CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	Comparative study of sugar extraction procedures for HPLC analysis and proposal of an ethanolic extraction method for plant-based high-protein ingredients
Authors	Hoehnel, Andrea;Salas García, Jairo;Coffey, Christine;Zannini, Emanuele;Arendt, Elke K.
Publication date	2021-03-11
Original Citation	Hoehnel, A., Salas García, J., Coffey, C., Zannini, E. and Arendt, E. K. (2021) 'Comparative study of sugar extraction procedures for HPLC analysis and proposal of an ethanolic extraction method for plant-based high-protein ingredients', Journal of the Science of Food and Agriculture, 102(12), pp.5055-5064. https://doi.org/10.1002/jsfa.11204
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1002/jsfa.11204
Rights	© 2021, Society of Chemical Industry. Published by John Wiley & Sons, Inc. All rights reserved. This is the peer reviewed version of the following article: Hoehnel, A., Salas García, J., Coffey, C., Zannini, E. and Arendt, E. K. (2021) 'Comparative study of sugar extraction procedures for HPLC analysis and proposal of an ethanolic extraction method for plant-based high-protein ingredients', Journal of the Science of Food and Agriculture, doi: 10.1002/jsfa.11204, which has been published in final form at: https://doi.org/10.1002/jsfa.11204. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.
Download date	2026-11-02 21:54:17
Item downloaded from	https://hdl.handle.net/10468/11219



University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Accepted Article

Hoehnel Andrea (Orcid ID: 0000-0002-2551-8776)
Zannini Emanuele (Orcid ID: 0000-0003-4144-4575)
Arendt Elke (Orcid ID: 0000-0003-4144-4575)

Comparative study of sugar extraction procedures for HPLC analysis and proposal of an ethanolic extraction method for plant-based high-protein ingredients

Sugar extraction from high-protein ingredients

Andrea Hoehnel[a], Jairo Salas García[a], Christine Coffey[a], Emanuele Zannini[a], Elke K. Arendt[a,b,*]

[a]*University College Cork, School of Food and Nutritional Sciences, College Road, Ireland*
[b]*APC Microbiome Ireland, Cork, Ireland*

Keywords:

plant protein, cereal, pseudocereal, legume, sugar quantification, sugar extraction, ethanolic extraction, HPLC analysis, RID

*Corresponding author. Tel: +353 21 490 2064; Fax: +353 21 427 0213; Email address: e.arendt@ucc.ie (E. K. Arendt)

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.11204

This article is protected by copyright. All rights reserved.

Accepted Article

Abstract

BACKGROUND: The increasing importance of plant-based proteins in the food sector makes a reliable compositional analysis of plant-based high-protein ingredients a necessity. Specifically, the quantification of short-chain carbohydrates is relevant for multiple areas, including food product development, food labelling and fundamental food chemistry and food technology research. Commonly used extraction procedures for subsequent HPLC separation and quantification of short-chain carbohydrates have been discussed controversially regarding a range of complications that can potentially lead to inaccurate sugar determination. The present study compares the sugar levels in wheat flour and wholemeal wheat flour determined with different aqueous and ethanolic extraction procedures. These procedures included measures to prevent enzyme activity and microbial growth, which represent two of the most relevant challenges in sugar extraction from food samples.

RESULTS: Differences in sugar levels (sum of sucrose/maltose, glucose and fructose) as high as 1.8 %DM (wheat flour) were observed between the employed extraction procedures. Ethanolic extraction (80 % ethanol in ultrapure water) with the use of the antimicrobial agent sodium azide but without Carrez clarification was identified as most promising for sugar determination in plant-based high-protein ingredients.

CONCLUSION: A screening of high-protein ingredients derived from cereals (wheat gluten), pseudocereals (quinoa, amaranth, buckwheat) and legumes (soy, pea, lupin, lentil, carob, chickpea, faba bean) concerning their levels of sucrose, maltose, glucose and fructose confirmed the applicability of the chosen extraction procedure.

Accepted Article

1. INTRODUCTION

Plant-based foods, and plant-based proteins in particular, are a growing sector in the food industry and one of the key trends in food, nutrition and health.[1] Research in this area focuses on nutritional and sensory characteristics but also on processing techniques to functionalise and optimise plant-based high-protein ingredients (HPIs) for multiple food applications. Promising raw materials that have received major research attention are cereals, pseudocereals and legumes. A reliable analysis of these ingredients' composition (and derived food products) builds the foundation for this research and becomes, thus, increasingly important. Specifically, the quantification of short-chain carbohydrates is relevant with regard to a range of different aspects. This includes the nutritional evaluation of foods, food labelling, the assessment of an ingredient's applicability to specific processing techniques (such as fermentation) and the understanding of compositional, techno-functional and structural changes during processing (such as protein isolation or incorporation in complex foods). Sugar analysis in plant-based HPIs with common aqueous extraction methods for subsequent HPLC separation, such as AACC 80-04.01, can bear challenges related to gelation and high water absorption of these samples. While an increase of the liquid/solid ratio during extraction promises improvement in this regard, it also results in less concentrated extracts. This negatively impacts the achievable limit of quantification (LOQ) and the ability to obtain meaningful results, especially when measuring with low sensitivity detectors such as refractive index detectors (RIDs). Moreover, a range of other complications with aqueous extractions has been reported and addressed in the literature. The primary concern refers to di-, oligo- and polysaccharide degrading enzymes present in the sample when extractions are carried out under conditions that allow their activity.[2] These can be endogenous plant-derived enzymes or microbial enzymes (from the natural microbial population of the raw materials or microbial

Accepted Article

contaminations). Additionally, enzymes originating from food additives, processing aids or other food ingredients (e.g. added malt in wheat flour) might be relevant. Further issues in sugar analysis can be related to microbial growth in the samples during or after extraction, as well as polysaccharide coextraction and the presence of proteins in the extracts.[3,4] Potential measures that aim to prevent enzyme activity, microbial growth, starch coextraction or to remove coextracted proteins are the following: sample pre-treatment with alcohol (methanol or ethanol), heat treatment, extraction with alcoholic solutions, use of antimicrobial agents (e.g. sodium azide; NaN_3) and performance of sample clean-up (e.g. Carrez clarification).[2–4] The present study's objective is the comparative investigation of selected aqueous and ethanolic extraction procedures with and without such measures. These are applied to wheat flour, representing a regular plant-based food ingredient, and to wholemeal wheat flour, representing a less homogenous and more complex food ingredient to challenge the extraction procedures. This will help identify a suitable method for the extraction and quantification of short-chain carbohydrates from plant-based HPIs derived from the following raw materials: wheat (gluten), quinoa, buckwheat, amaranth, soy, pea, lupin, lentil, carob, chickpea and faba bean.

2. EXPERIMENTAL

2.1. Materials

Wheat flour (baker's flour) was supplied by Whitworth Bros Ltd, UK and wholemeal wheat flour by Odlums, Valeo Foods, Ireland. The following commercial high-protein ingredients (HPIs) were used: soy protein isolate (Clarisoy 100; further referred to as "soy") supplied by ADM, Illinois, US; pea protein isolate (NUTRALYS PEA BF; further referred to as "pea") from Roquette, France; vital gluten (NUTRALYS W; further referred to as "gluten"); carob germ flour (GRINDSTED VEG PRO S1; further referred to as "carob") from Danisco, UK;

Accepted Article

and chickpea flour (GF gram flour; further referred to as "chickpea") from Doves Farm Foods Ltd, UK. Eight experimentally produced HPIs were provided by Fraunhofer Institute IVV, Germany: one regular flour – dehulled lentil flour (further referred to as "lentil"); five high-protein flours (fine fractions; obtained by air-classification) – quinoa high-protein flour (further referred to as "quinoa"), amaranth high-protein flour (further referred to as "amaranth"), buckwheat high-protein flour (further referred to as "buckwheat"), and faba bean high-protein flour (further referred to as "faba bean"); and three protein isolates – lentil protein isolate I (isolated by isoelectric precipitation; IEP; further referred to as "lentil IEP"), lentil protein isolate II (isolated by ultrafiltration; UF; further referred to as "lentil UF") and lupin protein isolate (from blue lupin; further referred to as "lupin"). The compositional data (analyses performed as indicated below) of all these ingredients are presented in Table 1.[5] Ultrapure water, with a resistivity of 18.2 MΩ·cm and a total organic carbon (TOC) content of < 5 ppb, was used for all extraction procedures and the preparation of eluents and reference standards. Sodium azide (NaN_3) was obtained from Thermo Fisher Scientific (Alfa Aesar, UK). HPLC-grade acetonitrile (MeCN), methanol (MeOH) and ethanol (EtOH) were purchased from Sigma-Aldrich (Missouri, USA). All other chemicals were also purchased from Sigma-Aldrich (Missouri, USA) unless stated otherwise.

2.2. *Compositional Analysis*

Results from compositional analysis were expressed as the percentage based on dry matter (%DM). Compositional analysis for wheat flour, wholemeal wheat flour and HPIs was performed as follows: moisture was determined according to the air-oven method AACC 44-15.02; fat based on AACC 30–25.01 (Soxhlet method using SoxCap and Soxtec (Foss UK Ltd, UK), including a digestion step with 4 M HCl and a subsequent extraction step); ash based on AOAC 923.03 (removal of organic matter in a muffle furnace at 550°C for 5 h); protein was based

Accepted Article

on MEBAK 1.5.2.1 (nitrogen-to-protein conversion factor 6.25). The analysis of total dietary fibre (TDF) was performed based on AOAC 991.43 (gravimetric method) by Concept Life Science Ltd, UK. Carbohydrate values were obtained by calculation (100% − [moisture% + fat% + ash% + protein% + TDF%]). Compositional analysis for wheat flour and wholemeal wheat flour was performed by Concept Life Sciences, UK, as previously specified by Hoehnel et al.[6].

2.3. *Comparison of Extraction Procedures*

Short-chain carbohydrates were extracted from wheat flour, wholemeal wheat flour and HPIs using nine different extraction procedures. Six of the procedures are based on aqueous extraction (A1 to A6); the other three extraction procedures are based on ethanolic extraction (E1 to E3). A2 to A6 are based on A1 and include small adaptions and additional treatments as indicated in the following sections. Similarly, E2 and E3 are based on E1 and include small adaptions and additional treatments, which are also specified below. Figure 1 provides an overview of all extraction procedures employed in this study (for comparison reasons, this figure also includes AACC 80-04.01, which was not used in the present study).

2.3.1. *A1 Extraction*

This aqueous extraction procedure is based on AACC 80-04.01 and was adapted according to laboratory equipment and practicability as follows: 8 mL of UPW (room temperature) were added to 2 g of sample in a 15 mL polypropylene (PP) test tube (conical bottom). The mixture was vortexed and shaken by hand (if necessary) until the powder was fully suspended. This was followed by 20 min of shaking at room temperature using an orbital shaker (UNI MAX 1010, Schwabach, Germany). After shaking, the sample was centrifuged at 1800 g for 10 min (20°C). The supernatant was quantitatively transferred into a 10 mL volumetric flask, and

Accepted Article

the volume adjusted to 10 mL with UPW. The extract was filtered through a syringe-driven polyamide (PA) filter with a pore size of 0.20 – 0.45 µm (Machery-Nagel, Germany) and analysed immediately (HPLC) or stored frozen in 2 mL screw-cap tubes with sealing ring until further analysis.

2.3.2. *A2 Extraction*

The extraction was performed according to the procedure specified for A1 with one modification. A dilute NaN_3 solution (50 ppm in UPW) was used instead of UPW in A1.

2.3.3. *A3 Extraction*

This extraction procedure includes two modifications of A1. Instead of 8 mL of UPW, the following solvents were added to the solid sample: 1 mL MeOH and 7 mL UPW. MeOH was added first to ensure the sample was soaked in MeOH before adding the UPW. Furthermore, instead of 20 min of shaking at room temperature (as specified in A1), the samples were subjected to a heat treatment at 80 °C for 10 min using a shaking incubator.

2.3.4. *A4 Extraction*

This extraction procedure was performed according to A3 but with extended heat treatment for 20 min instead of 10 min.

2.3.5. *A5 Extraction*

The extraction was performed according to procedure A4, but instead of UPW, a dilute NaN_3 solution (50 ppm) was used.

2.3.6. *A6 Extraction*

Accepted Article

The extraction was performed according to A1, with an additional Carrez clarification step. The Carrez solutions (Carrez I and II) were added after transferring the extract into the 10 mL volumetric flask and before adjusting the volume to 10 mL with UPW. First, 250 µL of Carrez I (15 g potassium hexacyanoferrate(II) trihydrate per 100 mL UPW) were added to the sample, followed by 250 µL Carrez II (23 g zinc acetate dihydrate per 100 mL UPW). The volume was then adjusted to 10 mL with UPW, the contents of the volumetric flask were transferred into a new 15 mL test tube and centrifuged at 1800 g for 10 min (20°C).

2.3.7. E1 Extraction

For the following extractions, alcohol/water mixtures with a high proportion of alcohol were used as extraction solvent. The alcohol is evaporated by vacuum centrifugation as part of the extraction procedures. EtOH was chosen due to the fact that it is less hazardous than MeOH. E1 was carried out as follows: 15 mL of 80/20 (v/v) ethanol/UPW (80 % EtOH), which was heated to 55 ± 5 °C, were added to 2 g of sample in a 50 mL PP test tube. The mixture was vortexed to make sure the powder was fully suspended. The sample was then sonicated (BANDELIN Sonoplus HD 3100 homogenizer with MS73 microtip) twice for 15 s with a 5 s break (75° amplitude) and centrifuged at 1800 g for 10 min (20°C). The supernatant was transferred to a new 50 mL PP tube and the remaining pellet reextracted by adding another 15 mL 80 % EtOH (55 ± 5°C). The mixture was vortexed until the pellet was resuspended and the sonication with subsequent centrifugation repeated as described above. The second supernatant was pooled with the first, and the entire sample extract was concentrated by vacuum centrifugation (Scanvac Scan Speed 32 with Scanvac VacSafe 15, Labogene ApS, Denmark) with the following conditions: run 1 - 1500 rpm, 45°C, 2 h; followed by run 2 (after replacement of ice in the cold trap and cold water in the pump) - 2000 rpm, 50°C. The concentrated extract (around 6 mL) was transferred into a 10 mL volumetric flask;

Accepted Article

filled up with UPW, filtered through a syringe driven PA filter with a pore size of 0.20 – 0.45 µm (Machery-Nagel, Germany) and analysed immediately (HPLC) or stored frozen in 2 mL screw-cap tubes with sealing ring until further analysis.

2.3.8. *E2 Extraction*

E2 represents an adaptation of E1. It was carried out as described above, but a dilute NaN_3 solution (50 ppm) was used instead of UPW to fill up the volumetric flask to 10 mL after concentration of the sample extract. This procedure was previously used and described by Hoehnel et al.[6] for the extraction of freeze-dried bread samples.

2.3.9. *E3 Extraction*

The extraction was performed according to the procedure described for E1 with one modification. After concentration of the sample extract and its transfer to the 10 mL volumetric flask, a Carrez clarification was realised by adding 250 µL of both Carrez I and Carrez II (as specified above for A6). The volumetric flask was then filled up with UPW, and the contents were transferred to a 15 mL test tube for centrifugation (1800 g for 10 min; 20°C) before filtration.

2.4. *Determination of α-Amylase and β-Amylase Activity*

The activity of both α-amylase (K-CERA) and β-amylase (K-BETA3) were determined using the indicated enzyme kits from Megazyme, Ireland. The enzyme activities were assessed to interpret differences observed between wheat flour and wholemeal wheat flour due to the different extraction procedures. The assays were carried out according to the manuals provided with the kits.

2.5. *Determination of Sugar Contents of Plant-Based High-Protein Ingredients*

A range of thirteen HPIs was screened for their contents of maltose/sucrose,

Accepted Article

glucose and fructose. The ethanolic extraction procedure E2 was chosen for the screening. This decision was based on the results obtained from comparing the different extraction procedures above and the fact that extracting HPIs is facilitated by using ethanolic solutions and higher volumes to minimise gelation related issues. Two different columns were used for the separation and quantification of the sugars, as specified below.

2.6. *Separation and Quantification of Mono-and Disaccharides*

Separation and quantification of mono- and disaccharides were achieved with an Agilent Infinity 1260 equipped with an RID. A dilute solution of calcium disodium ethylene diamine tetraacetate (CaEDTA; 0.0001 M) in UPW was used as eluent. The separation of sugars was achieved with a Sugar-Pak I (SP) column (300×6.5 mm, 10 µm; packed with a microparticulate cation-exchange gel in calcium form; Waters Corporation, MA, USA) and the following conditions: column temperature 80 °C; flow rate 0.5 mL/min; isocratic elution with 0.0001 M CaEDTA. A separation of sucrose and maltose was not achieved with this column. Therefore, a second column was used to allow for an individual quantification of sucrose and maltose for the screening of HPI sugar levels: High Performance Carbohydrate (HPC) column (4.6×250 mm, 4 µm spherical silica bonded with trifunctional amino propyl silane; Waters Corporation, MA, USA) with the following conditions: column temperature 40°C; flow rate 1.0 mL/min; isocratic elution with 78/22 (v/v) MeCN/UPW; sample extracts were mixed with MeCN in a ratio of 40/60 (v/v) extract/MeCN. Mixtures of reference standards (sucrose, maltose, glucose and fructose; all > 98 % purity) were used to quantify (standard concentrations ranging from 0.01 - 10 g/L for the SP column and from 0.1 - 3.5 g/L for the HPC column). Results of sugar levels in wheat flour, wholemeal wheat flour and HPIs are expressed in grams of analyte per 100 g of dry matter of the sample (%DM).

Accepted Article

2.7. *Statistical Analysis*

Extractions for the comparison of extraction procedures were carried out in duplicate and extractions for screening HPI sugar levels in triplicate. Data analysis was carried through with RStudio, v.1.2.1335 with R v.3.6.1 (RStudio Inc, USA; R Core Team, r-project). One-way analysis of variance (ANOVA) with post-hoc pairwise Tukey's test was utilised to determine significant differences ($p<0.05$).

3. RESULTS AND DISCUSSION

Figure 1 presents a condensed overview of the different extraction procedures (and their conditions) employed in this study to support the results' interpretation. The outcomes of both the comparison of extraction procedures and the screening of sugar levels in HPIs are visualised in Figures 2 and 3. The exact values (means, standard deviations), including results of one-way ANOVA, are enclosed in Tables A.1 and A.2 in the appendix.

3.1. *Comparison of Extraction Procedures*

Figure 2 reflects the large discrepancies in determined sugar levels that were observed using the different extraction procedure. Specifically, differences in sugar contents determined for wheat flour and wholemeal wheat flour, which were subjected to all nine extraction procedures, were found to be as high as 1.3 – 1.8 %DM (sum of sucrose/maltose, glucose and fructose). Soy could only be used to assess differences among the three ethanolic extraction procedures E1 – E3 since the aqueous extractions were not applicable to this ingredient (high water absorption/gelation).

3.1.1. *Use of Methanol and Heat Treatment*

Extraction procedures A3 and A4 include the replacement of 1 mL of the extraction solvent UPW by MeOH and a heat treatment. While shaking at 80 °C was

Accepted Article

carried out for only 10 min in A3, it was prolonged to a total of 20 min in A4. Significantly lower levels of sucrose/maltose, glucose and fructose were observed with A3 compared to A1 (except for sucrose/maltose in wholemeal wheat flour: slightly but significantly higher level with A3). Even smaller values than with A3 were obtained with A4 for all evaluated sugars. Sugar contents can be influenced by the degradation of di-, oligo- and polysaccharides present in the samples. Specifically, enzymatic degradation can occur. These enzymes can be endogenous plant-derived enzymes or microbial enzymes originating from the natural microbial population of the raw material or from microbial contamination during sample storage and sample preparation (if no antimicrobial agents used). A few examples of such enzymes that are relevant for carbohydrate degradation in cereal and legume matrices are the following: amylases (α- and β-amylases[7]), β-glucanases[8], invertases[9] and fructan exohydrolases[10,11] as well as α-galactosidases[12] and levansurases[13]. While, for example, for fructan degrading enzymes, it has been reported that their remaining activity is relatively small in mature wheat grains[10,11], it is also known that carbohydrate levels can change during storage of food ingredients and products, which was associated with potential enzyme activity or physicochemical processes.[14–17] Several different measures have been used to inactivate enzymes. Pre-treatment with alcohol (MeOH or EtOH) and heat treatment at 80 °C, similar to procedures A3 and A4, were applied during aqueous extraction of multiple different sample matrices[2,4,18] and has been shown to substantially reduce amylase activity in wholemeal wheat flour and to decrease measured levels of glucose.[4] Also, an increase of the determined sucrose level and a decrease in fructose were reported, which was attributed to the inactivation of potentially present invertase by the treatment.[4] This is in line with the lower levels of glucose and fructose determined in both wheat flour and wholemeal wheat flour when extracted with procedures A3 and A4 (compared to A1). The sucrose/maltose contents, however, were lower (wheat flour) or similar (wholemeal wheat flour) to A1 instead of

Accepted Article

higher (except A3 in wholemeal wheat flour: sucrose/maltose significantly higher than with A1). This observation might be caused by the lack of separation between sucrose and maltose and the determination of the sum of both sugars. In A1, where no measures against enzyme activity were taken, both a conversion of sucrose into glucose and fructose (potentially by invertases) as well as a degradation of starch into maltose and small amounts of glucose (amylases) is possible. The enzymatically released maltose would lead to high values for sucrose/maltose. An inhibition of both enzymes would prevent the degradation of sucrose but also the accumulation of maltose and thereby cause a reduction in the sum value for sucrose/maltose, which was observed for A3 and A4 in wheat flour (the exception of seemingly stable sucrose/maltose values in wholemeal wheat flour will be addressed separately below). Instead of an inactivation of enzymes, the generally lower sugar levels observed with A3 (compared to A1) could have been attributed to an incomplete extraction due to the shorter extraction time (but in spite of the increased temperature, which generally accelerates extraction). However, the fact that A4 resulted in even lower values for sucrose/maltose, glucose and fructose than A3 contradicts this theory and rather indicates that a more efficient enzyme inactivation was achieved with the prolonged heat treatment (20 min instead of 10 min in A3). A further extension of the heat treatment to 30 min did not lead to an additional decrease in sugar levels (data not shown). In aqueous extractions without or with insufficient measures to inhibit enzymes, their activity cannot only lead to carbohydrate degradation during extraction but also at any time after extraction (during sample handling at room temperature or above). This is particularly relevant for samples containing large amounts of polysaccharides (e.g. starch), which are partially coextracted when using UPW as extraction solvent.

3.1.2. *Aqueous vs Ethanolic Extraction*

Accepted Article

Besides alcohol (MeOH or EtOH) pre-treatment and heat treatment, alcohol/water mixtures with varying alcohol contents (30 – 80 %) have been described to minimise complications related to enzyme activity when used as extraction solvents for short-chain carbohydrates and are commonly used.[2,19] However, higher alcohol contents in the extraction solvent have been discussed controversially with regard to extractability of short-chain carbohydrates. Johansen et al.[2] and Xiaoli et al.[19] found that, while low levels of alcohol do not seem to reduce extraction yields, MeOH or EtOH levels of 50 % or higher cause a drastically reduced extractability of short-chain carbohydrates like galactooligosaccharides (GOS) and sucrose (unless extraction was performed at boiling point). On the other hand, other publications report no negative impact of the use of 80 % MeOH or EtOH (often in combination with elevated temperatures) on the extraction yield of short-chain carbohydrates.[14,20–24] In the present study, generally lower sugar levels were determined with the ethanolic extraction procedures than with the aqueous extractions (except for sucrose/maltose in wholemeal wheat flour). This might, at first, suggest that these methods do not provide satisfying extraction efficacy. However, a comparison of E1 with A4, which is not expected to show incomplete sugar extraction, reveals no significant differences in determined sugar levels. This indicates efficient enzyme inhibition and adequate sugar extractability rather than incomplete sugar extraction for E1. The fact that the ethanolic extractions in this study were performed with two consecutive extraction steps (re-extraction of the pellet obtained after first extraction and centrifugation) potentially contributes to this outcome. Furthermore, instead of the commonly used shaking (in aqueous extractions in this study) or stirring, sonication was applied to facilitate extraction. According to Machado et al.[25] and Karki et al.[26], sonication results in significantly more efficient sugar extraction than stirring. Therefore, the combination of 80 % EtOH with elevated temperature (55 °C), re-extraction of the pellet and sonication seems to achieve adequate sugar extraction. A comparison of determined sugar levels with

Accepted Article

values reported in the literature is difficult and bears limited validity due to natural variations in raw material composition (different cultivars, growing, processing and storage conditions) and differences in extraction procedures. However, the sugar contents in wheat flours determined by Ispiryan et al.[27] (glucose, fructose), Ziegler et al.[18] (glucose, fructose) and MacArthur and D'Appolonia[28] (sucrose, maltose, glucose, fructose) are generally better reflected by values obtained with the extraction procedures A4 and E1 in the present study. Slightly increased glucose values in the literature in comparison to values obtained with E1 could be associated with the expected starch coextraction and its possible degradation when using UPW as extraction solvent[18,27] (or the lack of using antimicrobial agents[18]). Starch from various plant materials has been described to be insoluble in 80 % EtOH which makes this a promising solvent to minimise starch coextraction.[29]

3.1.3. *Differences between Wheat Flour and Wholemeal Wheat Flour*

Wheat flour and wholemeal wheat flour show slightly different trends regarding the sugar levels obtained with the investigated extraction procedures. For wheat flour, A1 resulted in much higher sucrose/maltose values than A3, A4 and E1. For wholemeal wheat flour, on the other hand, A1 led to similar or even slightly lower sucrose/maltose values than A3, A4 and E1. In the case of glucose and fructose, the observed trends do not differ between wheat flour and wholemeal wheat flour. However, much higher glucose and fructose levels were determined with A1 in wholemeal wheat flour than in wheat flour. Also, the glucose/fructose ratio (values below 1 represent excess fructose) determined with A1 is much lower in wholemeal wheat flour (approx. 1.24; A1) than in wheat flour (approx. 0.76; A1). The fact that wholemeal wheat flour contains the outer parts of the wheat grain to a much higher degree than wheat flour consequently results in both a different pattern of present endogenous and microbial (inclusion of outer layers causes a larger microbial population[30]) enzymes as well as a higher content of non-

Accepted Article

starch oligo- and polysaccharides (such as fructans) which can act as substrates for these enzymes.[31] The activities of α- and β-amylase were determined in wheat flour and wholemeal wheat flour (Table 2) to support the results' interpretation. The activity of α-amylase accounted for 0.119 CU/g in wheat flour and 0.122 CU/g in wholemeal wheat flour. The determined values for β-amylase activity were 34.82 BU/g (wheat flour) and 35.38 BU/g (wholemeal wheat flour). While the determined activities of α- and β-amylase for wheat flour and wholemeal wheat flour were very similar (no significant differences), possibly invertase or other fructan degrading enzymes were more active in wholemeal wheat flour. Comparing A1 results of wheat flour and wholemeal wheat flour, this would explain the generally higher glucose and fructose levels, the lower glucose/fructose ratio (fructan degradation releases primarily fructose) and the relatively low sucrose/maltose levels in wholemeal wheat flour due to sucrose breakdown.

3.1.4. *Use of Sodium Azide*

The use of NaN_3 was investigated due to the potential impact of microbes present in the raw material or introduced by microbial contamination during sample storage or extraction. Growth of these microbes can lead to degradation (enzymatic; as discussed above) or consumption (metabolism) of sugars during sample preparation, storage and analysis at room temperature (e.g. HPLC autosampler without thermostat). This can negatively impact the accuracy and reproducibility of determined sugar levels, and the use of NaN_3 can, therefore, be seen as a preventative measure. The methods A2, A5 and E2 represent extraction procedures where a dilute solution of NaN_3 (50 ppm) in UPW was used as extraction solvent (A2, A5) or final solvent (E2). They were performed exactly like their counterparts without NaN_3, which are A1, A4 and E1, respectively. Therefore, a comparison of the results obtained for the following pairs allows for an assessment of the effect

Accepted Article

of NaN_3 on sugar extraction: A1/A2, A4/A5, E1/E2. Figure 2 reveals that no significant differences were found within these pairs for levels of sucrose/maltose, glucose and fructose. This implies that no microbial growth interfered in the samples without NaN_3. It also represents encouragement for the use of NaN_3 in sugar extractions since no negative impact on sugar determination was observed.

3.1.5. *Use of Carrez Clarification*

The methods A6 and E3 represent extraction procedures where a Carrez clarification was applied to the extracts just before adjusting the final volume. They were otherwise performed exactly like their counterparts without Carrez clarification, which are A1 and E1, respectively. No significant differences were detected between the sugar levels obtained with the ethanolic extractions E1 and E3. However, amongst the aqueous extractions, significantly smaller values were determined with A6 (compared to A1) for glucose and fructose in both wheat flour and wholemeal wheat flour. The sucrose/maltose levels observed with A6 were not significantly different from those of A1 in both wheat flour and wholemeal wheat flour (despite a small tendency towards higher values with A6). The slightly lower glucose and fructose contents determined with A6 could indicate a small decrease in enzyme activity due to co-precipitation of proteins (potentially including some carbohydrate degrading enzymes) and polysaccharides (potentially including enzyme substrates) with zinc hexacyanoferrate(II).[3] However, since the Carrez clarification was performed only at the end of the extraction procedure, determined glucose and fructose contents were still higher than with A4 and E1. Overall, no clear benefit of Carrez clarification concerning the determined sugar levels was observed in the present study. On the contrary, it was found that the abundance/height of matrix peaks (specifically at early retention times) in chromatograms (data not shown) of the separation with the cation-exchange column was largely increased when Carrez clarification was applied. This complicates peak

Accepted Article

integration of early eluting compounds (e.g. sucrose/maltose) and is probably related to the higher content of ions in these samples.[3]

3.2. *Screening of Sugar Contents in High-Protein Ingredients*

Based on the results presented above, extraction procedure E2 was selected to screen sugar contents in a range of HPIs. The sugar levels in E2 extracts of 7 HPIs with protein contents < 80 %DM (flours) and 6 HPIs with protein contents > 80 %DM (isolates) were determined with two different HPLC separation methods using two different columns. This allowed for both a direct comparison of these values with the results of the different extraction procedures and an individual quantification of sucrose and maltose. Generally, higher values for sucrose/maltose were determined in the extracts of HPIs with protein contents < 80 %DM. However, there are exceptions to this trend. Chickpea, buckwheat, faba bean and lentil contain with approx. 1-2 %DM relatively low amounts of sucrose/maltose when compared to the other HPIs with proteins contents < 80 %DM. Pea, gluten and lentil IEP represent HPIs with proteins contents > 80 %DM but exhibit similar levels of sucrose/maltose just below 1 %DM. The majority of the tested HPIs contains only sucrose and no maltose (not detected or in levels below the LOQ of 0.15 %DM) or sucrose in excess of maltose, which is the case for carob, chickpea, quinoa, amaranth, lentil and lentil IEP. Only gluten was found to contain with 0.78 %DM more maltose than sucrose (0.16 %DM), which might have been released from starch during gluten extraction from wheat. Glucose and fructose levels were below or approx. at 0.15 %DM for most of the HPIs. In quinoa, a slightly higher glucose level was found, which was indicated by both separation methods (SP and HPC column). The results obtained by separation on the HPC column largely confirm the results determined with the SP column. This provides reassurance for the identity of the quantified sugars since these columns rep-

Accepted Article

resent decisively different separation principles. However, discrepancies were observed for the glucose and fructose values in carob and chickpea. The glucose levels for carob (0.25 %DM) and chickpea (0.15 %DM) determined with the SP column were not confirmed by the values obtained with the HPC column, even though these levels exceed the HPC-LOQ of 0.15 %DM. Interestingly, fructose levels of 0.26 %DM (carob) and 0.15 %DM (chickpea) were determined with the HPC column as opposed to the much smaller levels of 0.07 %DM (carob) and 0.03 %DM (chickpea) with SP separation. These discrepancies could be explained by the presence of an unidentified compound that coelutes with glucose on the SP column but with fructose on the HPC column. The fact that similarly elevated levels for glucose on the one hand (SP column) and fructose on the other hand (HPC column) were measured in these two ingredients of related botanical origin supports this theory. The investigated samples lentil, lentil IEP and lentil UF are differently processed ingredients derived from the same lentil raw material. While the protein extraction procedure (isoelectric precipitation) applied to produce lentil IEP does not appear to have a big impact on the sugar content and sugar profile, the ultrafiltration used to produce lentil UF leads to a removal of the majority of short-chain carbohydrates quantified in this study. This was previously postulated by Alonso-Miravalles and Jeske et al.[32], who compared lentil IEP and lentil UF with regard to their physicochemical and techno-functional characteristics. Berrios et al.[23] applied sugar extraction procedures similar to E2 to determine mono-, di- and oligosaccharides in chickpea flour and lentil flour. The results reported in their study are largely in agreement with the findings of the present study. The sugar contents determined for carob, quinoa, amaranth, buckwheat and faba bean also correspond to previously reported results in the literature.[33–36] However, the high-protein flours of quinoa, amaranth and buckwheat analysed in this study appear to contain slightly higher levels of sucrose/maltose than reported for regular flours.

Accepted Article

While this might be related to natural variations, it could also indicate an accumulation of these sugars in the high-protein fraction during dry-fractionation.

4. CONCLUSION

The comparison of several aqueous and ethanolic extraction procedures revealed big differences in determined sugar levels for wheat flour and wholemeal wheat flour. These differences are likely associated with the activity of carbohydrate degrading enzymes (endogenous plant-derived or microbial), polysaccharide coextraction and the inhibition of both in some of the investigated extraction procedures. Ethanolic extraction procedures with a high volume of extraction solvent, which can minimise problems related to gelation and water absorption during the extraction of plant-based high-protein ingredients, generally resulted in lower determined sugar levels than aqueous extractions. While this could have suggested incomplete sugar extraction, the results of ethanolic extractions also resembled (no significant differences) those of aqueous extraction procedures where other measures for enzyme inhibition were applied (MeOH pre-treatment, heat treatment). Moreover, the presented results indicate incomplete enzyme inhibition in aqueous extractions with MeOH pre-treatment combined with a 10 min heat treatment as opposed to longer heat treatments (20 or 30 min). Ethanolic extraction with the application of NaN_3 but without Carrez clarification was found to be the most suitable (amongst the investigated extraction procedures in this study) to determine sugar contents in HPIs. Aqueous extractions without sufficient inhibition of enzymes can lead to an overestimation of short-chain carbohydrates (and potentially related underestimation of carbohydrates with a higher degree of polymerisation acting as substrates for these enzymes) by several grams per 100 grams of sample dry matter (in this study approx. 1.8 %DM for wheat flour and 1.3 %DM for wholemeal wheat flour). This can have implications for the assessment of suitable processing techniques for food ingredients (such as fermentation) as well as

Accepted Article

for food labelling and evaluation of the nutritional value of food products.

ACKNOWLEDGEMENTS

The authors want to thank Tom Hannon for technical support, Lilit Ispiryan for proofreading and fruitful scientific discussions, Concept Life Science Ltd. for performing fibre/compositional analysis and Fraunhofer Institute IVV for providing samples. The work for this study has been undertaken as part of the project PROTEIN2FOOD. This project has received funding from the European Union's Horizon 2020 research and innovation programme (grant agreement No 635727).

ABBREVIATIONS

HPI	High-protein ingredient
LOQ	Limit of quantification
RID	Refractive index detector
NaN_3	Sodium azide
IEP	Isoelectric precipitation
UF	Ultrafiltration
TDF	Total dietary fibre
%DM	Percentage based on dry matter
UPW	Ultrapure water
MeOH	Methanol
EtOH	Ethanol
MeCN	Acetonitrile
SP	Sugar-Pak I column
HPC	High performance carbohydrate column
HT	Heat treatment
CT	Concentration treatment
PP	Polypropylene
Carrez I	15 g per 100 mL of ultrapure water, potassium hexacyanoferrate(II) trihydrate
Carrez II	23 g per 100 mL of ultrapure water, zinc acetate dihydrate
80 % EtOH	80/20 (v/v) ethanol/ultrapure water
CaEDTA	Calcium disodium ethylene diamine tetraacetate
ANOVA	Analysis of variance

REFERENCES

1 Mellentin J. 10 Key Trends in Food, Nutrition and Health 2020. Vol. 25. NewNutrition Business; 2019.

2 Johansen HN, Glitsø V, Bach Knudsen KE. Influence of extraction solvent and temperature on the quantitative determination of oligosaccharides from plant materials by high-performance liquid chromatography. *J Agric Food Chem.* **44**(6):1470–4 (1996).

3 Eliasson A-C, editor. Carbohydrates in food. Second edi. Carbohydrates in Food. Taylor & Francis Group; 1–640 p.2017.

4 Ispiryan L, Heitmann M, Hoehnel A, Zannini E, Arendt EK. Optimization and Validation of an HPAEC-

Accepted Article

PAD Method for the Quantification of FODMAPs in Cereals and Cereal-Based Products. *J Agric Food Chem*. **67**(15):4384–92 (2019).

5 Hoehnel A, Axel C, Bez J, Arendt EK, Zannini E. Comparative analysis of plant-based high-protein ingredients and their impact on quality of high-protein bread. *J Cereal Sci*. **89**:1–8 (2019).

6 Hoehnel A, Bez J, Petersen IL, Amarowicz R, Juśkiewicz J, Arendt EK, et al. Enhancing the nutritional profile of regular wheat bread while maintaining technological quality and adequate sensory attributes. *Food Funct*. **11**:4732–51 (2020).

7 Zhou W, Hui YH, editors. Bakery Products Science and Technology. Bakery Products: Science and Technology. Wiley Blackwell; 3–68 p.2014.

8 Vatandoust A, Ragaee S, Wood PJ, Tosh SM, Seetharaman K. Detection, localization, and variability of endogenous β-glucanase in wheat kernels. *Cereal Chem*. **89**(1):59–64 (2012).

9 Atzler JJ, Ispiryan L, Gallagher E, Sahin AW, Zannini E, Arendt EK. Enzymatic degradation of FODMAPS via application of β-fructofuranosidases and α-galactosidases- A fundamental study. *J Cereal Sci*. **95**(May):102993 (2020).

10 Verspreet J, Cimini S, Vergauwen R, Dornez E, Locato V, Le Roy K, et al. Fructan metabolism in developing wheat (*Triticum aestivum* L.) kernels. *Plant Cell Physiol*. **54**(12):2047–57 (2013).

11 Cimini S, Locato V, Vergauwen R, Paradiso A, Cecchini C, Vandenpoel L, et al. Fructan biosynthesis and degradation as part of plant metabolism controlling sugar fluxes during durum wheat kernel maturation. *Front Plant Sci*. **6**(FEB):1–10 (2015).

12 Dey M, Pridham JB, Holloway R. Purification and Properties of α-Galactosidases from *Vicia faba* Seeds. *Biochem J*. **113**(49):49–55 (1969).

13 Gänzle MG, Follador R. Metabolism of Oligosaccharides and Starch in Lactobacilli: A Review. *Front Microbiol*. **3**:1–15 (2012).

14 Li BW, Schuhmann PJ, Wolf WR. Chromatographic Determinations of Sugars and Starch in a Diet Composite Reference Material. *J Agric Food Chem*. **33**(3):531–6 (1985).

15 El-Refai AA, Harras HM, El-Nemr KM, Noaman MA. Chemical and technological studies on faba bean seeds. I-Effect of storage on some physical and chemical properties. *Food Chem*. **29**(1):27–39 (1988).

16 Nyakuni GA, Kikafunda JK, Muyonga JH, Kyamuhangire WM, Nakimbugwe D, Ugen M. Chemical and nutritional changes associated with the development of the hard-to-cook defect in common. *Int J Food Sci Nutr*. **59**(7–8):652–9 (2008).

17 Saldivar X, Wang YJ, Chen P, Mauromoustakos A. Effects of blanching and storage conditions on soluble sugar contents in vegetable soybean. *LWT - Food Sci Technol*. **43**(9):1368–72 (2010).

18 Ziegler JU, Steiner D, Longin FH, Würschum T, Schweiggert RM, Carle R. Wheat and the irritable bowel syndrome – FODMAP levels of modern and ancient species and their retention during bread making. *J Funct Foods*. **25**:257–66 (2016).

Accepted Article

19 Xiaoli X, Liyi Y, Shuang H, Wei L, Yi S, Hao M, et al. Determination of oligosaccharide contents in 19 cultivars of chickpea (*Cicer arietinum* L) seeds by high performance liquid chromatography. *Food Chem*. **111**(1):215–9 (2008).

20 Knudsen IM. High-performance liquid chromatographic determination of oligosaccharides in leguminous seeds. *J Sci Food Agric*. **37**(6):560–6 (1986).

21 Conkerton EJ, Parrish FW, Chaptial DC, Ory RL. Isolation of a Stachyose-Sucrose Complex from Soybeans and Peanuts. *J Food Sci*. **48**(4):1269–71 (1983).

22 Li BW, Schuhmann PJ. Gas-Liquid Chromatographic Analysis of Sugars in Ready-To-Eat Breakfast Cereals. *J Food Sci*. **45**:138–41 (1980).

23 Berrios JDJ, Morales P, Cámara M, Sánchez-Mata MC. Carbohydrate composition of raw and extruded pulse flours. *Food Res Int*. **43**(2):531–6 (2010).

24 Sánchez-Mata MC, Peñuela-Teruel MJ, Cámara-Hurtado M, Díez-Marqués C, Torija-Isasa ME. Determination of Mono-, Di-, and Oligosaccharides in Legumes by High-Performance Liquid Chromatography Using an Amino-Bonded Silica Column. *J Agric Food Chem*. **46**(9):3648–52 (1998).

25 Machado MTC, Eça KS, Vieira GS, Menegalli FC, Martínez J, Hubinger MD. Prebiotic oligosaccharides from artichoke industrial waste: Evaluation of different extraction methods. *Ind Crops Prod*. **76**:141–8 (2015).

26 Karki B, Lamsal BP, Jung S, van Leeuwen J (Hans), Pometto AL, Grewell D, et al. Enhancing protein and sugar release from defatted soy flakes using ultrasound technology. *J Food Eng*. **96**(2):270–8 (2010).

27 Ispiryan L, Zannini E, Arendt EK. Characterization of the FODMAP-profile in cereal-product ingredients. *J Cereal Sci*. **92**:1–10 (2020).

28 MacArthur L a., D'Appolonia BL. Comparison of Oat and Wheat Carbohydrates. I. Sugars. *Cereal Chem*. **56**(5):455–7 (1979).

29 Ranwala AP, Miller WB. Analysis of nonstructural carbohydrates in storage organs of 30 ornamental geophytes by high-performance anion-exchange chromatography with pulsed amperometric detection. *New Phytol*. **180**(2):421–33 (2008).

30 Berghofer LK, Hocking AD, Miskelly D, Jansson E. Microbiology of wheat and flour milling in Australia. *Int J Food Microbiol*. **85**(1–2):137–49 (2003).

31 Hasså L, Nyman M, Andersson R. Distribution and characterisation of fructan in wheat milling fractions. *J Cereal Sci*. **48**(3):768–74 (2008).

32 Alonso-Miravalles L, Jeske S, Bez J, Detzel A, Busch M, Krueger M, et al. Membrane filtration and isoelectric precipitation technological approaches for the preparation of novel, functional and sustainable protein isolate from lentils. *Eur Food Res Technol*. **245**(9):1855–69 (2019).

33 Avallone R, Plessi M, Baraldi M, Monzani A. Determination of chemical composition of carob (*Ceratonia siliqua*): Protein, fat, carbohydrates, and tannins. *J Food Compos Anal*. **10**(2):166–72 (1997).

34 Schoenlechner R, Siebenhandl S, Bergho E. Pseudocereals. 2008.

35 Martínez-Villaluenga C, Peñas E, Hernández-Ledesma B. Pseudocereal grains: Nutritional value, health benefits and current applications for the development of gluten-free foods. *Food Chem Toxicol*. **137**:1–26 (2020).

36 Hoehnel A, Bez J, Sahin AW, Coffey A, Arendt EK, Zannini E. *Leuconostoc citreum* TR116 as a Microbial Cell Factory to Functionalise High-Protein Faba Bean Ingredients for Bakery Applications. *Foods*. **9**(11):1–39 (2020).

Accepted Article

Accepted Article

Tables

Table 1: Composition of wheat flour, wholemeal wheat flour and HPIs

Ingredient	Moisture [%]	Protein [%]	Fat [%]	Ash [%]	TDF [%]	Carbohydrates [%]†
Wheat flour	12.60	14.76	0.98	0.80	2.29	81.17
Wholemeal wheat flour	12.80	12.61	1.54	1.38	2.75	81.72
Soy*	5.72	96.78	0.30	0.25	< 0.1	2.67
Pea*	9.73	80.19	6.45	5.90	2.88	4.58
Lupin*	6.45	94.51	2.94	5.62	< 0.1	-
Gluten*	8.20	83.11	0.72	0.87	< 0.1	15.31
Lentil IEP	4.87	86.91	4.72	5.74	1.89	2.63
Lentil UF	5.63	99.29	4.66	3.72	< 0.1	-
Carob*	6.06	55.04	0.20	7.04	17.67	20.05
Chickpea	10.24	23.65	6.17	2.59	6.24	61.35
Quinoa	10.38	37.44	18.06	4.29	3.35	36.86
Amaranth	11.71	40.95	18.12	7.40	14.61	18.92
Buckwheat	10.49	22.52	2.78	3.22	1.56	69.91
Faba bean*	13.07	61.25	3.81	5.43	0.35	29.17
Lentil	11.24	28.29	1.34	3.16	4.51	62.70

* Compositional data previously reported by Hoehnel et al. (2019)

† Calculated by subtraction.

This article is protected by copyright. All rights reserved.

Accepted Article

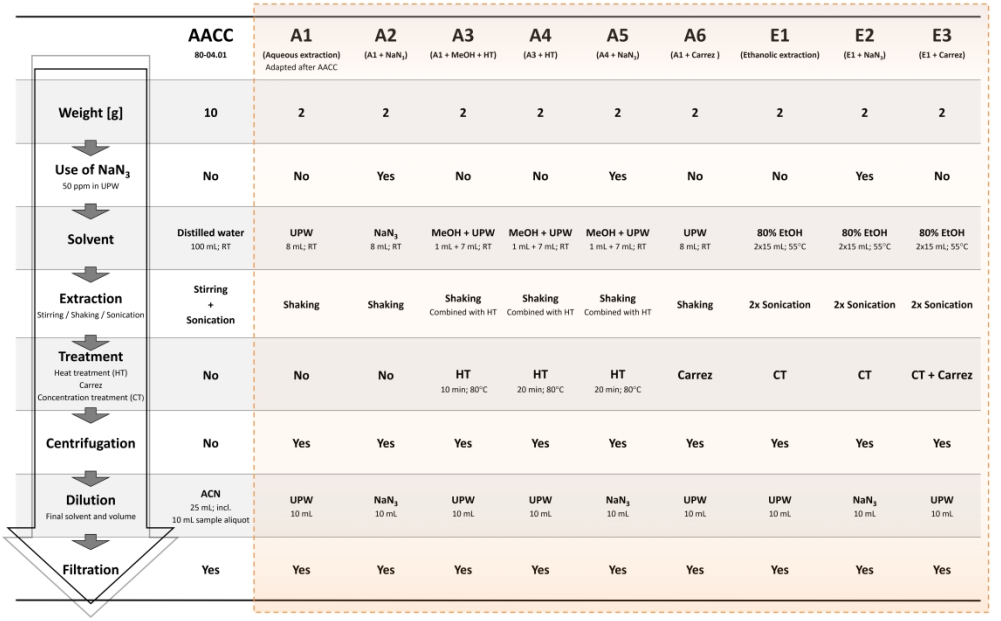
Table 2: Activity of α- and β-amylase determined for wheat flour and wholemeal wheat flour.

Enzyme activity	Wheat flour	Wholemeal wheat flour
α-Amylase activity [CU/g]	0.119 ± 0.006^{a}	0.122 ± 0.016^{a}
β-Amylase activity [BU/g]	34.82 ± 0.57^{a}	35.38 ± 0.35^{a}

Means ± standard deviation with different letters in the same row indicate significant differences at $p < 0.05$

This article is protected by copyright. All rights reserved.

Accepted Article



	AACC 80-04.01	A1 (Aqueous extraction) Adapted after AACC	A2 (A1 + NaN_3)	A3 (A1 + MeOH + HT)	A4 (A3 + HT)	A5 (A4 + NaN_3)	A6 (A1 + Carrez)	E1 (Ethanolic extraction)	E2 (E1 + NaN_3)	E3 (E1 + Carrez)
Weight [g]	10	2	2	2	2	2	2	2	2	2
Use of NaN_3 50 ppm in UPW	No	No	Yes	No	No	Yes	No	No	Yes	No
Solvent	Distilled water 100 mL; RT	UPW 8 mL; RT	NaN_3 8 mL; RT	MeOH + UPW 1 mL + 7 mL; RT	MeOH + UPW 1 mL + 7 mL; RT	MeOH + UPW 1 mL + 7 mL; RT	UPW 8 mL; RT	80% EtOH 2x15 mL; 55°C	80% EtOH 2x15 mL; 55°C	80% EtOH 2x15 mL; 55°C
Extraction Stirring / Shaking / Sonication	Stirring + Sonication	Shaking	Shaking	Shaking Combined with HT	Shaking Combined with HT	Shaking Combined with HT	Shaking	2x Sonication	2x Sonication	2x Sonication
Treatment Heat treatment (HT) Carrez Concentration treatment (CT)	No	No	No	HT 10 min; 80°C	HT 20 min; 80°C	HT 20 min; 80°C	Carrez	CT	CT	CT + Carrez
Centrifugation	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Dilution Final solvent and volume	ACN 25 mL; incl. 10 mL sample aliquot	UPW 10 mL	NaN_3 10 mL	UPW 10 mL	UPW 10 mL	NaN_3 10 mL	UPW 10 mL	UPW 10 mL	NaN_3 10 mL	UPW 10 mL
Filtration	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Figure 1: Overview of extraction methods employed in this study (highlighted in orange) and comparison to AACC method 80-04.01.

This article is protected by copyright. All rights reserved.

Accepted Article

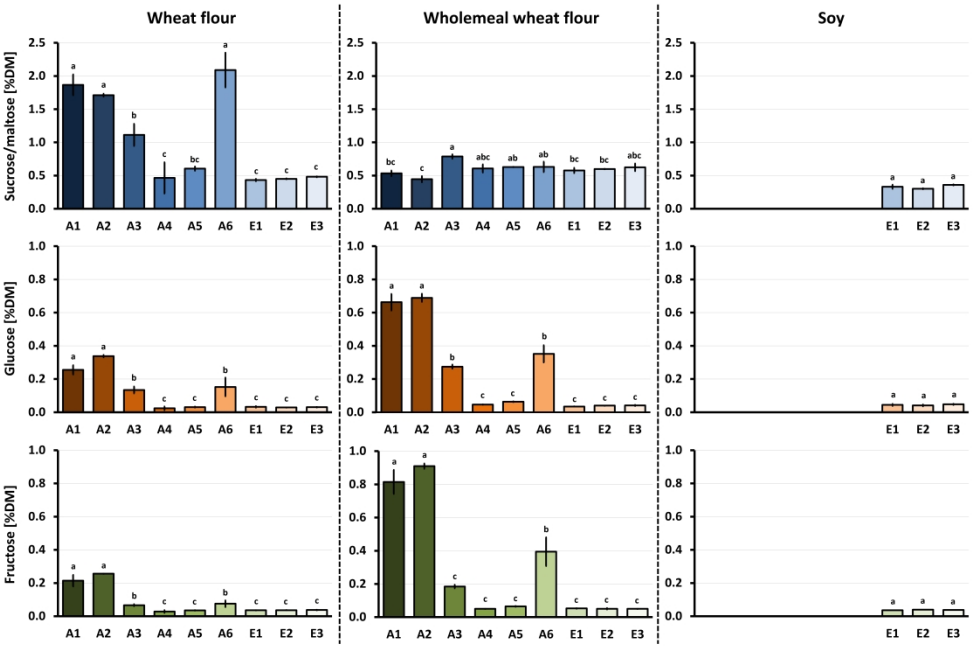


Figure 2: Maltose/sucrose (blue), glucose (orange) and fructose (green) levels determined for wheat flour, wholemeal wheat flour and soy protein isolate with the different extraction procedures A1 – A6 and E1 – E3. Bars within the same individual charts and with different letters represent significantly different sugar levels ($p < 0.05$).

This article is protected by copyright. All rights reserved.

Accepted Article

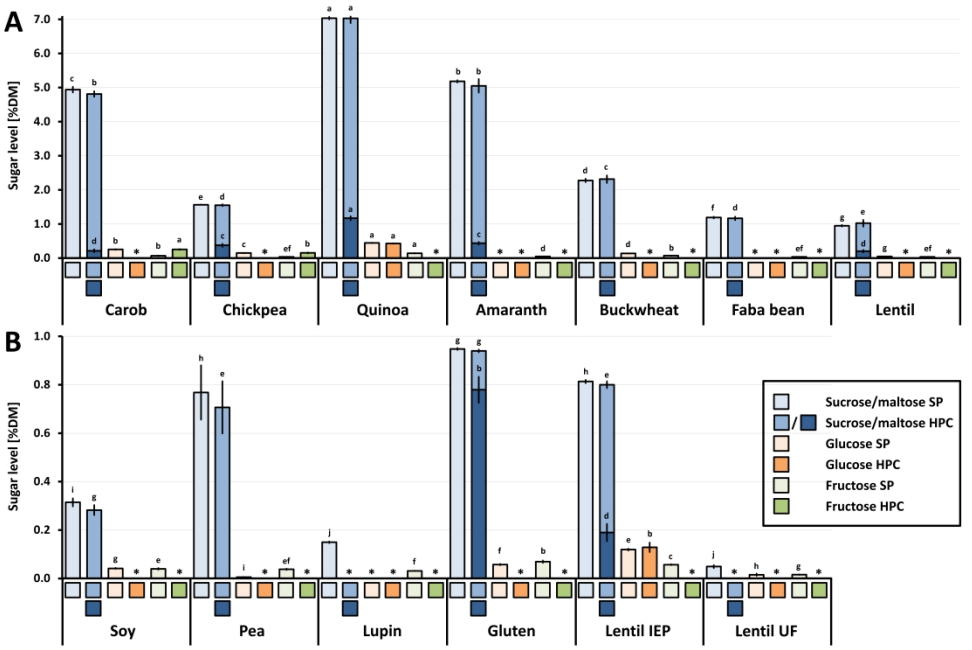


Figure 3: Sugar levels determined in HPIs using extraction procedure E2 and HPLC separation with two different columns (SP: Sugar-Pak I column, Waters Corporation, MA, USA; HPC: High-Performance Carbohydrate column, Waters Corporation, MA, USA) to obtain individual values for sucrose and maltose. * Asterisks indicate where sugars were not detected or below 0.01 %DM (SP) or 0.15 %DM (HPC). Bars with the same colour (across A and B) and different letters represent significantly different sugar levels ($p < 0.05$). A - HPIs with protein contents < 80 %DM; B - HPIs with protein contents > 80 %DM.

This article is protected by copyright. All rights reserved.