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

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1 **ABSTRACT**: This study presents an analytical method for the quantification of fermentable 2 oligo-, di-, monosaccharides and polyols (FODMAPs) in cereals and cereal-based products, 3 considering diverse ingredients, such as different cereals in addition to wheat, pulses or 4 pseudocereals. All carbohydrates have been separated, identified and quantified with a high-5 performance anion exchange chromatographic system coupled with pulsed amperometric 6 detection (HPAEC-PAD). The total fructan content and the average degree of polymerization 7 (DPav) have been determined after enzymatic hydrolysis to the monomers glucose and 8 fructose, based on the principle of the official method for fructan quantification in food 9 products, AOAC 997.08. Extraction, separation and detection as well as fructan determination 10 methods are based on several other studies and were modified in order to minimize 11 interferences in the analysis. The method has been validated with regard to the limits of 12 detection and quantification, linearity, repeatability and accuracy as well as the DP_{av} of the 13 fructans.

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

16 **INTRODUCTION**

17 Fermentable oligo-, di-, monosaccharides and polyols (FODMAPs) are carbohydrates often 18 associated with symptoms of irritable bowel syndrome (IBS). They comprise 19 galactooligosaccharides, fructans and fructooligosaccharides (FOS), lactose, fructose in 20 excess of glucose and polyols. GOS (often in not FODMAP related literature referred to as 21 raffinose family oligosaccharides, RFO) are α -galactose derivates (1 \rightarrow 6 linked) from sucrose 22 (α -glucose 1 \rightarrow 2 linked to β -fructose) and found especially in pulses, but also in different 23 grains such as wheat, barley or rye.^{1,2} Fructans are composed of fructose-chains, containing 24 one glucose residue. Depending on the fructan-source different types are classified. Inulin-25 type and levan-type fructans are linear β (2 \rightarrow 1) or β (2 \rightarrow 6) fructosyl-fructose chains with 26 one final glucose residue. The branched group, called graminan-type fructans, contains both 27 types of linkages (β (2 \rightarrow 1) or β (2 \rightarrow 6)) and is commonly found in cereals. As humans do 28 not possess the enzymes α -galactosidase or fructanases, fructans as well as GOS are not 29 digested in the small intestine, delivered to the large intestine and fermented by colonic 30 bacteria, inducing gas formation.³ Also the β (1 \rightarrow 4) linked galactosyl-glucose disaccharide, 31 lactose, is not tolerated by some individuals, due to the lack of the enzyme lactase. Lactose 32 is the major FODMAP in dairy products. If the monosaccharide fructose appears in excess of 33 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 34 35 sugars in their chemical structure, also called sugar alcohols are as well poorly absorbed and 36 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 43 of the products may be very diverse, in particular due to beneficial techno-functional or
44 nutritional properties of specific novel ingredients.

45 The successfully applied analytical methodology, described by Muir et al. (2007) comprises 46 the liquid chromatographic separation coupled with evaporative light scattering detection 47 (HPLC-ELSD) for the guantification of the smaller FODMAPs, combined with the photometric 48 determination of glucose and fructose after enzymatic hydrolysis of fructans, using an enzyme 49 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 50 51 information about the average degree of polymerization (DP_{av}) of fructans. Knowledge about 52 chain length is important as putative beneficial properties or induction of IBS-symptoms may 53 relate to the DP of fructan molecules.¹² Muir et al. (2007) proposed a modification of the HK-54 FRUC assay, which enables an estimation of the DP_{av}.¹⁰ Yet, the photometric determination 55 has a low analytical sensitivity and thus high detection limits, leading to unreliable results if 56 fructan levels are below 1 % on dry weight basis. Furthermore, the combination of different 57 analytical methods may be laborious for many samples. Also, both fructan assays must be 58 amended with the additional GOS correction step (incubation of the sample extracts with α -59 galactosidase) as fructose released from GOS by the enzyme inulinase, included in the assay, 60 leads to an overestimation of the total fructan content, which increases the complication of the 61 assays. Another study, conducted by Chumpitazi et al. (2018), quantified all FODMAPs using 62 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

67 Ziegler et al. (2016) proposed an analytical method based on HPAEC-PAD for the 68 quantification of FODMAPs in wheat.⁵ However, their approach for the quantification of 69 fructans is not suitable if other fructan sources than wheat or a combination of different sources 70 (diverse recipes of cereal-products) are considered. Generally, there are two approaches to 71 quantify fructans, classified as "direct" and "indirect" method by Stöber et al. (2004).¹⁵ The 72 direct method comprises the evaluation of each peak in the chromatogram belonging to fructan 73 molecules of different chain lengths and linkages. The reliability of this approach is limited due 74 to several factors. On the one hand, only few reference standards of fructans are available to 75 ensure an accurate quantification. On the other hand, the identification and assignment of 76 peaks as fructans for different sources may be very laborious and interfered by other oligo-77 and polysaccharides than fructans. Thus, for example Haskå et al. (2008) hydrolyzed sample 78 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 79 80 sources but also dependent on storage conditions or processing of the fructan containing 81 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}

89

90 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 ScientificTMDionexTMIC PureTM
Water purification system (Sunnyvale, CA, USA). Extra pure, 50 % w/w sodium hydroxide
(NaOH) solution (in water) was purchased from Thermo Fisher Scientific (ACROS OrganicsTM;
Dublin, Ireland). Electrochemical-grade sodium acetate (NaOAc) was purchased from Thermo

Scientific[™] (Dionex[™] AAA-Direct Reagents; Dublin, Ireland). HPLC-grade acetonitrile 97 98 (CH₃CN) as well as methanol (MeOH) were purchased from Sigma-Aldrich (Darmstadt, Germany) and glacial acetic acid (AcOH) from fisher scientific (J.T. Baker[™]; Loughborough, 99 100 UK). Sodiumazide (NaN₃) was obtained from Thermo Fisher Scientific (Alfa Aesar; 101 Lancashire, UK). Potassium hexacyanoferrate (II) trihydrate (Carrez I, K₄[Fe(CN)₆]*3H₂O) and 102 zinc acetate dihydrate (Carrez II, Zn(OAc)₂*2H₂O) were purchased from Sigma-Aldrich 103 (Darmstadt, Germany). D-chiro-inositol was purchased from Carbosynth (Compton, UK), the 104 fructooligosaccharides 1-kestotriose (1-kestose), 1,1-kestotetraose (nystose) and 1,1,1-105 kestopentaose (1F-fructofuranosylnysose) from FUJIFILM Wako Pure Chemicals (Neuss, 106 Germany), verbascose from Megazyme (Bray, Ireland) and glucose, galactose, fructose, 107 sucrose, melibiose, lactose monohydrate, raffinose pentahydrate, stachyose tetrahydrate as 108 well as the sugar alcohol kit (arabitol, dulcitol, erythritol, mannitol, maltitol, adonitol, xylitol, 109 sorbitol) from Sigma-Aldrich (Darmstadt, Germany). All carbohydrate reference standards 110 were of > 98 % purity, except for 1,1,1-kestopentaose (80 % purity). Amyloglucosidase (E-111 AMGFR), α -galactosidase (E-AGLANP) and inulinase (E-FRMXPD), the fructan assay kit (K-112 FRUC) as well as the alpha amylase assay kit (K-CERA) were purchased from Megazyme 113 (Bray, Ireland).

114 HPAEC-PAD. Separation and quantification of all carbohydrates was performed on a 115 Dionex[™] ICS-5000⁺ system (Sunnyvale, CA, USA), equipped with a SP Single Pump 116 (analytical gradient pump), AS-AP Autosampler, a 10 µl injection loop (full loop injection used) 117 and ED Electrochemical Detector cell with a gold working electrode and a PdH reference 118 electrode. The gold carbo quad waveform, which has been shown to be most suitable for 119 reproducible results in the analyses of carbohydrates has been applied. The pulsed potential 120 starts with a period (0.2 s) that allows the charging current to decay at +0.1 V, the detection 121 period (0.2 s) measuring the current from the analyte oxidation at 0.1 V, followed by reductive 122 cleaning at -2.0 V (0.01 s), activation and further cleaning of the working electrode surface by 123 Au-oxide formation at +0.6 V (0.01 s) and reduction at -0.1 V (0.06 s).²¹

124 The eluents, purified water (A), 225 mM NaOH (B) and 500 mM NaOAc (C; vacuum filtered 125 through 0.2 µm filter) as well as the syringe wash solution 5 % CH₃CN (D), were kept under 126 N₂-atmosphere using a direct connection to the Peak Scientific (Inchinnan, UK) Corona Air 127 Compressor and Corona Nitrogen Generator (constant pressure 4.5 - 5 bar). The separation 128 of mono- and disaccharides, fructans and GOS was performed on a Thermo 129 Scientific[™]Dionex[™]CarboPac[™]PA200 analytical column (3 x 250 mm) with the 130 corresponding guard column applying gradient elution, according to Ziegler et al. (2016) with 131 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, 132 133 glucose and galactose as well as melibiose and fructose were coeluting on that column. The 134 separation was carried out using a Thermo Scientific[™]Dionex[™]CarboPac[™]PA1 analytical 135 column (2 x 250 mm) with the corresponding guard column (hereafter referred to as CarboPac 136 PA1 or CarboPac PA200, respectively), applying an isocratic elution with 18 mM NaOH. The 137 composition of the mobile phase, for the chromatographic methods on both columns is 138 presented in Table S1 (Supporting Information). Separation and detection were carried out at 139 25 °C and 0.25 ml/min flow rate. The columns were washed with 500 mM NaOAc followed by 140 225 mM NaOH after each separation run; the latter condition promoted additionally the 141 removal of potential carbonate contamination on the column and a clean-up of the working 142 electrode surface, avoiding a loss of reproducibility in peak area due to oxidized products on 143 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 cereal151 product raw material or powder from the lyophilized product was mixed thoroughly with 1 ml 152 MeOH and left for 5 min in a closed reaction tube, in order to inactivate interfering, native 153 enzymes from the samples such as α -amylases from cereals. Subsequently, 100 µl internal 154 standard rhamnose (9 mg/ml) and 20 ml 80 °C H₂O, containing 50 mg/l NaN₃, were added 155 and the mixture was subjected to the first extraction step, using the BANDELIN Sonoplus HD 156 3100 homogenizer (Berlin, Germany) equipped with a MS73 microtip and operated at 75° 157 amplitude for 2 x 15 s. Hot H₂O was used in order to enhance the solubilization of fructans 158 and denature native cereal enzymes. The intermixture of NaN₃ prevents the carbohydrates 159 from microbial degradation during sample preparation, storage and analysis at room 160 temperature. After centrifugation at 1520 g for 5 min the supernatant was transferred into a 161 100 ml volumetric flask and the extraction was repeated with 20 ml 80 °C H₂O (containing 162 50 mg/l NaN₃). The supernatants were combined, cooled to room temperature, and the 163 proteins were precipitated by adding 200 µl Carrez I (15 g/100 ml) and Carrez II (23 g/100 ml), 164 respectively. After adjustment to 100 ml, the extract was centrifuged at 3000 g for 10 min and 165 filtered through 0.2 µm polyamide syringe filter (Chromafil AO-20/25, Machery Nagel, Düren, 166 Germany). If extracts were frozen prior to analysis they were reheated to 80 °C in a Stuart 167 Scientific SHT 1D test tube heater (UK) in order to redissolve precipitated fructans. Samples 168 were extracted in duplicates.

169 **Identification of carbohydrates.** The retention times of the reference standards and the 170 peaks in the chromatograms have been compared. Additionally, sample extracts have been 171 spiked with reference standards. Furthermore, enzymatic degradations with α -galactosidase, 172 inulinase and amyloglucosidase have been conducted to confirm the identification and the 173 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

180 Determination of total fructan content and average degree of polymerization. The 181 procedure for the enzymatic hydrolysis for the fructan quantification was based on the method 182 described by Huynh et al. (2008) and the principle of the official method AOAC 997.08 for 183 fructan analysis in food products.^{17,18} Two 500 µl aliquots of the diluted sample extract (usually 184 5 to 20 fold dilution, taking into consideration the substrate to enzyme ratio and the high 185 glucose amounts resulting from the glucose release by amyloglucosidase from co-extracted 186 starch and dextrins) were subjected to two separate enzymatic treatments (with enzyme 187 mixture A and B). 150 µl of the enzyme mixtures were added to the samples. Enzyme mixture 188 A contained a 1:1:1 mixture of amyloglucosidase, α -galactosidase and 0.1 M NaOAc-buffer, 189 latter was replaced by inulinase in mixture B. The lyophilized enzymes were diluted in 0.1 M 190 NaOAc-buffer at pH 4.5 (prepared according to AOAC 997.08) to 220 U/ml, resulting in 191 11 U/150 µl for each enzyme in the mixtures A and B.¹⁷ The reaction mixtures were incubated, 192 in a water bath, in 2 ml screw cap micro tubes for 30 min at 60 °C. Subsequently, the enzymes 193 were inactivated in a test tube heater at 100 °C for 40 min. After cooling to room temperature, 194 350 µl H₂O was added to the hydrolysate, to bring the volume to 1 ml. The precipitated 195 enzymes were removed by centrifugation at 10000 g for 2 min. The hydrolysates were 196 measured via HPAEC-PAD with the CarboPac PA200 column. The total fructan content and 197 the average degree of polymerization (DP_{av}) were calculated based on the results of free 198 (hydrolysate A) and released (hydrolysate B) glucose, fructose and sucrose, based on Huynh 199 et al. (2007).¹⁸ The concentration of glucose (G_f) and fructose (F_f) released from fructans was 200 calculated according to equations 1 and 2, whereas $G_{A\!/\!B},\,F_{A\!/\!B}$ and S_A are the determined 201 glucose, fructose and sucrose concentrations from the hydrolysates A and B in µmol/l, 180.16 202 is the molecular weight of glucose or fructose, DF is the dilution factor (dilution factor from 203 extract dilution before hydrolysis multiplied by 2 from dilution of hydrolysate to 1 ml), V_E is the 204 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)
$$F_f[\%] = \frac{(F_B - F_A - S_A) * 180.16 * DF * V_E}{10\,000 * M_s}$$

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

235 Wholemeal flour was spiked with the reference standards in 5 different concentrations ([0.5 – 236 12] mg/l). Each level was spiked and extracted in a triplicate. The LOD and LOQ have been 237 determined with the signal to noise ratio (S/N, 3 and 10, respectively), from the analytes in the 238 matrix. The wholemeal flour was extracted and analyzed in 6 replicates and in two additional 239 duplicates from three different analysts, respectively, indicating the repeatability of the 240 method. For the validation of the DP_{av} determination of fructans, wheat starch has been spiked 241 with the reference standards kestose (DP3) and nystose (DP4) in 4 different concentrations 242 in triplicates ([0.1 - 0.7] % based on the weight of wheat starch), and additionally 0.2 % 243 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.

250 **Determination of** α **-amylase-activity.** The impact of the extraction temperature and the initial 251 addition of MeOH (cf. procedure for FODMAP extraction) on the activity of the native enzyme 252 α -amylase in wheat, has been investigated, using the Ceralpha Method with the Megazyme 253 alpha amylase assay kit. The enzyme extraction procedure has been modified in order to 254 obtain indications about the impact of the extraction temperature and the addition of MeOH. 255 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.

259 **Statistical analysis.** The statistical evaluation of the validation experiments as well as the 260 investigation of the suitability of the linear and the quadratic regression for a calibration model, 261 applying the Mandel's fitting test, has been carried out with MS excel 2010.

262 **RESULTS AND DISCUSSION**

263 Sample preparation – extraction of FODMAPs. For the extraction of carbohydrates from 264 cereals different extraction media are described in literature. For instance, Pico et al. (2015), 265 Haskå et al. (2008) and Verspreet et al. (2012) have tested H₂O and EtOH as pure extraction 266 agents and in different combinations.^{16,19,24} Whereas the use of 80 % EtOH is known to be 267 advantageous to minimize the co-extraction of starch, it leads to an incomplete extraction of 268 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 269 comparison to 100 % H₂O.²⁴ In order to achieve a complete extraction of carbohydrates, 270 271 especially the higher DP fructans, a second extraction step with H₂O of the remaining pellet 272 from the EtOH extraction is necessary.^{16,19} Before the supernatants can be combined, the 273 EtOH must be fully evaporated and the residues recovered in aqueous solution, in particular 274 if enzymatic hydrolysis is applied for fructan analysis. The simplest, efficient extraction medium 275 appears to be 100 % H₂O. Verspreet et al. (2012) have shown no significant difference 276 between the fructan results obtained from EtOH/ H₂O supernatants compared to only H₂O 277 supernatants. However, their method applies mild acid hydrolysis for the quantification of 278 fructans, which is less sensitive to the interference of co-extracted starch. Stöber et al. (2004) 279 have shown a strong interference of starch with the fructan analysis, leading to an 280 overestimation of the glucose amount released from fructans (G_f, cf. equation 1 and 5) as 281 commercial fructan degrading enzyme-preparations release glucose from non-fructan 282 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 thisstudy.

285 Another important parameter is the temperature of the extraction medium. Whereas smaller 286 carbohydrates are easily extracted at room temperature, fructans, especially longer chains, 287 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 H₂O has been compared in 288 289 preliminary work for this study, using the K-FRUC assay. Only 70 % of the fructans extracted 290 with hot H₂O 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 291 292 be enhanced due to the suspension of the sample in MeOH prior to the extraction. The 293 analysis of the native α-amylase activity (K-CERA) in commercial whole wheat flour and milled 294 wheat grains, after extraction with MeOH-addition to the extraction buffer and heating of 295 extraction buffer and the mixture of both, confirmed this assumption (Figure S1, Supporting 296 Information).

297 The determined α -amylase activities correlated with the glucose amounts in the commercial 298 whole wheat flour, obtained from the HPAEC-PAD profiles of the extracts with the different 299 conditions. Furthermore, the amounts of fructose, glucose and sucrose gave indication of 300 sucrose-hydrolyzing invertase activity (potentially from microbial contamination, as preliminary trials did not include NaN₃ addition). Samples extracted at room temperature, without the 301 302 addition of MeOH contained the highest amounts of glucose and fructose and the lowest 303 amounts of sucrose, whereas the addition of MeOH and extraction with H₂O heated to 80 °C 304 led to the lowest levels of glucose and fructose and highest sucrose levels. The values 305 obtained from the extraction at room temperature with the addition of MeOH were inbetween 306 the latter described amounts. This indicates the enhancing effect of enzyme inhibition of the 307 hot extraction medium. The high difference in the glucose amounts derived from the different 308 extraction conditions compared to the difference in the sucrose and fructose amounts 309 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 H₂O 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.

319 All compounds in the HPAEC-PAD profiles have been either identified comparing the retention 320 times of reference standards (pure and added to the sample matrix), or if commercial 321 standards were not available, by comparison with other studies and hydrolysis of sample 322 extracts with different enzymes. Peaks in the chromatogram, which disappeared after 323 incubation of the sample extracts with inulinase, could be assigned as fructans, sucrose, 324 fructosylraffinose and GOS. The hydrolysis led to an increase of fructose and glucose and the 325 appearance of the degradation-products from fructosylraffinose, raffinose/ stachyose (not 326 separated on CarboPac PA200, separation on CarboPac PA1 not shown) and verbascose, 327 resulting in melibiose (confirmed with reference standard; separated on CarboPac PA1, on 328 CarboPac PA200 coeluting with fructose), manninotriose and manninotetraose, respectively 329 (Figure 2). Incubations with α -galactosidase degraded GOS and fructosylraffinose and led to 330 an increase of glucose/ galactose (only separated on CarboPac PA1 column), sucrose and 331 kestose (Figure 2). Furthermore, incubations with amyloglucosidase degraded maltose, 332 maltotriose and higher malto-dextrins from co-extracted and partially hydrolyzed starch 333 (retention times on corresponding columns summarized in Table S2, Supporting Information). 334 These hydrolysis trials gave also indication about the purity of the peaks, excluding on the one 335 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 336

337 same area on the CarboPac PA200 (after 13 min onwards), are very difficult to assign clearly 338 to one of those oligo- and polysaccharide groups.^{16,25} Hence, any quantitative or semi-339 quantitative evaluation, including the fingerprint of the fructan profile or the integration of peaks 340 assigned to one DP, can only be carried out if the sample extracts were initially incubated with 341 amyloglucosidase, as described by Rakha et al.(2010).²⁵ Furthermore, if divers samples, in 342 particular products containing different cereals/ pseudocereals/ pulses as ingredients, the 343 chromatographic profiles, especially in the higher DP area may be very difficult to identify (cf. 344 Figure S3, Supporting Information, showing Error! Reference source not found.profiles from 345 different typical cereal-product ingredients). In order to evaluate peaks from that area further 346 studies, focused on the characterization of the specific ingredients, would be required, such 347 as those conducted by Haskå et al. (2008) or Rhaka et al. (2010)., for wheat and rye.^{16,25} Thus, 348 this study approached the fructan quantification and determination of the DP_{av} only after 349 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.

353 The linearity of a method is referred to the whole method, including all steps of the sample 354 preparation. Hence replicates of reference standard dilutions would not indicate the linearity 355 of a method.²⁷ It describes the relation of analytical signal and concentration of analytes. 356 However, the term linearity itself is misleading and might suggest a linear relation is required. 357 In reality, the impact of sample matrix and the principle of analytical measurement, often lead 358 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 359 360 the quantification of carbohydrates a quadratic function may reveal a better fit in a calibration 361 model, depending on analytical system, sample type and analytes investigated.^{20,29} In this 362 study, the calibration model was evaluated using the Mandel's fitting test. Correlation 363 coefficient of the calibration curve and visual comparison of linear and quadratic regression

364 functions as well as residual plots (Figure 3; Table 1) may give first indication about the 365 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 366 367 of the linear function is a sign for an incorrect model. However, the Mandel's fitting test allows 368 a better evidence, determining a potential significant difference in residual variances from linear and guadratic calibration.^{27,30} According to equation 6 the test variable (TV) is calculated 369 370 and compared to the corresponding value of the F-distribution with 1 and n-3 degrees of freedom at the significance level of α = 0.05 (F_{0.05, 1, n-3}). 371

372 (6)
$$TV = \frac{(n-2) * s_{y_1}^2 - (n-3) * s_{y_2}^2}{s_{y_2}^2}$$

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

373 (7)
$$s_{y_1} = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \hat{y_i})^2}{n-2}}$$

374

375 (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 H₀ (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 $[0.5 - 10] \mu g/l$ and $[2 - 5*10^{1}] \mu g/l$, 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 389 wheat flour replicates on the same day and on different days, revealed an acceptable 390 reproducibility of the method, with RSD ranging between [2.3 - 10.3] %. The highest variation 391 in the results was observed for the analysis of glucose, in the replicates on different days, 392 conducted by different analysts. As discussed above, this deviation is due to partially 393 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.

404 Enzymatic fructan determination. The determination of the total fructan content via HAPEC-405 PAD has been compared with the results obtained from the photometric fructan assay (K-406 FRUC). Both methods are based on the measurement of enzymatically released glucose and 407 fructose monomers from fructans. In accordance to other studies ^{16,19}, the total fructan 408 amounts obtained from the enzymatic assay, were overestimated due to GOS if the additional 409 α -galactosidase was not included in the procedure (data not shown), since inulinases release 410 fructose from those non-fructan compounds (Figure 2). Thus, this correction was included in 411 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

17

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.

423 **Application of the method.** This method has been applied for the FODMAP quantification in 424 different commercial cereal-based products and delivered results, assessed to be reasonable, 425 based on the product's ingredients and comparable literature data (data not shown).^{1,2} Further 426 studies will concentrate on the FODMAP-characterization of cereal-product ingredients and 427 the development of products with a lowered FODMAP content using the presented method as 428 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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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.

	LOD/ LOQª	RSD _{interday} / RSD _{intraday} [%]	SD _{interday} / Recovery ^a D _{intraday} [%] [%]		Fit regression model ^a		
	[µg/l]	(n=6) ^{b,c}	(n=3, m=5)	R² _{linear} / R² _{quad} ď	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	

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

^{*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 R² represents the correlation coefficient

 $^{\rm e}~s_{y1}/s_{y2}$ 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)

	$DP_{av} \pm standard deviation$		
	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	

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

^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 [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 2













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.

213x133mm (150 x 150 DPI)





193x164mm (150 x 150 DPI)



Table of Contents Graphic

260x176mm (150 x 150 DPI)

Supporting Information

(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	
50	26.5	27.5	46	separation of
70	15.3	27.5	57.2	analytes
75	0	27.5	72.5	
80	0	100	0	
85	0	100	0	column cleanup
86	0	0	100	
101	0	0	100	regeneration gold surface
102	70	27.5	2.5	
117	70	27.5	2.5	re-equilibration
(2) Isocratio	c method	d on Car	boPac P	A1 ^a
time [min]	A ^b [%]	B¢ [%]	C ^d [%]	
0	91.9	8.1	0	separation of
25	91.9	8.1	0	analytes
26	0	0	100	
31	0	0	100	column cleanup
32	0	100	0	
47	0	100	0	regeneration gold surface
48	91.9	8.1	0	
60	91.9	8.1	0	re-equilibration

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

^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 H₂O heated to 80 °C with MeOH addition. Error bars represent standard deviations of triplicates.

	Rt [min]	Rt [min]	
Compound	CarboPac	CarboPac	
	PA200 ^a	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 ^{<i>b</i>}	53 ^{b,c}	
Lactose	6.4	39.5 ^c	
Raffinose	7.8	52.5°	
Stachyose	7.8	61.9 ^c	
Verbascose	8.7	-	
Kestose	8.8	-	
Nystose	13.0	-	
Kestopentaose	16.3	-	
	1		

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

^aOnly peaks with retention times printed in bold have been used for quantitative analyses. ^bIdentification based on enzymatic hydrolysis; reference standards were not available. ^cExtended 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