of the assays. Another study, conducted by Chumpitazi et al. (2018), quantified all FODMAPs using a number of different enzyme assay kits.[13]

High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) has increasingly gained popularity for the analysis of carbohydrates, due to its ability to separate different classes of carbohydrates (sugar alcohols, mono-, di- and oligosaccharides as well as polysaccharides) and its high sensitivity.[14]

Ziegler et al. (2016) proposed an analytical method based on HPAEC-PAD for the quantification of FODMAPs in wheat.[5] However, their approach for the quantification of fructans is not suitable if other fructan sources than wheat or a combination of different sources

(diverse recipes of cereal-products) are considered. Generally, there are two approaches to quantify fructans, classified as “direct” and “indirect” method by Stöber et al. (2004).[15] The direct method comprises the evaluation of each peak in the chromatogram belonging to fructan molecules of different chain lengths and linkages. The reliability of this approach is limited due to several factors. On the one hand, only few reference standards of fructans are available to ensure an accurate quantification. On the other hand, the identification and assignment of peaks as fructans for different sources may be very laborious and interfered by other oligo- and polysaccharides than fructans. Thus, for example Haskå et al. (2008) hydrolyzed sample extracts with the enzyme amyloglucosidase, to remove coeluting malto-dextrins from the fructans’ fingerprint.[16] However, the fingerprint may be very different for varying fructan sources but also dependent on storage conditions or processing of the fructan containing material; this requires a tedious characterization of each material.

Hence, the indirect approach for the determination of the total fructan content is simpler and considered as more reliable. Thereby, fructans are hydrolyzed to their monomers fructose and glucose. The amounts of those sugars are used for the calculation of the total fructans amount and the DP_{av}. Two different approaches are described, the acid and the enzymatic hydrolysis of the fructans.[15,17–20] This study applied an adapted version of the method described by Huynh et al. (2008) for the quantification of fructans after extraction and separation of all FODMAPs via HPAEC-PAD using a modified version of the method described by Ziegler et al. (2016).[5,18]

MATERIALS AND METHODS

Materials. Ultrapure water, with a resistivity of 18.2 MΩ*cm and a total organic carbon (TOC) content < 5 ppb (ASTM Type I), used for the preparation of HPAEC-PAD eluents, all standard solutions and sample preparation, was obtained from a Thermo Scientific™Dionex™IC Pure™ Water purification system (Sunnyvale, CA, USA). Extra pure, 50 % w/w sodium hydroxide (NaOH) solution (in water) was purchased from Thermo Fisher Scientific (ACROS Organics™; Dublin, Ireland). Electrochemical-grade sodium acetate (NaOAc) was purchased from Thermo

Scientific™ (Dionex™ AAA-Direct Reagents; Dublin, Ireland). HPLC-grade acetonitrile (CH_3CN) as well as methanol (MeOH) were purchased from Sigma-Aldrich (Darmstadt, Germany) and glacial acetic acid (AcOH) from fisher scientific (J.T. Baker™; Loughborough, UK). Sodiumazide (NaN_3) was obtained from Thermo Fisher Scientific (Alfa Aesar; Lancashire, UK). Potassium hexacyanoferrate (II) trihydrate (Carrez I, $K_4[Fe(CN)_6]*3H_2O$) and zinc acetate dihydrate (Carrez II, $Zn(OAc)_2*2H_2O$) were purchased from Sigma-Aldrich (Darmstadt, Germany). D-chiro-inositol was purchased from Carbosynth (Compton, UK), the fructooligosaccharides 1-kestotriose (1-kestose), 1,1-kestotetraose (nystose) and 1,1,1-kestopentaose (1F-fructofuranosylnysose) from FUJIFILM Wako Pure Chemicals (Neuss, Germany), verbascose from Megazyme (Bray, Ireland) and glucose, galactose, fructose, sucrose, melibiose, lactose monohydrate, raffinose pentahydrate, stachyose tetrahydrate as well as the sugar alcohol kit (arabitol, dulcitol, erythritol, mannitol, maltitol, adonitol, xylitol, sorbitol) from Sigma-Aldrich (Darmstadt, Germany). All carbohydrate reference standards were of > 98 % purity, except for 1,1,1-kestopentaose (80 % purity). Amyloglucosidase (E-AMGFR), α-galactosidase (E-AGLANP) and inulinase (E-FRMXPD), the fructan assay kit (K-FRUC) as well as the alpha amylase assay kit (K-CERA) were purchased from Megazyme (Bray, Ireland).

HPAEC-PAD. Separation and quantification of all carbohydrates was performed on a Dionex™ ICS-5000$^+$ system (Sunnyvale, CA, USA), equipped with a SP Single Pump (analytical gradient pump), AS-AP Autosampler, a 10 µl injection loop (full loop injection used) and ED Electrochemical Detector cell with a gold working electrode and a PdH reference electrode. The gold carbo quad waveform, which has been shown to be most suitable for reproducible results in the analyses of carbohydrates has been applied. The pulsed potential starts with a period (0.2 s) that allows the charging current to decay at +0.1 V, the detection period (0.2 s) measuring the current from the analyte oxidation at 0.1 V, followed by reductive cleaning at -2.0 V (0.01 s), activation and further cleaning of the working electrode surface by Au-oxide formation at +0.6 V (0.01 s) and reduction at -0.1 V (0.06 s).[21]

The eluents, purified water (A), 225 mM NaOH (B) and 500 mM NaOAc (C; vacuum filtered through 0.2 µm filter) as well as the syringe wash solution 5 % CH_3CN (D), were kept under N_2-atmosphere using a direct connection to the Peak Scientific (Inchinnan, UK) Corona Air Compressor and Corona Nitrogen Generator (constant pressure 4.5 – 5 bar). The separation of mono- and disaccharides, fructans and GOS was performed on a Thermo Scientific™Dionex™CarboPac™PA200 analytical column (3 x 250 mm) with the corresponding guard column applying gradient elution, according to Ziegler et al. (2016) with some modifications. This column is specifically applied for the separation of oligo- and polysaccharides, thus not fully separating some simple sugars.[14] In particular sugar alcohols, glucose and galactose as well as melibiose and fructose were coeluting on that column. The separation was carried out using a Thermo Scientific™Dionex™CarboPac™PA1 analytical column (2 x 250 mm) with the corresponding guard column (hereafter referred to as CarboPac PA1 or CarboPac PA200, respectively), applying an isocratic elution with 18 mM NaOH. The composition of the mobile phase, for the chromatographic methods on both columns is presented in Table S1 (Supporting Information). Separation and detection were carried out at 25 °C and 0.25 ml/min flow rate. The columns were washed with 500 mM NaOAc followed by 225 mM NaOH after each separation run; the latter condition promoted additionally the removal of potential carbonate contamination on the column and a clean-up of the working electrode surface, avoiding a loss of reproducibility in peak area due to oxidized products on the detector surface.

Sample preparation and FODMAP extraction. Whole grains were milled with a Bühler laboratory disc mill (Brauchschweig, Germany) or disrupted using a QIAGEN Tissue Lyser II (Hilden, Germany) to a particle size ≤ 0,5 mm. Baked products, including bread and biscuits as well as cooked pasta, were freeze-dried and ground to a fine powder. The extraction of the carbohydrates was based on the method described by Ziegler et al. (2016) with different changes and supplementations deduced from the official method for fructan analysis in food products, AOAC 997.08, and Huynh et al. (2008).[5,17,18] An aliquot of [400 ± 0.5] mg of cereal-

product raw material or powder from the lyophilized product was mixed thoroughly with 1 ml MeOH and left for 5 min in a closed reaction tube, in order to inactivate interfering, native enzymes from the samples such as α-amylases from cereals. Subsequently, 100 µl internal standard rhamnose (9 mg/ml) and 20 ml 80 °C H_2O, containing 50 mg/l NaN_3, were added and the mixture was subjected to the first extraction step, using the BANDELIN Sonoplus HD 3100 homogenizer (Berlin, Germany) equipped with a MS73 microtip and operated at 75° amplitude for 2 x 15 s. Hot H_2O was used in order to enhance the solubilization of fructans and denature native cereal enzymes. The intermixture of NaN_3 prevents the carbohydrates from microbial degradation during sample preparation, storage and analysis at room temperature. After centrifugation at 1520 g for 5 min the supernatant was transferred into a 100 ml volumetric flask and the extraction was repeated with 20 ml 80 °C H_2O (containing 50 mg/l NaN_3). The supernatants were combined, cooled to room temperature, and the proteins were precipitated by adding 200 µl Carrez I (15 g/100 ml) and Carrez II (23 g/100 ml), respectively. After adjustment to 100 ml, the extract was centrifuged at 3000 g for 10 min and filtered through 0.2 µm polyamide syringe filter (Chromafil AO-20/25, Machery Nagel, Düren, Germany). If extracts were frozen prior to analysis they were reheated to 80 °C in a Stuart Scientific SHT 1D test tube heater (UK) in order to redissolve precipitated fructans. Samples were extracted in duplicates.

Identification of carbohydrates. The retention times of the reference standards and the peaks in the chromatograms have been compared. Additionally, sample extracts have been spiked with reference standards. Furthermore, enzymatic degradations with α-galactosidase, inulinase and amyloglucosidase have been conducted to confirm the identification and the purity of peaks (not all data shown).

Quantification of mono-/di-/oligosachharides and polyols. Extracts were diluted and analyzed via HPAEC-PAD. The quantification of the carbohydrates was conducted using mixtures of the reference standards in the ranges between [0.1-1] mg/l and [1-20] mg/l. The software Chromeleon 7.2 was used for data acquisition and processing. Analytical results

were calculated to g analyte per 100 g dry matter of the sample. The dry matter was determined according to AACC 44-15.02.[22]

Determination of total fructan content and average degree of polymerization. The procedure for the enzymatic hydrolysis for the fructan quantification was based on the method described by Huynh et al. (2008) and the principle of the official method AOAC 997.08 for fructan analysis in food products.[17,18] Two 500 µl aliquots of the diluted sample extract (usually 5 to 20 fold dilution, taking into consideration the substrate to enzyme ratio and the high glucose amounts resulting from the glucose release by amyloglucosidase from co-extracted starch and dextrins) were subjected to two separate enzymatic treatments (with enzyme mixture A and B). 150 µl of the enzyme mixtures were added to the samples. Enzyme mixture A contained a 1:1:1 mixture of amyloglucosidase, α-galactosidase and 0.1 M NaOAc-buffer, latter was replaced by inulinase in mixture B. The lyophilized enzymes were diluted in 0.1 M NaOAc-buffer at pH 4.5 (prepared according to AOAC 997.08) to 220 U/ml, resulting in 11 U/150 µl for each enzyme in the mixtures A and B.[17] The reaction mixtures were incubated, in a water bath, in 2 ml screw cap micro tubes for 30 min at 60 °C. Subsequently, the enzymes were inactivated in a test tube heater at 100 °C for 40 min. After cooling to room temperature, 350 µl H_2O was added to the hydrolysate, to bring the volume to 1 ml. The precipitated enzymes were removed by centrifugation at 10·000 g for 2 min. The hydrolysates were measured via HPAEC-PAD with the CarboPac PA200 column. The total fructan content and the average degree of polymerization (DP_{av}) were calculated based on the results of free (hydrolysate A) and released (hydrolysate B) glucose, fructose and sucrose, based on Huynh et al. (2007).[18] The concentration of glucose (G_f) and fructose (F_f) released from fructans was calculated according to equations 1 and 2, whereas $G_{A/B}$, $F_{A/B}$ and S_A are the determined glucose, fructose and sucrose concentrations from the hydrolysates A and B in µmol/l, 180.16 is the molecular weight of glucose or fructose, DF is the dilution factor (dilution factor from extract dilution before hydrolysis multiplied by 2 from dilution of hydrolysate to 1 ml), V_E is the extract volume (100 ml) and M_S is the sample mass ([400 ± 0.5] mg).

205 (1) $$G_f[\%] = \frac{(G_B - G_A - S_A) * 180.16 * DF * V_E}{10\,000 * M_s}$$

206 (2) $$F_f[\%] = \frac{(F_B - F_A - S_A) * 180.16 * DF * V_E}{10\,000 * M_s}$$

207 The DP_{av} is calculated according to equation 3. The fructan content is finally calculated
208 according to equation 5, whereas k (equation 4) is the water correction factor (water uptake
209 during hydrolysis, dependent on chain length of the fructans). This calculation is suitable for
210 native cereal fructans, such as graminan-type or neo-levan-type fructans (references), with
211 one glucose residue in each fructan molecule.

212 (3) $$DP_{av} = \frac{F_f}{G_f} + 1$$

213 (4) $$k = \frac{180 + 162 * (DP_{av} - 1)}{180 * DP_{av}}$$

214 (5) $$Fructan\ [\%] = k * (G_f + F_f)$$

215 However, the calculation of the fructan concentration for partially hydrolyzed fructans is slightly
216 different and does not deliver information about the average degree of polymerization. If longer
217 chains of fructans are partially hydrolyzed not each of the shorter chains will contain a glucose
218 residue, as the native fructan contains only one glucose moiety. However, equation 3,
219 presumes one glucose residue per fructan molecule. Thus, the average degree of
220 polymerization would be overestimated, if most molecules actually are FOS without glucose.
221 Subsequently the total amount of fructan would be underestimated. This error can only be
222 eluded if the average degree of polymerization is known or can be estimated, enabling a more
223 accurate calculation of the water correction. As fructan (inulin or FOS) isolates are often food
224 additives, their structure is well known. A FOS standard with a DP_{av} of 2-8 has been analyzed
225 in 3 different concentrations, in duplicates, which supports this hypothesis (data not shown).
226 If information about the average degree of polymeriziation is not available, this can be obtained
227 using the fructan-fingerprint in the chromatogram of the sample hydrolyzed with
228 amyloglucosidase as described by Haskå et al. (2008) and Nemeth et al. (2014).[16,23]

Validation. The HPAEC-PAD method for the quantification of FODMAPs has been validated with regard to the limits of detection and quantification (LOD, LOQ), linearity, repeatability and accuracy. Furthermore, the determination of the average degree of polymerization of the fructans in the wheat matrix has been validated according to Verspreet et al. (2012).[19] Therefore, different spiking experiments and replications of extractions with wheat wholemeal flour and wheat starch have been conducted.

Wholemeal flour was spiked with the reference standards in 5 different concentrations ([0.5 – 12] mg/l). Each level was spiked and extracted in a triplicate. The LOD and LOQ have been determined with the signal to noise ratio (S/N, 3 and 10, respectively), from the analytes in the matrix. The wholemeal flour was extracted and analyzed in 6 replicates and in two additional duplicates from three different analysts, respectively, indicating the repeatability of the method. For the validation of the DP_{av} determination of fructans, wheat starch has been spiked with the reference standards kestose (DP3) and nystose (DP4) in 4 different concentrations in triplicates ([0.1 - 0.7] % based on the weight of wheat starch), and additionally 0.2 % raffinose and 0.4 % sucrose were added, to mimic the interfering components from wheat.

Total fructan determination via enzyme assay. The Megazyme fructan assay kit, K-FRUC, has been used as reference method for the determination of the total fructan content. The interference of GOS (mentioned in the assay procedure as raffinose family oligosaccharides, RFO) is taken into account by incubation of the sample extracts with α-galactosidase prior to the degradation of starch, malto-dextrins and sucrose, as described in the controls and precautions of the assay procedure.

Determination of α-amylase-activity. The impact of the extraction temperature and the initial addition of MeOH (cf. procedure for FODMAP extraction) on the activity of the native enzyme α-amylase in wheat, has been investigated, using the Ceralpha Method with the Megazyme alpha amylase assay kit. The enzyme extraction procedure has been modified in order to obtain indications about the impact of the extraction temperature and the addition of MeOH. The α-amalyse activity was determined after enzyme extraction according to the assay

manual. Furthermore, the activities were determined after extraction with heated (80 °C) extraction buffer, with a mixture of extraction buffer and MeOH at room temperature and finally with the heated (80 °C) mixture of MeOH and buffer.

Statistical analysis. The statistical evaluation of the validation experiments as well as the investigation of the suitability of the linear and the quadratic regression for a calibration model, applying the Mandel's fitting test, has been carried out with MS excel 2010.

RESULTS AND DISCUSSION

Sample preparation – extraction of FODMAPs. For the extraction of carbohydrates from cereals different extraction media are described in literature. For instance, Pico et al. (2015), Haskå et al. (2008) and Verspreet et al. (2012) have tested H_2O and EtOH as pure extraction agents and in different combinations.[16,19,24] Whereas the use of 80 % EtOH is known to be advantageous to minimize the co-extraction of starch, it leads to an incomplete extraction of fructans according to Haskå et al. (2008).[16] Also 10 % EtOH and 90 % EtOH have been shown to have a lower extraction efficiency for different mono-, di- and oligosaccharides in comparison to 100 % H_2O.[24] In order to achieve a complete extraction of carbohydrates, especially the higher DP fructans, a second extraction step with H_2O of the remaining pellet from the EtOH extraction is necessary.[16,19] Before the supernatants can be combined, the EtOH must be fully evaporated and the residues recovered in aqueous solution, in particular if enzymatic hydrolysis is applied for fructan analysis. The simplest, efficient extraction medium appears to be 100 % H_2O. Verspreet et al. (2012) have shown no significant difference between the fructan results obtained from EtOH/ H_2O supernatants compared to only H_2O supernatants. However, their method applies mild acid hydrolysis for the quantification of fructans, which is less sensitive to the interference of co-extracted starch. Stöber et al. (2004) have shown a strong interference of starch with the fructan analysis, leading to an overestimation of the glucose amount released from fructans (G_f, cf. equation 1 and 5) as commercial fructan degrading enzyme-preparations release glucose from non-fructan compounds, such as starch and malto-dextrins.[15] This inaccuracy can be conquered if a

starch-degrading enzyme (amyloglucosidase) is included, as described in the method of this study.

Another important parameter is the temperature of the extraction medium. Whereas smaller carbohydrates are easily extracted at room temperature, fructans, especially longer chains, solubilize better at higher temperatures.[16] Most methods described for fructan-analysis are at 80 °C.[11,15–19,25] The extraction with heated and room-tempered H_2O has been compared in preliminary work for this study, using the K-FRUC assay. Only 70 % of the fructans extracted with hot H_2O could be extracted with room-tempered water (data not shown). Furthermore, the use of hot water inactivates interfering native enzymes of the sample.[5,19] This effect could be enhanced due to the suspension of the sample in MeOH prior to the extraction. The analysis of the native α-amylase activity (K-CERA) in commercial whole wheat flour and milled wheat grains, after extraction with MeOH-addition to the extraction buffer and heating of extraction buffer and the mixture of both, confirmed this assumption (Figure S1, Supporting Information).

The determined α-amylase activities correlated with the glucose amounts in the commercial whole wheat flour, obtained from the HPAEC-PAD profiles of the extracts with the different conditions. Furthermore, the amounts of fructose, glucose and sucrose gave indication of sucrose-hydrolyzing invertase activity (potentially from microbial contamination, as preliminary trials did not include NaN_3 addition). Samples extracted at room temperature, without the addition of MeOH contained the highest amounts of glucose and fructose and the lowest amounts of sucrose, whereas the addition of MeOH and extraction with H_2O heated to 80 °C led to the lowest levels of glucose and fructose and highest sucrose levels. The values obtained from the extraction at room temperature with the addition of MeOH were inbetween the latter described amounts. This indicates the enhancing effect of enzyme inhibition of the hot extraction medium. The high difference in the glucose amounts derived from the different extraction conditions compared to the difference in the sucrose and fructose amounts evidenced the higher amylase activity in samples extracted at room temperature without the

addition of MeOH (Figure S2, Supporting Information). This overestimation leads to a misinterpretation of the ratio of glucose to fructose, which is important for the determination of FODMAPs, as fructose can act as FODMAP if it occurs in higher levels than glucose in the consumed product.[4,26] However, it should be kept in mind, using H_2O as extraction medium for samples which are high in starch, will lead to co-extraction of the starch; the partial hydrolysis can be minimized by the actions undertaken in this study but not fully excluded.

Identification of FODMAPs. The identification of the carbohydrates separated on both columns, CarboPac PA200 and CarboPac PA1, is shown in Figure 1 with wheat whole meal extract as an example.

All compounds in the HPAEC-PAD profiles have been either identified comparing the retention times of reference standards (pure and added to the sample matrix), or if commercial standards were not available, by comparison with other studies and hydrolysis of sample extracts with different enzymes. Peaks in the chromatogram, which disappeared after incubation of the sample extracts with inulinase, could be assigned as fructans, sucrose, fructosylraffinose and GOS. The hydrolysis led to an increase of fructose and glucose and the appearance of the degradation-products from fructosylraffinose, raffinose/ stachyose (not separated on CarboPac PA200, separation on CarboPac PA1 not shown) and verbascose, resulting in melibiose (confirmed with reference standard; separated on CarboPac PA1, on CarboPac PA200 coeluting with fructose), manninotriose and manninotetraose, respectively (Figure 2). Incubations with α-galactosidase degraded GOS and fructosylraffinose and led to an increase of glucose/ galactose (only separated on CarboPac PA1 column), sucrose and kestose (Figure 2). Furthermore, incubations with amyloglucosidase degraded maltose, maltotriose and higher malto-dextrins from co-extracted and partially hydrolyzed starch (retention times on corresponding columns summarized in Table S2, Supporting Information). These hydrolysis trials gave also indication about the purity of the peaks, excluding on the one hand coelution of other compounds with those of interest. On the other hand, it could be seen, in accordance with other studies, that higher DP fructans and malto-dextrins, eluting in the

same area on the CarboPac PA200 (after 13 min onwards), are very difficult to assign clearly to one of those oligo- and polysaccharide groups.[16,25] Hence, any quantitative or semi-quantitative evaluation, including the fingerprint of the fructan profile or the integration of peaks assigned to one DP, can only be carried out if the sample extracts were initially incubated with amyloglucosidase, as described by Rakha et al.(2010).[25] Furthermore, if divers samples, in particular products containing different cereals/ pseudocereals/ pulses as ingredients, the chromatographic profiles, especially in the higher DP area may be very difficult to identify (cf. Figure S3, Supporting Information, showing **Error! Reference source not found.**profiles from different typical cereal-product ingredients). In order to evaluate peaks from that area further studies, focused on the characterization of the specific ingredients, would be required, such as those conducted by Haskå et al. (2008) or Rhaka et al. (2010)., for wheat and rye.[16,25] Thus, this study approached the fructan quantification and determination of the DP_{av} only after enzymatic hydrolysis to the monomers.

Validity of the method. The method presented for the quantification of specific carbohydrates, considered as FODMAPs, has been checked and validated regarding its linearity, accuracy and repeatability as well as the limits of detection and quantification.

The linearity of a method is referred to the whole method, including all steps of the sample preparation. Hence replicates of reference standard dilutions would not indicate the linearity of a method.[27] It describes the relation of analytical signal and concentration of analytes. However, the term linearity itself is misleading and might suggest a linear relation is required. In reality, the impact of sample matrix and the principle of analytical measurement, often lead to the fact, that a different regression than linear is required to describe the mathematical correlation of signal and concentration.[27,28] With regard to existing HPAEC-PAD methods for the quantification of carbohydrates a quadratic function may reveal a better fit in a calibration model, depending on analytical system, sample type and analytes investigated.[20,29] In this study, the calibration model was evaluated using the Mandel's fitting test. Correlation coefficient of the calibration curve and visual comparison of linear and quadratic regression

functions as well as residual plots (Figure 3; Table 1) may give first indication about the suitability of a regression model. If the mathematical model is chosen correctly, the residuals should be distributed normally around the zero level; on the contrary the pattern of residuals of the linear function is a sign for an incorrect model. However, the Mandel's fitting test allows a better evidence, determining a potential significant difference in residual variances from linear and quadratic calibration.[27,30] According to equation 6 the test variable (TV) is calculated and compared to the corresponding value of the F-distribution with 1 and n-3 degrees of freedom at the significance level of $\alpha = 0.05$ ($F_{0.05, 1, n-3}$).

(6) $$TV = \frac{(n-2) * s_{y_1}^2 - (n-3) * s_{y_2}^2}{s_{y_2}^2}$$

(7) $$s_{y_1} = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n-2}}$$

$$\hat{y}_1 = a + bx$$

(8) $$s_{y_2} = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n-3}}$$

$$\hat{y}_2 = a + bx + cx^2$$

The hypothesis H_0 (no significant difference between the residual variances) is rejected, if $TV > F_{0.05, 1, n-3}$. Residual standard deviations of linear and quadratic regressions (s_{y1} and s_{y2}) are calculated according to equation 7 and 8, respectively. As presented in Table 1, for all analytes of interest a second order polynomial equation was better suitable to describe the correlation of signal and concentration (always TV > 4.6).

The LOD and LOQ of the method ranged between [0.5 - 10] μg/l and [2 - 5*10[1]] μg/l, respectively. In comparison to other analytical methods, HPAEC-PAD is known to be very sensitive; for instance, Muir et al. (2009) determined LOD of [0.05 - 0.1] g/l for the FODMAP quantification via HPLC-ELSD. However, the main advantage weighs for the more accurate and sensitive electrochemical fructan determination in contrast to the photometric enzyme assays.[1,19] Carbohydrates spiked to wheat whole meal flour were recovered to [94.1 - 101.4] % which indicates a good accuracy and selectivity of the method. The analysis of

wheat flour replicates on the same day and on different days, revealed an acceptable reproducibility of the method, with RSD ranging between [2.3 - 10.3] %. The highest variation in the results was observed for the analysis of glucose, in the replicates on different days, conducted by different analysts. As discussed above, this deviation is due to partially hydrolyzed, co-extracted starch.

In order to estimate the validity of the method to determine the DP_{av} of fructans, wheat starch was spiked with different concentrations of a kestose (DP3) – nystose (DP4) mixture, as well as sucrose and raffinose (to mimic the natural interference in wheat).

The DP_{av} determined for the rye and whole wheat flour as well as for the inulin reference standard from the K-FRUC assay (Table 2) were in accordance to other studies.[19,25] The DP_{av} resulting from the wheat starch spiking experiment was close to the actual DP_{av} of kestose and nystose (Table 2), indicating a good suitability of the method to calculate the DP_{av} after enzymatic hydrolysis. However, this method is limited to the determination of the DP_{av} of fructans containing one glucose residue, as explained in the calculation in Materials and Methods.

Enzymatic fructan determination. The determination of the total fructan content via HAPEC-PAD has been compared with the results obtained from the photometric fructan assay (K-FRUC). Both methods are based on the measurement of enzymatically released glucose and fructose monomers from fructans. In accordance to other studies [16,19], the total fructan amounts obtained from the enzymatic assay, were overestimated due to GOS if the additional α-galactosidase was not included in the procedure (data not shown), since inulinases release fructose from those non-fructan compounds (Figure 2). Thus, this correction was included in the enzymatic assay.

In contrast to the total fructan determination via HPAEC-PAD after acid hydrolysis, as described by Verspreet et al. (2012), the calculation after enzymatic hydrolysis did not require any further corrections, as the inclusion of α-galactosidase in both enzyme mixtures (cf. Materials and Methods) avoided any GOS interferences.[19] The calculation is based on the

difference of hydrolysate A and B. In hydrolysate A GOS are degraded to galactose and sucrose, as B contains additionally inulinase, GOS are fully degraded to the monomers galactose, glucose and fructose (Figure 2), whereas fructose and glucose released from GOS are considered in the calculation as sucrose from A (equations 1 and 2).

The determination via HPAEC-PAD resulted in higher fructan values than the photometric determination, which is considered to be due to the higher sensitivity of the analytical methodology.

Application of the method. This method has been applied for the FODMAP quantification in different commercial cereal-based products and delivered results, assessed to be reasonable, based on the product's ingredients and comparable literature data (data not shown).[1,2] Further studies will concentrate on the FODMAP-characterization of cereal-product ingredients and the development of products with a lowered FODMAP content using the presented method as analytical tool.

ASSOCIATED CONTENT

Supporting Information. Table S1 presenting composition of mobile phases for chromatographic separation. Figure S1 showing impact of extraction-temperature and MeOH addition on α-amylase activity in wheat. Figure S2 showing the impact of extraction conditions on glucose, fructose, sucrose determination in whole wheat flour. Table S2 presenting the retention times of the analytes on CarboPac PA1 and CarboPac PA200 columns. Figure S3 showing the higher DP area in CarboPac PA200 profiles of different flour extracts. This material is available free of charge via the Internet at http://pubs.acs.org

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All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

FODMAP, fermentable oligo-, di, monosaccharides and polyols; HPAEC-PAD, high performance anion exchange chromatography coupled with pulsed amperometric detection; FOS, fructooligosaccharides; GOS, galactooligosaccharides; DP_{av}, average degree of polymerization; TV, test variable; RFO, raffinose family oligosaccharides; RSD, relative standard deviation; LOQ/LOD, limit of quantification/ detection.

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Figure captions

Figure 1. HPAEC-PAD (A) CarboPac PA200 and (B) CarboPac PA1 profiles of (a) wheat whole meal extract overlaid with (b) standard mixture: (1) xylitol, (2) sorbitol, (3) mannitol, (4) rhamnose, (5) arabinose, (6*) galactose, (6) glucose, (6**) xylose, (7) fructose, (7*) melibiose, (8) sucrose, (9) lactose, (10) raffinose, (11) verbascose, (12) 1-kestose, (13) fructosylraffinose, (14) maltose, (15) unidentified fructan of DP3, (16) nystose, (17) maltotriose, (18) 1,1,1-kestopentaose. Numbers marked with an asterisk (*/ **) in CarboPacPA1 profile are not separated from the corresponding numbers without the asterisk on the CarboPac PA200.

Figure 2. Enzymatic hydrolysis of fructans, GOS and malto-dextrins; conducted with wheat extract and standard solutions to obtain additional information for peak identification.

Figure 3. (A) Linear (B) quadratic regression with corresponding residual plots on the example of sorbitol spiked to the wheat matrix.

Table 1. Validation of analytical method regarding LOD, LOQ, Recovery, Repeatability (RSD) and fit to a regression model (Mandel's test)

	LOD/ LOQ[a] [µg/l]	$RSD_{interday}$/ $RSD_{intraday}$ [%] (n=6)[b,c]	Recovery[a] [%] (n=3, m=5)[c]	Fit regression model[a]		
				R^2_{linear}/ R^2_{quad}[d]	s_{y1}/s_{y2}[e]	TV $(0.05,1,12)$[f]
Xylitol	2 / 7	-	100.7 ± 1.5	0.979 / 0.998	1.15 / 0.34	131.67
Sorbitol	2 / 3	6.6 / 4.6	100.1 ± 3.7	0.989 / 0.998	0.99 / 0.40	68.43
Mannitol	1 / 2	3.8 / 4.0	97.4 ± 3.7	0.992 / 0.999	0.97 / 0.43	55.45
Rhamnose	1 / 3	3.9 / 6.9	100.1 ± 1.2	0.995 / 0.999	0.57 / 0.30	33.93
Glucose	1 / 3	2.3 / 10.3	96.3 ± 3.9	0.994 / 0.998	1.12 / 0.59	34.77
Fructose	0.5 / 2	3.0 / 5.5	94.1 ± 4.5	0.997 / 0.998	0.27 / 0.19	13.13
Lactose	1 / 6	-	100.8 ± 2.4	0.997 / 0.997	0.38 / 0.33	4.96
Raffinose	4 / $4*10^1$	3.3 / 4.5	97.6 ± 5.3	0.989 / 0.990	0.28 / 0.24	5.91
Verbascose	$1*10^1$/ $4*10^1$	-	94.5 ± 2.9	0.992 / 0.992	0.15 / 0.13	4.91
Kestose	5 / $5*10^1$	2.3 / 7.7	101.4 ± 5.7	0.989 / 0.991	0.38 / 0.30	8.71
Nystose	3 / $4*10^1$	4.7 / 6.0	97.7 ± 2.2	0.998 / 0.998	0.13 / 0.11	5.28
Kestopentaose	$1*10^1$ / $5*10^1$	-	97.6 ± 1.8	0.998 / 0.998	0.09 / 0.08	5.79

[a] limits of detection and quantification (LOD/LOQ), recovery (± standard deviation) and fit to a regression model determined with data obtained from wheat whole meal flour spiking (each compound added at five levels between [0.5 – 12] mg/l in triplicate)
[b] replications of whole wheat flour extractions on the same day were used to determine the intraday repeatability, duplicates on three different days were used to determine the interday repeatability
[c] n represents number of replications, m represents number of spiking concentrations
[d] R^2 represents the correlation coefficient
[e] s_{y1}/s_{y2} represent the residual standard deviations from the linear and the quadratic regressions, respectively
[f] TV represents the test variable by means of the Mandel's test. TV is compared to 4.6 resulting from the F-distribution, at α=0.05, for 1 and 12 degrees of freedom, to reject or accept the H0 (no significant difference between the residual variances of the linear and quadratic regressions)

Table 2. Validation of DP_{av} determination after enzymatic hydrolysis

	DP_{av} ± standard deviation	
	experimental	expected
rye flour [a]	9.1 ± 0.1	
whole wheat flour [a]	5.5 ± 0.1	
inulin [a]	28.4 ± 0.6	>25
spiked wheat starch [b]	3.3 ± 0.1	3.5

[a] rye flour, whole wheat flour and inulin have been analyzed in duplicates
[b] kestose and nystose added to wheat starch in 4 levels between [0.1-0.7] % of sample weight in triplicate

Table 3. Enzymatic total fructan determination via HPAEC-PAD and assay kit

	Fructan [g/100g] ± standard deviation	
	K-FRUC	HPAEC-PAD
inulin [a]	26.82 ± 0.13	27.84 ± 0.34
plain wheat flour [a,b]	1.28 ± 0.03	1.38 ± 0.03
whole wheat flour [a,b]	1.83 ± 0	1.92 ± 0.03

[a] analyses carried out in duplicates,
[b] results based on dry matter

Figure 1

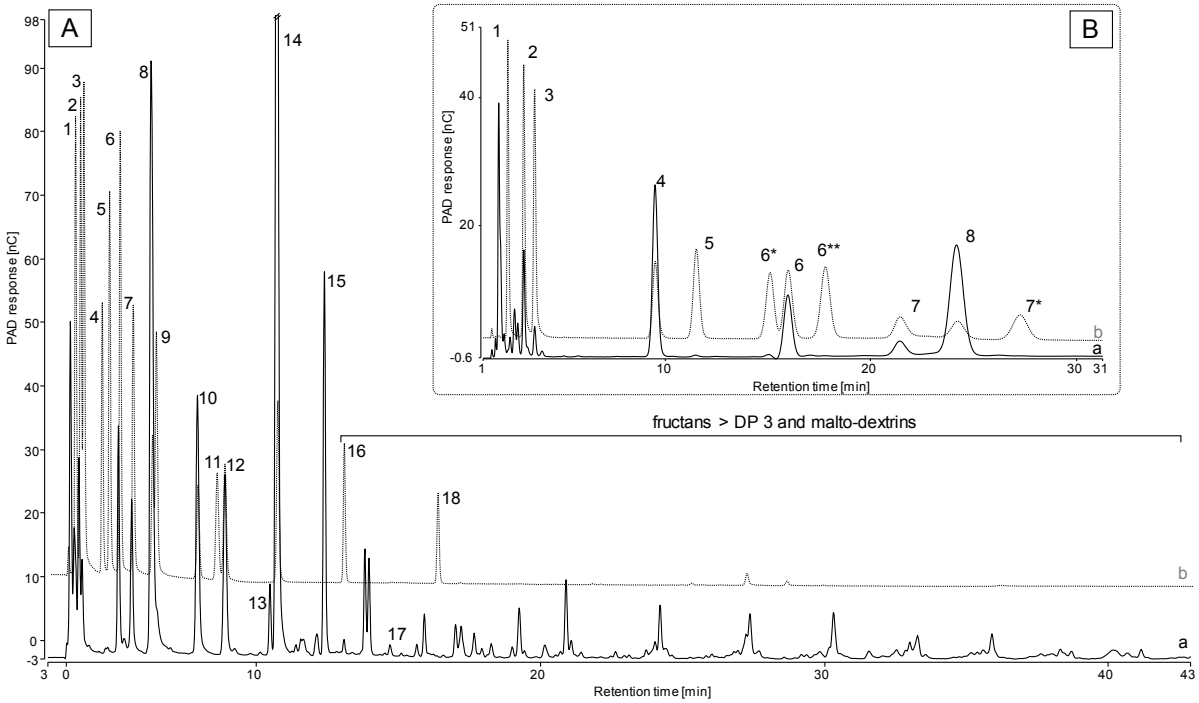


Figure 2

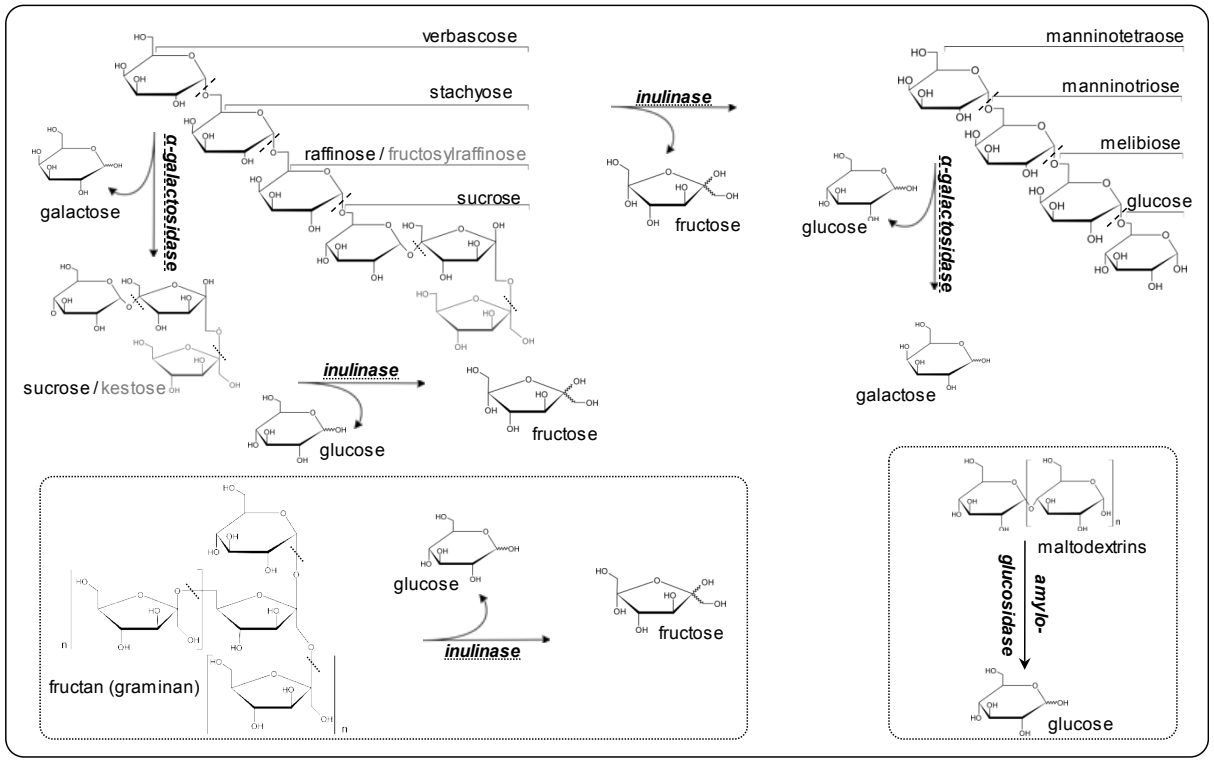
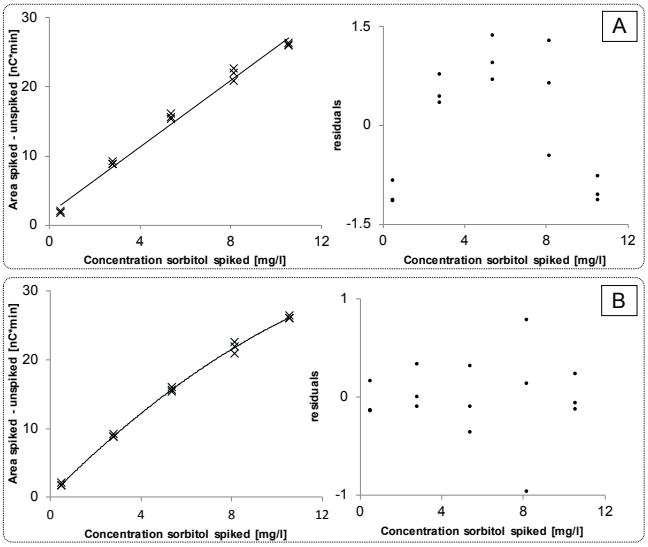
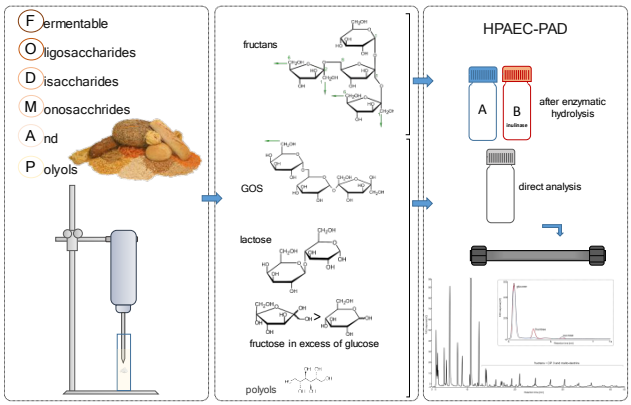


Figure 3


A
Area spiked - unspiked [nC*min]
Concentration sorbitol spiked [mg/l]
residuals
Concentration sorbitol spiked [mg/l]
B
Area spiked - unspiked [nC*min]
Concentration sorbitol spiked [mg/l]
residuals
Concentration sorbitol spiked [mg/l]

TOC


Fermentable
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inulinase
after enzymatic hydrolysis
direct analysis

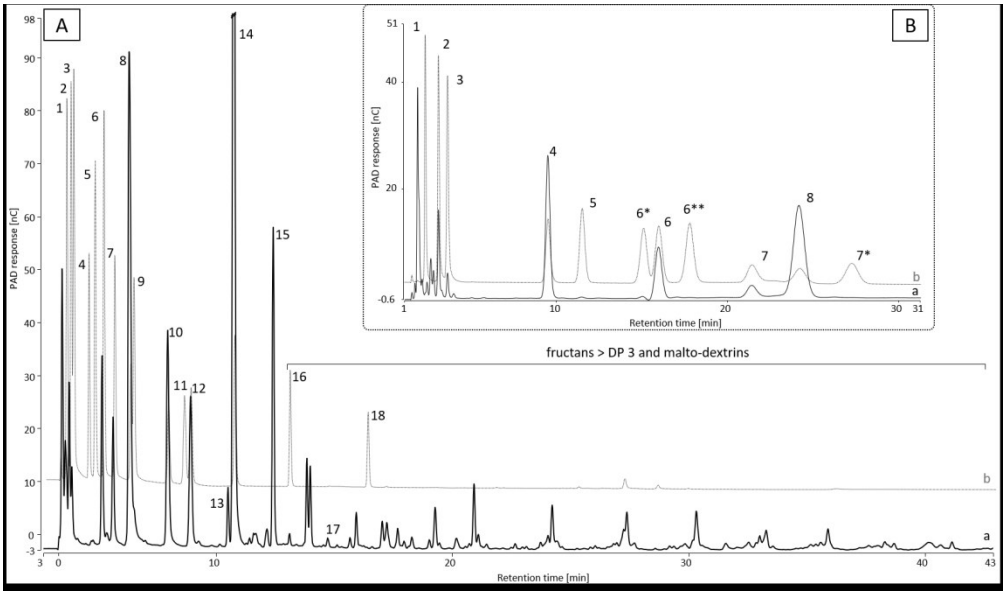

Figure 1. HPAEC-PAD (A) CarboPac PA200 and (B) CarboPac PA1 profiles of (a) wheat whole meal extract overlaid with (b) standard mixture: (1) xylitol, (2) sorbitol, (3) mannitol, (4) rhamnose, (5) arabinose, (6*) galactose, (6) glucose, (6**) xylose, (7) fructose, (7*) melibiose, (8) sucrose, (9) lactose, (10) raffinose, (11) verbascose, (12) 1-kestose, (13) fructosylraffinose, (14) maltose, (15) unidentified fructan of DP3, (16) nystose, (17) maltotriose, (18) 1,1,1-kestopentaose. Numbers marked with an asterisk (*/ **) in CarboPacPA1 profile are not separated from the corresponding numbers without the asterisk on the CarboPac PA200.

293x172mm (150 x 150 DPI)

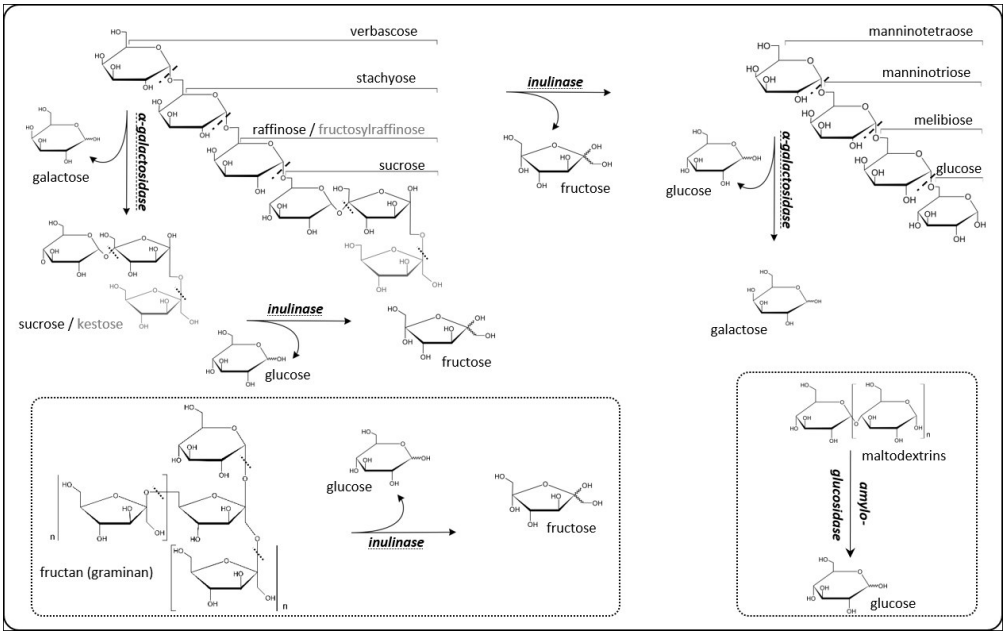

Figure 2. Enzymatic hydrolysis of fructans, GOS and malto-dextrins; conducted with wheat extract and standard solutions to obtain additional information for peak identification.

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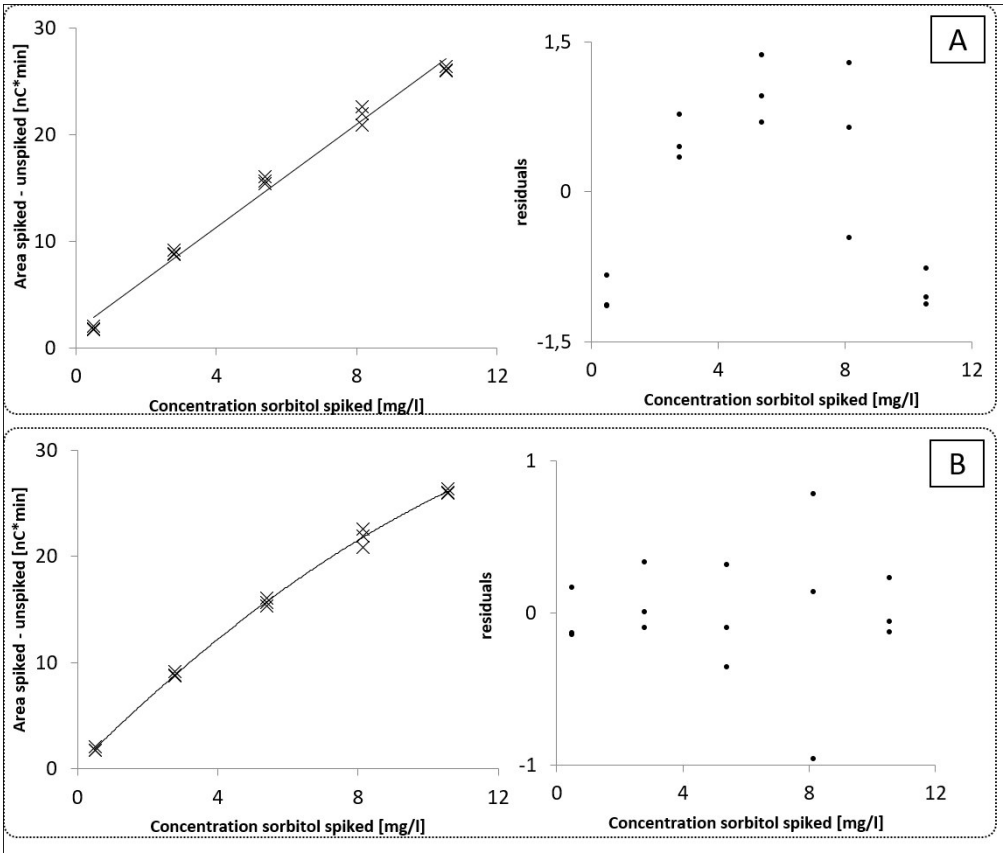

Figure 3. (A) Linear (B) quadratic regression with corresponding residual plots on the example of sorbitol spiked to the wheat matrix.

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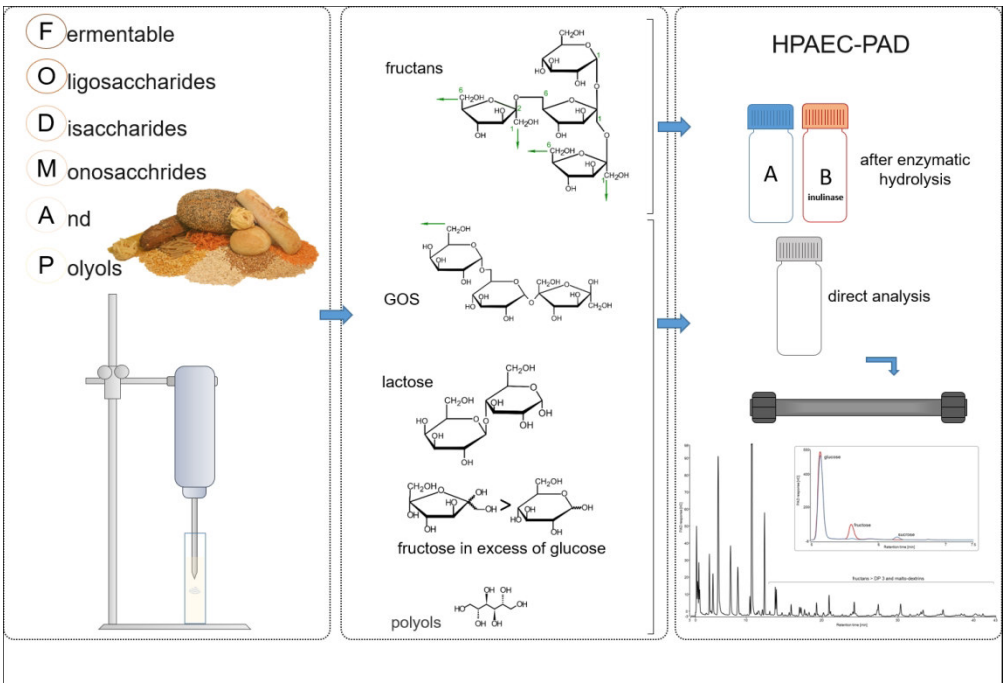

Table of Contents Graphic

260x176mm (150 x 150 DPI)

Supporting Information

Table S 1. Composition of mobile phase for chromatographic separation on CarboPac PA200 (1) and CarboPac PA1 (2)

(1) Gradient method on CarboPac PA200[a]				
time [min]	A[b] [%]	B[c] [%]	C[d] [%]	
0	70	27.5	2.5	
30	42.5	27.5	30	separation of
50	26.5	27.5	46	analytes
70	15.3	27.5	57.2	
75	0	27.5	72.5	
80	0	100	0	column cleanup
85	0	100	0	
86	0	0	100	regeneration gold surface
101	0	0	100	
102	70	27.5	2.5	re-equilibration
117	70	27.5	2.5	
(2) Isocratic method on CarboPac PA1[a]				
time [min]	A[b] [%]	B[c] [%]	C[d] [%]	
0	91.9	8.1	0	separation of
25	91.9	8.1	0	analytes
26	0	0	100	column cleanup
31	0	0	100	
32	0	100	0	regeneration gold surface
47	0	100	0	
48	91.9	8.1	0	re-equilibration
60	91.9	8.1	0	

[a] flow rate 0.25 ml/min, column temperature 25 °C.
[b] purified water.
[c] 225 mM NaOH.
[d] 500 mM NaOAc.

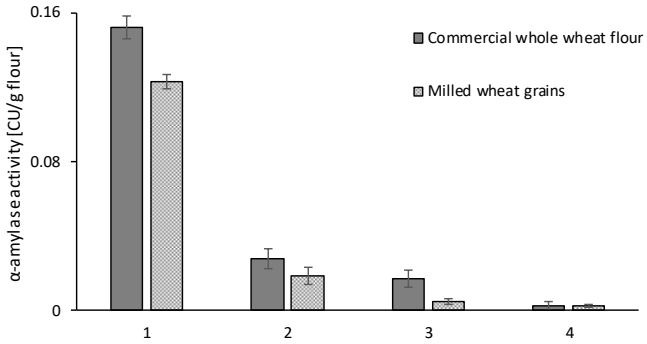

Figure S 1. Impact of extraction-temperature and MeOH addition on α-amylase activity in wheat. (1) Extraction according to K-CERA procedure with room-temperature buffer, (2) Extraction with buffer heated to 80 °C, (3) Extraction with a mixture of MeOH and buffer, (4) Extraction with a mixture of MeOH and buffer heated to 80 °C. Error bars represent standard deviations of triplicates.

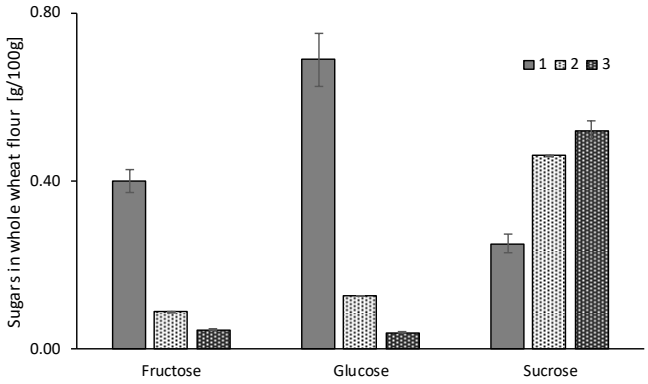

Figure S 2. Impact of extraction conditions on glucose, fructose, and sucrose determination in whole-wheat flour (HPAEC-PAD analysis). (1) Extraction at room temperature without MeOH addition, (2) Extraction at room temperature with MeOH addition, (3) Extraction with H_2O heated to 80 °C with MeOH addition. Error bars represent standard deviations of triplicates.

Table S 2. Retention times of carbohydrates for CarboPac PA200 and CarboPac PA1

Compound	Rt [min] CarboPac PA200[a]	Rt [min] CarboPac PA1[a]
Xylitol	3.6	**2.4**
Sorbitol	3.7	**3.1**
Mannitol	3.9	**3.7**
Maltitol	4.7	**10.0**
Rhamnose	**4.5**	**9.5**
Galactose	**5.1**	**15.2**
Glucose	**5.1**	15.9
Fructose	**5.6**	21.6
Melibiose	5.6	**27.5**
Sucrose	**6.3**	24.4
Manninotriose	5.8[b]	34.6[b]
Manninotetraose	6.1[b]	53[b,c]
Lactose	**6.4**	39.5[c]
Raffinose	**7.8**	52.5[c]
Stachyose	**7.8**	61.9[c]
Verbascose	**8.7**	-
Kestose	**8.8**	-
Nystose	**13.0**	-
Kestopentaose	**16.3**	-

[a]Only peaks with retention times printed in bold have been used for quantitative analyses.
[b]Identification based on enzymatic hydrolysis; reference standards were not available.
[c]Extended instrument method for CarboPac PA1 was only used for qualitative identification.

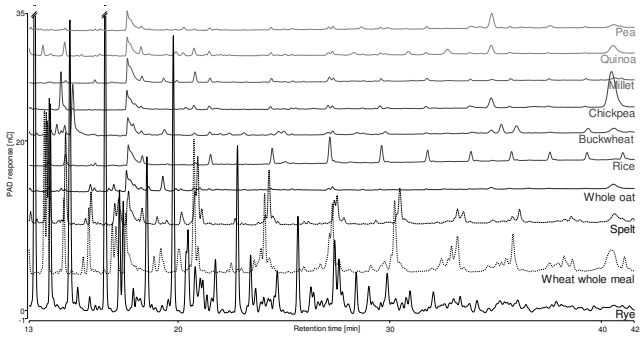

Figure S 3. HPAEC-PAD CarboPac PA200 profile in higher-DP area from different flour extracts showing the diversity and complexity of composition of different material