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HealthFerm

Deliverable No. 2.1
Analytical toolbox to evaluate raw material and food fermentations

WP2

Grant Agreement no.: 101060247

Project Title: Innovative pulse and cereal-based food fermentations for human health and sustainable diets

Contractual Submission Date: 31/08/2023

Actual Submission Date: 31/08/2023

Responsible partner: P1-KU Leuven

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Deliverable number	**D2.1**
Deliverable title	**Analytical toolbox to evaluate raw material and food fermentations**
Type[1]	R
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Work package number	WP2
Work package leader	Prof. Kati Katina (P2-UH)
Author(s)	Prof. Arno Wouters (P1-KU Leuven), Prof. Christophe Courtin (P1-KU Leuven)
Keywords	Analytics, toolbox, characterisation

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Checklist	Y (Yes), N (No)
1) The deliverable has been sent to all relevant partners for review (i.e. in the same task/WP):	N
2) The deliverable has been endorsed by the WP lead and co-lead:	Y
3) Input from other WPs has been sought, if applicable: ***Please specify:***	N

[1] **Type**: Use one of the following codes (in consistence with the Description of the Action):
R: Document, report (excluding the periodic and final reports)
DEM: Demonstrator, pilot, prototype, plan designs
DEC: Websites, patents filing, press & media actions, videos, etc.

[2] **Dissemination level**: Use one of the following codes (in consistence with the Description of the Action)
PU: Public, fully open, e.g. web
SEN: Sensitive, limited under conditions of the Grant Agreement

Table of Contents

Publishable Summary

The overall objective of WP 2 of the HealthFerm project is to understand the impact of fermentation on grain-based raw materials and foods for developing novel foods optimized for health benefits without compromising flavour, sustainability and safety. An important requirement for achieving this objective is the availability of a broadly applicable analytical toolbox that allows in-depth characterization of changes in the chemical structure of the many constituents that occur in grain-based raw materials and foods. The aim of Task 2.1 within WP2 is to establish such an analytical toolbox, which all involved partners can make use of and which can facilitate interactions and collaboration within the consortium. Under the supervision of KU Leuven, the lead partner for this task and associated deliverable, several steps were taken within the first project year (1 September 2022 – 31 August 2023) leading to the successful development of the mentioned analytical toolbox. First, inventories were made on (i) the required raw materials as well as the target (model) food products for all partners within WP 2 and (ii) the analytical capabilities of all partners. Next, the scope of the analytical toolbox was defined and discussed in several meetings involving all WP 2 partners. A decision was made to establish the analytical toolbox in the form of a publishable 'Book of Methods', focusing on chemical characterisation of constituents present in the raw materials used in HealthFerm and changes therein as the result of fermentation processes. Then, the different partners of WP 2 were made responsible for specific chapters of the Book of Methods, and discussions among partners with compatible analytical capabilities were set up, after which draft texts for the different chapters were prepared. This has led to the compilation of a first draft of a complete 'Book of Methods' for WP 2 of the HealthFerm project, attached to this document and available to all consortium members, representing the successful completion of Deliverable 2.1. In follow-up steps, internal peer review and final formatting will be undertaken, with the final goal of achieving an open access publication of the 'Book of Methods'.

1. Introduction

The overall objective of WP2 is to understand the impact of fermentation on grain-based raw materials and foods for developing novel foods optimized for health benefits without compromising flavour, sustainability and safety. Achieving this objective involves a variety of challenges, one of which is the need for a broadly applicable analytical toolbox that allows in-depth characterization of changes in the chemical structure of the many constituents that occur in grain-based raw materials and foods. Within WP2, different partners will focus on different raw materials (oat, wheat, yellow pea and faba bean-based), and on different (model) foods (liquid, semi-solid and solid products). This implies a wide variability in target constituents, matrix properties and characterization needs but also a wealth of analytical capabilities, distributed over the different WP 2 partners. The aim of Task 2.1 within WP 2 is to establish an analytical toolbox, which all involved partners can make use of and which can facilitate interactions and collaboration within the consortium.

2. Description of Activities

As **the first step** towards establishing an analytical toolbox, **two inventories** were made:

- An inventory of the required raw materials, as well as the target (model) food product for all partners. The aim of setting up this inventory was to facilitate (i) alignment and coordination between partners working on similar raw materials, and (ii) collaboration between partners working on topics.
- An inventory of the analytical capabilities of the different partners. This served as the basis for setting up the analytical toolbox subsequently.

These inventories were prepared and **made available to all involved partners via the Teams platform** by KU Leuven, and then completed by all partners. They served as a basis for discussions in several online WP2 meetings.

As **the second step**, the scope of the analytical toolbox was proposed by KU Leuven and discussed in the Consortium meeting of September 2022 and in an online WP2 meeting. Several strategic choices were made.

- The analytical toolbox will be established **as a 'HealthFerm book of methods'** (HF-BoM).
- The HF-BoM will focus on the **chemical characterisation of constituents** present in the raw materials within the scope of HealthFerm (oats, wheat, yellow pea, faba bean). Assessment of technological, biological and nutritional functionalities, although still relevant within HealthFerm as a whole, is considered out of scope for the HF-BoM.
- The HF-BoM will comprise different chapters, each representing a target constituent, describing:
    - An introduction to the target constituent, its chemical structure, its relevance for nutrition and food processing and the impact of fermentation thereon
    - A description of the principle behind the described method.
    - A detailed step-by-step description of the experimental protocol.
    - A description of the required steps for data analysis and interpretation.
- **Omics technologies will not be included within the HF-BoM**. Even though various types of omics technologies will be key in the HealthFerm project as a whole, they require specific expertise, and especially the data processing afterwards is considered challenging and requires tailored approaches depending on the application. Therefore, a generalised description of such techniques was deemed useless.

As **a third step**, a proposal of a **table of contents** for the HF-BoM was made by KU Leuven, which was discussed and agreed upon in a WP2 meeting. Table 1 in Section 3 of this document provides an overview of the HF-BoM table of contents. Each chapter was assigned to one of the partners involved in WP2. This **partner was designated as responsible for coordinating the writing efforts**. This involved taking the initiative in writing drafts, but also setting up meetings with partners with similar analytical capabilities and possibly involving them in the writing process.

In **the fourth step,** KU Leuven prepared templates for draft texts for the HF-BoM chapters. These were made available to all partners. The first drafts of the different chapters were completed by the end of June 2023. Texts are available and can be consulted by all partners via the Teams platform. They constitute the availability of an analytical toolbox and, hence, Deliverable 2.1.

Follow-up steps to achieve publication of the HF-BoM include:

- An internal peer review process. Draft texts will be divided among the WP2 partners for internal peer review so that texts can be further fine-tuned.
- Formatting of the HF-BoM.
- Identification of a suitable open-access publishing partner. A possible option is to publish the book via Leuven University Press.

3. Results

The final version of the HF-BoM will be made available as a published book. Table 1 shows the table of contents, as well as the partner within WP2 responsible for coordinating the efforts on each chapter.

Table 1: HealthFerm Book of Methods table of contents.

	Title	Responsible partner
Part 1: Proteins		
Chapter 1	(Free) amino acid composition	VTT
Chapter 2	Extractable protein content	VTT
Chapter 3	Degree of hydrolysis and apparent molecular weight distribution	KU Leuven
Part 2: Carbohydrates		
Chapter 4	Starch	SLU
Chapter 5	Mono-, di-, and oligosaccharides, FODMAPs and raffinose family oligosaccharides	UBZ
Chapter 6	Arabinoxylan	KU Leuven
Chapter 7	Cereal β-D-glucan	ETHZ
Chapter 8	Exopolysaccharides	UBZ
Part 3: Minor grain constituents		
Chapter 9	Phytate and minerals	KU Leuven
Chapter 10	Saponins and tannins	UH
Chapter 11	(Con)vicine	UH
Chapter 12	Vitamin B12	UH
Chapter 13	Free fatty acids	UH
Part 4: Fermentation metabolites and intermediates		
Chapter 14	Organic acids	VUB
Chapter 15	Biogenic amines	VUB
Chapter 16	Ethanol	VUB
Chapter 17	Sugar alcohols	VUB
Chapter 18	Volatile compounds	UH

The currently available text of the HF-BoM is attached to this document as Appendix A. Although this draft document is not fully finalised and requires further editing and formatting to prepare it for publishing, full descriptions of all methods are included and can be consulted by all partners via the Teams platform.

4. Conclusion

Assessing the impact of fermentation on grain-based raw materials and food products requires a state-of-the-art analytical toolbox to investigate changes in molecular structure and composition as a result of fermentation. A clear framework for such an analytical toolbox for the HealthFerm project, with a focus on the work in WP2, was established within its first 12 months. A strategic choice was made to realise this toolbox as an open-access publishable “Book of Methods”. This HF-BoM will comprise different chapters, each focusing on a specific chemical constituent. Each chapter will describe the structure of the target constituent, its relevance for nutrition and processing, the possible impact of fermentation thereon, a description of the principle of a suitable state-of-the-art analytical technique, a detailed step-by-step experimental protocol and a description of data processing and interpretation. A major advantage of this choice is that not only the HealthFerm partners but also others will be able to consult this analytical toolbox, thereby establishing a reference work for the chemical analysis of

fermented grain-based raw materials and foods. A draft text for the HF-BoM is available as Annex A to this document. Although several steps still need to be taken to finalise this HF-BoM, all methods of the analytical toolbox are available to all involved partners via the Teams platform, thereby meeting the objective put forward for this Deliverable 2.1.

5. Deviations, if applicable

A first deviation to what was described in the project proposal is the choice to not include a description of omics-based techniques (specifically metabolomics) in the HF-BoM. This choice was made based on a discussion with all partners involved in WP2, in which it became clear that particularly the post analysis data processing is challenging and requires a tailored approach for a specific application/research question. A generalised description in this regard would not be useful. It should still be mentioned that omics technologies will still be carried out within HealthFerm and still represent an important strategic tool to assess the impact of fermentation on grain-based raw materials and foods. As such, no concrete impact on other work packages, deliverables or milestones is expected.

The choice to publish the analytical toolbox as a 'Book of Methods' implies a timeframe beyond that for completing this Deliverable 2.1. However, texts for all chapters are available to all partners within the Teams platform. This implies that the objectives put forward for this deliverable (Deliverable 2.1) have been met, regardless of the fact that the final HF-BoM will be finalised later. Therefore, no impact on other work packages, deliverables or milestones is expected.

6. References

Not applicable.

7. Annexes

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HealthFerm

Book of Methods

Appendix to

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HealthFerm

Table of Contents

Chapter 1
(Free) Amino acid composition and content

Heli Nygren, Riikka Juvonen, Nesli Sözer (VTT, Finland)

Summary

- **Raw material**: Plant-based
- **Relevant applications:** Determination of nutritive value; characterization of peptidolytic and proteolytic activities of microorganisms in laboratory media and plant matrix.
- **Fermentation-induced changes**: Changes in amino acid profile of proteins and free amino acid content due to 1) hydrolysis of peptides, proteins, or both by plant endogenous or microbial peptidases, proteases, or both, 2) microbial synthesis and metabolic conversions of amino acids during fermentation.
- **Analytical methods**:
- Analysis of free amino acids
    - Extraction of free amino acids from plant based materials
    - Quantitative analysis of free amino acids using UHPLC-MS/MS method
- Analysis of total amino acid content and composition of plant based material
    - Acidic hydrolysis
    - Alkaline hydrolysis
    - Derivatization of amino acids
    - Determination of amino acid composition: UHPLC analysis

1. Introduction

Analysis of total amino acid composition, including protein bound and free amino acids is an important tool for the assessment of protein nutritional quality, especially the content of the essential amino acids (EAA). The Food and Agriculture Organization of the United Nations (FAO) has established a standard for protein quality evaluation and set recommended minimum intake of EAA (per gram protein) for different age groups (FAO, 2013). Plant proteins are considered an incomplete source of proteins since they lack some of the EAA. Cereals are typically deficient in lysine but rich in sulfur-containing amino acids. On the contrary, legumes provide a good source of lysine (64 mg/g of protein) and threonine (38 mg/g of protein) but are low in sulfur-containing amino acids methionine, cysteine, and tryptophan (Havemeier, et al., 2017)

1.1 Exploring the amino acid puzzle in plant based raw materials

There are 20 typical proteinogenic amino acids: alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp) cysteine (Cys), glutamine (Gln) glutamate (Glu) glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), Phenylalanine (Phe), proline (Pro), Serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val). Amino acids that cannot be synthesized by the body to supply the demand and must therefore come from the diet are called as essential amino acids. The nine essential amino acids for humans are valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, threonine, histidine, and lysine. FAA are involved in many physiological functions, they can enhance immune system, prevent liver damage, balance blood sugar levels, act as neurotransmitters and have many other effects (FAO, 2013).

Gamma-aminobutyric acid (GABA) is a four-carbon free amino acid widely distributed in nature and considered a potent bioactive compound with numerous and important physiological functions, such as anti-hypertensive and antidepressant activities (Cui, et al., 2020).

1.2 How can fermentation affect amino acid composition and free amino acid content?

Fermentation can modify the free amino acid content and amino acid composition in plant materials, their fractions and processing products. The specific outcome depends on the microorganisms, substrate, and fermentation conditions. Fermentation typically leads to partial protein hydrolysis into peptides and free amino acids. Some microorganisms, such as certain lactic acid bacteria (LAB) and many filamentous fungi, secrete proteolytic and peptidolytic enzymes that hydrolyze plant proteins into peptides and free amino acids. Acidification during fermentation can also activate plant endogenous enzymes releasing free amino acids from the plant matrix (Rizzello, et al., 2019). Several studies have shown an increase in total free amino acids in various legumes, seeds, and cereals after fungal or lactic fermentation, which improves bioavailability of amino acids. FAA take part in many physiological functions (Garrido-Galand, et al., 2021; Emkani, et al., 2022; Harper, et al., 2022). Fermentation can also result in synthesis of new amino acids and amino acid derivatives (Gänzle, 2015). FAA are involved in many physiological functions, they can enhance immune system, prevent liver damage, balance blood sugar levels, act as neurotransmitters and have many other effects (FAO, 2013).

Fermentation of cereals, such as maize, can significantly enhance the quality of protein and especially the concentrations of certain essential amino acids such as lysine (Singh, et al., 2015). Many studies have also shown an improvement in the content of essential amino acids in various legumes after fermentation (Verni, et al., 2019; Sozer, et al., 2019; Emkani, et al., 2022).

Microorganisms can also reduce the total free amino acid content or the level of specific amino acids, when using them for growth and various metabolic reactions, which negatively affects the nutritional value. For instance, LAB may also consume specific amino acids (arginine and glutamate) in acidic conditions to improve the acid resistance (Cui, et al., 2020). The action of microorganisms on amino acids can produce biogenic amines which can cause at high concentrations headache, nausea, rash, giddiness, and hypo- or hypertension.

The free amino acids and peptides released during fermentation are important in the development of flavour and aroma characteristics of the fermented plant products (Emkani, et al., 2022; Zhao, et al., 2016). They can directly to contribute sweet, bitter, sour, and umami characteristics to foods (Kato, et al., 1989; Zhao, et al., 2016). Free amino acids are also precursors of various volatile flavour compounds and can be converted into various alcohols, aldehydes, acids, esters, and sulphur compounds via oxidative deamination and/or transamination reactions.

2. Method(s) of analysis

2.1 Analysis of free amino acids

2.1.1 Reagents

- Acetonitrile LC-MS Ultra,
- Formic acid
- Ammonium formate
- Amino Acid Standard mixture solution (Sigma-Aldrich)
- Labelled Internal standards: (DL-Alanine-2,3,3,3,-d4, DL-Glutamic acid-2,3,3,4,4-d5, DL-Valine-2,3,4,4,4,5,5,5-d8) obtained from Sigma-Aldrich (St. Luis, Missouri, USA). Labelled Internal standards L-Phenyl-d5-alanine, L-Leucine-d10, DL-Cystine-2,2,3,3,3,3-d6, DL-2-Aminobutyric-d6 Acid, DL-Histidine-α,β,β-d3, 4-Aminobutyric-2,2.3,3,4,4,-d6, DL-Aspartic-2,3,3-d3 Acid, Glycine-d5, DL-Methionine-3,3,4,4-d4 obtained from C/D/N Isotopes Inc.(Quebec, Canada).

2.1.2 Extraction of free amino acids from plant based materials

The protocol is developed for dry, powdered plant-based samples for the determination of free amino acids and other usual amines. First 10-15 mg samples of freeze-dried biomass are accurately weighed. The samples are extracted with 750 µL of 30% ethanol by first mixing thoroughly and then by sonicating for 2 h. Dissolved proteins are precipitated with sulfosalicylic acid (final concentration of 5 %, w/v), and the samples are centrifuged. The supernatant is transferred to a new tube and samples are diluted with 10 mM ammonium formate.

2.1.3 Quantitative analysis of free amino acids using UHPLC-MS/MS method

Analysis of the samples (1 µl) is performed on an Acquity UHPLC system, Waters (Milford, MA, USA) and Waters Xevo TQ-XS MS (Manchester, UK) using an ACQUITY UPLC BEH Amide Column, 130Å, 1.7 µm, 2.1 mm X 100 mm (Waters), kept at 35 ºC. Separation is performed using gradient elution with 10mM ammonium formate and 0.5% formic acid in water (A solvent) and 10mM ammonium formate and 0.5% formic acid in 90% acetonitrile (B solvent) at a flow rate of 0.5 ml/min.

Mass spectrometry is performed in positive polarity using the capillary voltage of 1,0 kV, desolvation temperature of 550 ºC, and source temperature of 150 ºC. Analytes are detected using multiple reaction monitoring (MRM) using auto dwell time function. Quantification is performed using internal standard method. Additional details of the method are presented in Table 2.

***Table 2**. Free amino acid and related compounds included in the analysis, their precursor and product ions used for MRM and the internal standards.*

	Component	Abr	Precursor ion m/z	Product ion, m/z	ISTD
1	Alanine	Ala	90.1	44.1	L-Ala-d4
2	β-Alanine	β-Ala	90.0	30.1	L-Ala-d4
3	a-Aminobutyric acid	AABA	104.0	58.1	AABA-d6
4	g-Aminobutyric acid	GABA	104.0	69.0	GABA-d6
5	Arginine	Arg	175.1	115.9	His-d3
6	Aspargine	Asn	133.0	74.0	L-Ala-d4
7	Aspartic acid	Asp	134.0	74.0	AspA-d3
8	Citrulline	Cit	176.1	159.1	L-Glu-d5
9	Cystathionine	Cyst	223.1	134.0	Cys-d6
10	Cysteine	Cys	122.0	76.0	Cys-d6
11	Ethanolamine	EA	62.0	44.1	Met-d4
12	Glutamic acid	Glu	148.0	84.0	L-Glu-d5
13	Glutamine	Gln	147.1	84.1	L-Glu-d5
14	Glycine	Gly	76.0	30.1	Gly-d5
15	Histidine	His	156.1	110.0	His-d3
16	Isoleucine	Ile	132.1	86.1	L-Leu-d10
17	Leucine	Leu	132.1	86.1	L-Leu-d10
18	Lysine	Lys	147.1	84.1	His-d3
19	Methionine	Met	150.0	104.0	Met-d4
20	Ornithine	Orn	133.1	70.1	His-d3
21	Phenylalanine	Phe	166.1	120.1	L-Phe-d5
22	Proline	Pro	116.0	70.1	L-Ala-d4
23	Serine	Ser	106.0	60.0	L-Ala-d4
24	Threonine	Thr	120.0	74.1	Met-d4
25	Tryptophan	Trp	205.1	146.0	L-Leu-d10
26	Tyrosine	Tyr	182.1	136.1	Met-d4
27	Valine	Val	118.1	72.1	DL-Val-d8

2.2 Analysis of total amino acid content and composition of plant based material

2.2.1 General principle

The general principle of the total amino acid content and composition determination is presented in Figure 2. The derivatization step and UPC analysis are performed according to the validated Waters AccQ-Tag™ method. AQC reagent converts both primary and secondary amino acids to stable derivatives.

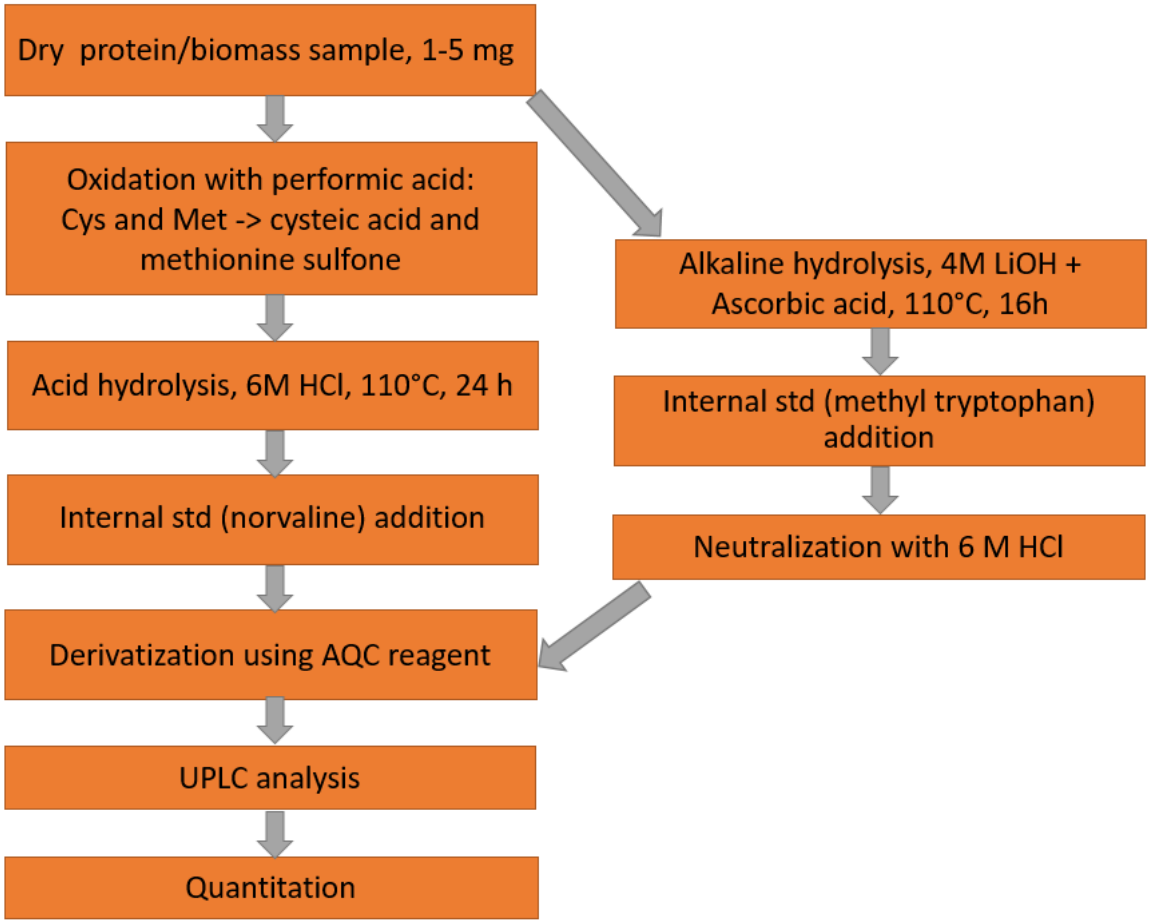

Figure 2. General principle of amino acid composition analysis.

2.2.2 Reagents

- Hydrogen peroxide, w = 30%.
- Formic acid, w = 98-100 %.
- Phenol
- Sodium hydroxide
- Hydrochloric acid
- Lithium hydroxide
- AccQ-Tag reagent kit (Waters) including Mass TRAK Amino Acid Analysis concentrate A and eluent B (Waters,Milford, MA, USA)
- Amino Acid Standard mixture solution(Sigma-AldrichSt. Luis, Missouri, USA).
- Norvaline, or other compound suitable for use as internal standard
- Nitrogen gas (< 10 ppm oxygen).

2.2.3 Acidic hydrolysis

For the determination of total amino acid content, the samples (5 mg, accurately weighed) are first oxidized with fresh performic acid solution (0.5 ml) prepared by mixing 5 ml of 30% hydrogen peroxide (Sigma-Aldrich) with 45 ml of formic acid–phenol solution (88% formic acid and 0.5 % phenol). After incubation at 0 ºC for 12 h ice-cold H2O (1 ml) is added and the mixture is evaporated to dryness under nitrogen flow. Subsequently, 1 ml of 6 N HCl containing 0.1 % phenol is added and the samples are hydrolysed at 110ºC for 20h. After hydrolysis samples are evaporated to dryness, reconstituted in 1000µl of H2O and further diluted 1/10. Finally, the internal standard norvaline is added.

2.2.4 Alkaline hydrolysis

For the determination of tryptophan, samples are hydrolyzed in alkaline conditions with 4 M lithium hydroxide (LiOH) solution containing ascorbic acid (95 mM). The lyophilized samples (2 mg, accurately weighed) are dissolved in 0.9 ml of freshly prepared hydrolysis solution. A vacuum is applied to the hydrolysis tubes followed by nitrogen replacement to the headspace. The samples are hydrolysed at 110ºC for 16 h. After hydrolysis, samples are neutralized by adding 600 ml of hydrochloric acid (6 M) and methyl-tryptophan internal standard 1s added.

2.2.5 Derivatization of amino acids

After acidic and alkaline hydrolysis 10 µl of the hydrolysis solution is taken and derivatization is performed according to Waters (Milford, MA, USA) AccQ-Tag method. Briefly, MassTrakTM AAA borate buffer and AQC (i.e. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) reagent are added, and sample mixture was instantly vortexed before incubation at 55 ºC for 10 min. Amino acid standard mixtures were derivatized as the samples.

2.2.6 Determination of amino acid composition: UHPLC analysis

Analysis is performed on an Acquity UPLC system, Waters (Milford, MA, USA) with diode array detector. For chromatography an Acquity Mass TRAK^{tm} (2.1 x 100 mm, 1.7 µm) column, Waters (Milford, USA) is used and the column temperature is kept at 43 ºC. Injection volume is 1 µL. Separation was performed using gradient elution with 10% (v/v) Amino Acid Analysis concentrate A in water (A) and Amino Acid Analysis eluent B (B) at a flow rate of 0.4 mL/min using a gradient elution program. Signal was detected at 260 nm (2.4 nm resolution, 20 points/second).

2.2.7 Calculations

With this method His, Ser, Arg Gly, Asp, Glu, Thr, Ala, Pro, Cys, Lys, Tyr, Met, Val, Ile, Leu, Phe and Trp can be quantified in the samples. In acid hydrolysis Asn is converted to Asp and Gln to Glu. Cysteine and methionine were determined as cysteic acid and methionine sulfone after the oxidation procedure. Protein content of the samples can be estimated based on the sum of the determined amino acids.

3. Tips and tricks

As amino acid composition analysis consists of many steps, there are several possible sources of errors, originating from hydrolysis and derivatization steps. The optimal pH range of AQC reagent used for amino acid derivatization, is from 8.2 to 10. To ensure complete derivatization, the acid hydrolysate must be properly buffered. A control sample, e.g. a pure, known protein such as BSA (bovine serum albumin) is recommended to be hydrolyzed and derivatized in parallel to follow the recoveries of amino acids.

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Chapter 2
Soluble or extractable protein content

Martina Lille, Riikka Juvonen (VTT, Finland)

Frederik Janssen, Arno G.B. Wouters (KU Leuven, Belgium)

Anja Herneke, Saeid Karkehabadi, Mathias Johansson, Jing Lu (SLU, Sweden)

Summary

- **Raw material**: Cereals and legumes
- **Relevant applications:** e.g. dairy and meat analogues
- **Fermentation-induced changes**: Protein extractability and/or solubility affected by enzyme-induced cell wall degradation and proteolysis, pH change and phytic acid degradation
- **Analytical method**:
    - Centrifugation of aqueous dispersion of plant material and analysis of protein/nitrogen content in resulting supernatant

1. Introduction

1.1 Plant proteins

Proteins are complex macromolecules that are essential to the structure and function of all living organisms. They are made up of long chains of amino acids, which are linked together by peptide bonds to form a polypeptide chain. Polypeptide chains can have varying numbers of amino acid residues, but a typical range falls between 50 and 2000 (Stryer, 1995). The arrangement of amino acids within a protein plays a crucial role in its folding into distinct secondary and tertiary structures, which in turn dictates its specific function and activity. Proteins are incredibly diverse in their function and structure. They can act as enzymes, transporters, hormones, antibodies, and structural components, among other roles (Stryer, 1995).

In plants, proteins that are consumed by humans are primarily found in the seeds and grains of the plants, where they exist as storage proteins. These storage proteins accumulate in the endosperm and other seed tissues during seed development (Shewry & Hey, 2015), and can make up a significant proportion of the dry weight of the seed or grain. Plant sources used for human consumption, such as cereals, legumes, and oilseeds, contain varying amounts of protein, usually ranging from 7-40%. Wheat typically has a protein content of 10-15% (Shewry & Hey, 2015), oat's protein content falls within the range of 10-22% (Webster & Wood, 2011), pea contains 20-31% protein (Day, 2013), faba bean has a protein content of 22-38% (Mayer Labba, et al., 2021), while rapeseed contains about 17-26% protein (Day, 2013).

1.2 Protein solubility and extractability

Plant proteins can be broadly categorised into four groups based on their solubility in different solvents. Albumins are metabolic proteins that dissolve in water, while globulins are salt-soluble storage proteins. Prolamins can be extracted from plant sources using concentrated aqueous alcohol solutions, and glutelins can be extracted using dilute aqueous acid or alkali solutions (Day, 2013; Lam, et al., 2018). The proportion of each protein class in plants varies depending on the source.

The solubility of proteins is dictated by their surface properties, which are influenced by the quantity and arrangement of hydrophilic and hydrophobic amino acid components on the protein surface. In water, solubility is hampered if hydrophobic patches remain on the protein surface. Additionally, solvent pH, ionic strength, and temperature are important factors affecting protein solubility (Lam, et al., 2018).

The functional properties and bioavailability of plant proteins in liquid and semi-solid food products are, however, not solely determined by the solubility of plant proteins. Equally important is the extractability of these proteins from the plant tissue. In plants, proteins are commonly enclosed within cellular structures that require disintegration to release the proteins. Processing of plant materials can have an impact on both the extractability and the solubility of plant proteins.

To determine the extractability/solubility of plant proteins, a typical approach involves dispersing the plant material in a solvent (commonly water or a buffer solution), followed by centrifugation of the dispersion. The protein content in the resulting supernatant is then analysed. Due to significant variations in the specific methods employed, comparing results obtained from different studies becomes challenging. The quantity of protein present in the supernatant is significantly influenced by the magnitude of the centrifugation force applied to the dispersion. It is also important to note that some of the protein present in the supernatant may not be entirely soluble but rather exists in a colloidal state. Consequently, in many cases, it is more accurate to refer to protein extractability rather than protein solubility.

1.3 How can fermentation affect protein extractability and solubility?

Fermentation can affect both the extractability and solubility of proteins from plant materials. The specific outcome depends on the microorganisms, substrate, and fermentation conditions. Fermentation may cause degradation of the cell tissue through the activity of hydrolytic enzymes produced by starter cultures added to the medium or by microorganisms already present in the raw material. The plant materials may also contain cell wall hydrolysing endogenous enzymes that can be activated during the fermentation process. The degradation of the cell tissue during fermentation may enhance the extractability of proteins from the plant material. Fermentation is also known to induce degradation of phytic acid through the activity of phytase produced by the microorganisms present in the matrix, which may also enhance the extractability of proteins from the plant tissue (Sharma, et al., 2020).

Some microorganisms, such as certain lactic acid bacteria (LAB) and many filamentous fungi, secrete proteolytic and peptidolytic enzymes that hydrolyse plant proteins into peptides and free amino acids, which is assumed to increase the solubility of the extracted proteins. For legumes, however, contradictory results have been reported on the effects of fermentation on protein solubility, which may partly be attributed to the decrease in pH during fermentation (Emkani;Oliele;& Saurel, Effect of lactic acid fermentation on legume protein properties, a review, 2022). Many plant proteins have a low solubility at acidic pH-values.

2. Method(s) of analysis

2.1 General principle

The evaluation of protein extractability/solubility is generally conducted using dry powders of plant ingredients. The analysis procedure typically consists of the following main steps:

1. Dispersion of the powder in an extraction medium
2. Centrifugation of the dispersion
3. Separation of the resulting supernatant
4. Analysing the protein content of the supernatant

Protein extractability is often simply expressed as the ratio of the protein content in the supernatant to the protein content in the initial dispersion. An alternative and more precise method to quantify

protein extractability, is to divide the total mass of protein extracted into the supernatant with the mass of protein in the initial powdered sample. However, this method requires accurate collection and weighing of the entire supernatant, which can sometimes be challenging in practice.
The methods employed in scientific research for protein extractability (or solubility) exhibit significant variations in how specific steps are executed, posing challenges when attempting to compare results obtained from different studies. Although two standardized AACC methods for protein extractability exist, namely the "Nitrogen Solubility Index (AACC 46-23-01)" and "Protein dispersibility index (AACC 46-24-01)," they are not frequently employed in scientific research. The main difference between these two methods is the level of shear applied to the dispersion prior to centrifugation.
While the analysis method may appear straightforward, in reality, it is more complex due to various factors in the applied procedures that can influence the final outcome. These factors are discussed in detail in the following chapter. Sample properties, such as composition and processing history, which cannot be altered during the analysis phase, are not addressed here.

2.2 Factors affecting analysis outcome

2.2.1 Sample preparation

The extractability (or solubility) analysis is most often conducted for plant materials in dry, powdered form. The extractability of proteins from powdered plant material is greatly influenced by the particle size of the material. To ensure accurate comparisons of extractability results across different ingredients, it is advisable to standardize the particle size to a specific level. When milling a sample before analysis, it is, however, important to consider that the sample may experience heating during the milling process, potentially leading to protein denaturation and other changes which may affect the quantity and quality of the extracted protein. To minimise heat-induced alterations in the sample, it is recommended to mix the sample with dry ice prior to grinding.
In the “Nitrogen Solubility Index” method (AACC 46-23-01), it is for example advised to grind the sample with an impact-type laboratory mill equipped with screen (tweed type), so that at least 95% of sample will go through a 100-mesh screen.
In a study with defatted oat whole meal (Janssen, et al., 2023), it was showed that reducing the particle size (D50) by ball milling from 34 to 16 µm increased the protein extractability from 18% to 35%.
When analysing the effect of processing, such as fermentation, on the extractability of proteins from plant materials, the sample of interest may contain a substantial amount of water. These kinds of samples can be analysed as such (diluted if necessary) or dried and milled before the extractability analysis. It has to be noted that the drying process may have an effect on the extractability and solubility of the proteins.

2.2.2 Dispersion medium

In food research, water (deionised) is the most commonly employed solvent in protein extractability studies. The solubility of proteins is influenced by factors such as pH and the presence of salts. Therefore, it is often of interest to study the extractability of proteins at specific pH values or salt concentrations. The pH can be controlled by utilising buffer solutions with specific pH values or by adjusting the pH of the sample dispersion through the addition of HCl or NaOH. When using HCl or NaOH, the quantities added must be considered in the result calculations. It is important to note that adjusting the pH also simultaneously modifies the ionic strength of the dispersion. Fermentation may alter the pH of the studied matrix, which must be taken into account in the protein extractability assay.

2.2.3 Dispersing conditions

To obtain consistent and reproducible results during the dispersion of the sample into the extraction medium, it is essential to control many variables. First, it is important to fix the sample concentration in the extraction medium to a level that does not lead to a significant increase in the viscosity of the dispersion. Excessive viscosity can hinder effective mixing of the dispersion, particularly when

employing low-shear agitation methods like magnetic stirring. Additionally, a high viscosity can impede the extraction of proteins into the extraction medium during centrifugation. A common practice is to disperse the sample into the extraction medium at a 1:10 w/v ratio (or 10% w/w), however, this concentration may be too high for ingredients with a high water-binding capacity.
Temperature is another variable that needs to be controlled during the dispersion phase. It is important to be aware that plant-based materials may contain endogenous enzymes, such as proteases, which can be activated at specific temperatures during the dispersion phase, especially when long dispersion times are used. To prevent this activation, suitable enzyme inhibitors can be added to the dispersion. Sometimes also preservatives such as sodium azide are added to prevent microbial growth during the analysis.
Both the intensity of shear applied during mixing and the duration of shear have significant impacts on protein extractability. Various types of mixing, such as magnetic stirring, mechanical shaking, mixing with a blade or Waring blender, have been applied to disperse the powdered sample into the extraction medium. Mixing times generally range from a few minutes to up to 2 hours. Longer mixing times are typically required when adjusting the pH with HCl or NaOH to allow for the pH to stabilize at a constant value. When high-shear mixing is employed, it may be necessary to include an antifoaming agent in the dispersion to prevent excessive foaming.

2.2.4 Centrifugation conditions

The amount of extracted proteins is influenced by the magnitude of the applied centrifugation force, as well as the temperature and duration of the centrifugation process. Centrifugation forces applied in protein extractability (solubility) studies can vary widely, with reported values typically ranging from 1 000 to 20 000 x g. The amount of colloidal protein in the supernatant will presumably decrease with an increase in centrifugation force. For the determination of "truly" soluble protein, ultracentrifugation can be employed. For example, in a study involving oat protein, ultracentrifugation at a force of 170 000 x g was applied (Runyon, et al., 2015).
In addition to the centrifugation force, the way the centrifugation process is halted (with or without breaking) can impact the results. Also, the size of the centrifugation container (tube) and the centrifuged sample volume must be standardised.

2.2.5 Protein content determination method

During protein extractability analysis, it is necessary to determine the protein content in both the initial sample powder and the supernatant obtained after centrifugation. Various protein assays can be utilised, including direct measurement of the amino acid content, indirect determination of proteinaceous nitrogen, or spectrophotometric assays (McClements, et al., 2021).
The protein content of the initial powdered sample is typically determined by analysing the nitrogen content of the material with combustion-based methos such as the Dumas or Kjeldahl. When these methods are used, it must be noted that nitrogen from both protein and nonprotein sources, such as NH_3, NH_4^+, nucleic acids, free amino acids, peptides, and phospholipids, can be present in the samples (Lam, et al., 2018; McClements, et al., 2021). For the calculation of the protein content from the nitrogen content, a nitrogen-to-protein conversion factor is used. This factor can be determined by analysing the amino acid content of the material. In the amino acid analysis, care must be taken that the amino acids (tryptophan, cysteine, and methionine) that are degraded during the typically applied acid hydrolysis are quantified with separate assays (McClements, et al., 2021). If the nitrogen-to-protein conversion factor is unknown, it is recommended to refer to nitrogen extractability/solubility instead of protein extractability/solubility.
The protein content of the liquid supernatant obtained after centrifugation is commonly determined using spectrophotometric protein assays, such as the Lowry or Bradford methods (Maehre, et al., 2018). These assays require calibration with a suitable reference material and may be influenced by interfering substances. The measured absorbance is influenced by the amino acid composition of the protein, making these methods less suitable for comparing the extractability/solubility of protein from different sources.

To ensure consistency and comparability, it is recommended to employ the same protein assay method, such as Dumas or Kjeldahl, for analysing the protein content of both the initial powdered sample and the supernatant. This approach helps mitigate potential discrepancies arising from fundamental differences between various protein assay methods.

2.3 Example of detailed protocol

In the following, an example of a protein extractability method currently in use at VTT is given. Another example can be found in a recent publication from KUL (Janssen, et al., 2023).

2.3.1 Sample preparation

This protocol is developed for plant-based samples in dry, powdered form. Powdered samples can be analysed as such or after milling to a standardised particle size.

2.3.2 Reagents

- 1M NaOH
- 1M HCl
- Ultrapure water

2.3.3 Protocol

- Analyse the dry matter content of the samples
- Analyse the nitrogen content of the samples with the DUMAS method
- Select the number of pH values at which solubility will be studied: e.g. 5 (pH 4, 5, 6, native and 8)
- Prepare (number of pH values) x 40 g (e.g. 5 x 40 g = 200 g) of 5% sample dispersion (based on dry matter)
- Agitate on magnetic stirrer for 30 min, avoid excessive foam formation
- Measure native pH
- Divide dispersion to (number of pH values) x 35 g batches into small decanter glasses (100 mL) and place decanter glasses on magnetic stirrer
- Adjust pH of four samples with HCl or NaOH to desired pH values (do not add anything to native pH sample) under continuous magnetic stirring and write down the consumed amount of HCl or NaOH
- Stir for another 30 min and adjust pH again if needed and write down consumed amount of HCl or NaOH
- Stir again for another 30 min and write down the consumed amount of HCl or NaOH
- Pour 35 g of the dispersions into 50 mL Falcon tubes and centrifuge samples at 10 000 x g for 15 min at 22 °C
- Collect at least 10 mL of the supernatant with a pipette
- Store the supernatants in the freezer (-20 °C)
- Analyse the protein content of the thawed supernatants with the DUMAS method in triplicate

2.3.3.1 Calculations

- Calculate the nitrogen content (%) in the initial dispersion, take into account the added amounts of HCl and NaOH
- Protein solubility (%) = 100 * (nitrogen content of supernatant)/(nitrogen content of initial dispersion)

3. Example of results

Results obtained with the above described method are typically presented as shown in Figure 1, i.e. protein extractability as function of pH. Figure 1 reveals that the extractability of protein from pea and faba bean protein is remarkably higher than that from oat protein concentrate especially at pH values above 6.

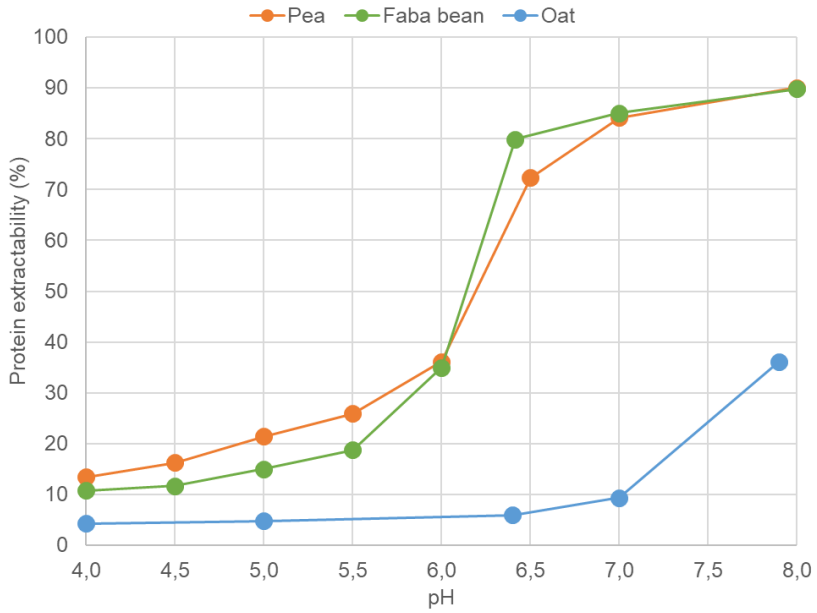

***Figure 1**. Extractability of proteins from pea, faba bean and oat protein concentrates at various pH values.*

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Chapter 3
Analysis of protein degree of hydrolysis and apparent molecular weight distribution

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Summary

- **Raw materials**: Wheat, oats
- **Relevant applications:** Meat and dairy alternatives, sourdough, digesta
- **Fermentation-induced changes**: Protein enzymatic hydrolysis, increased protein solubility, altered protein hydrophobicity, increased protein digestibility, ..
- **Analytical methods**:
    - Protein degree of hydrolysis
        - Ortho-pthalaldehyde (OPA) assay
    - Protein apparent molecular weight distribution
        - Size exclusion high performance liquid chromatography (SE-HPLC)

1. Introduction

1.1 The proteins in wheat and oats

Proteins are ubiquitous in nature and are large molecules made up of chains of amino acids. The specific sequence of these amino acids in a protein determines its unique three-dimensional structure and functionality. Cereals and legumes are two of the main protein sources in the human diet. Wheat and oats contain 12-15% (Hermans et al., 2021) and 15-20% (Sunilkumar et al., 2017) proteins, respectively.

The majority of wheat proteins are prolamins (i.e. gliadins) and glutelins (i.e. glutenins) (together called gluten proteins). Gliadins are monomeric proteins with molecular masses between 30 and 60 kDa, while glutenins are polymeric proteins with molecular masses that range from <100 to several thousands kDa (Delcour et al., 2012). Wheat gluten proteins can develop a viscoelastic network when hydrated and kneaded and provide structure and texture to i.a. (sourdough) bread (Delcour et al., 2012). They are also often used in the production of meat analogues (Pietsch et al., 2018). To extend the applicability of gluten proteins to other plant-based foods, structural modification is necessary to increase their solubility in aqueous media (Janssen, Monterde, et al., 2023).

Oat proteins consist of up to 12% albumins and up to 80% globulins (Klose & Arendt, 2012; Mäkinen et al., 2016). The latter fraction comprises 3S, 7S and 12S globulins, with the latter as the main fraction (Mäkinen et al., 2016). Oat 12S globulins are 320 kDa hexamers, comprised of six monomers, each consisting of an α- and a β-subunit linked by a disulfide bond (Mäkinen et al., 2016; Peterson, 1978). Oats have traditionally been used as whole meal or flakes, but the ongoing shift from animal protein to plant protein based foods has extended the applicability of oat protein to e.g. dairy alternatives (Spaen & Silva, 2021). The solubility and functionality of oat proteins have recently been reviewed by several authors (Mel & Malalgoda, 2022; Spaen & Silva, 2021). Overall, however, the extractability of oat proteins in aqueous media is low. This can be ascribed to the low solubility of oat 12S globulins in water (Janssen, Lambrechts, et al., 2023), the physical unavailability of oat proteins as a result of their

encapsulation in intact cell wall structures (Miller & Fulcher, 2011), and the heat treatment ('kilning') that is typically applied to oat groats to ensure lipase inactivation (Runyon et al., 2015).

1.2 How can food fermentation affect the molecular structure of proteins?

Food fermentation micro-organisms, often lactic acid bacteria, can grow by degrading substrates from a food matrix by the action of catabolic enzymes, including proteases (Marco et al., 2021). At the same time, organic acids (typically lactic acid) are produced during food fermentation, which can cause a pH-induced activation of the proteases inherently present in the food matrix (Arte et al., 2015). In the specific case of oats, it should be noted that the latter effect is probably limited when using kilned oats, as this prior heat treatment likely not only inactivates endogenous lipases but also proteases (Loponen et al., 2007). Still, proteases, either microbial or endogenous in origin, can be active during a food fermentation process. Proteases can hydrolyse native proteins in a food matrix into lower molecular weight peptides and amino acids (**Figure 1**). These peptides can have structures varying in among others molecular weight, hydrophobicity and charge density (Wouters et al., 2016). All this can have major consequences along the entire food value chain**.**

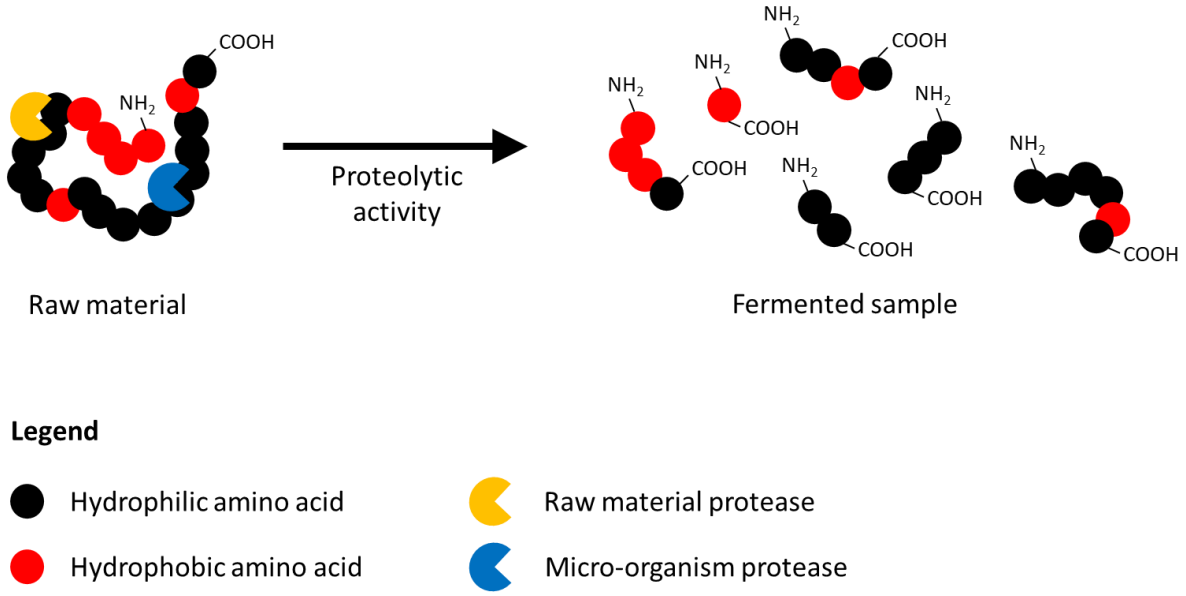

Figure 1*. Possible impact of food fermentation on the molecular structure of wheat and oat proteins.*

As mentioned, wheat gluten and oat proteins have low extractabilities in aqueous media, because of several reasons. Enzymatic protein hydrolysis can result in an increased protein solubility (Wouters et al., 2016) and, hence, also protein extractability (Arte et al., 2016), leading to a more efficient use of plant proteins in food applications made from a 'liquid base' such as plant-based alternatives to milk and yoghurt. Increasing the amount of protein in such liquid or semi-solid foods would be beneficial from a nutritional point of view, as many commercially available plant-based dairy alternatives have rather low protein contents (Walther et al., 2022). In addition, partial protein hydrolysis can lead to higher digestibility of the protein (Joye, 2019), thus increasing the bioavailability of essential amino acids. At the same time, the impact of enzymatic hydrolysis on the technofunctional contribution of oat and wheat proteins in food systems must be considered. Increased solubility in general is beneficial for specific functionalities such as foaming, emulsifying or gelling properties. However, extensive proteolysis can also have an adverse effect on protein functionality. For instance, extensive hydrolysis of oat or wheat gluten protein can result in emulsions with lower stability (Guan et al., 2007; Kong et al., 2007). Another example is that the gas cell stabilizing potential of sourdough and the

overall quality of sourdough bread depends on the degree of enzymatic gluten protein degradation (Gänzle et al., 2008).
In conclusion, the molecular structure of proteins can be significantly affected as the result of proteolytic activity during a food fermentation process, which in turn can affect the nutritional and sensory properties of the final food. It is therefore crucial to be able to dispose over analytical methods which allow assessing such changes in molecular structure of proteins.

2. Method(s) of analysis

2.1 Advantages and disadvantages of analytical methods

The most common approach to investigate proteolysis is to determine a 'degree of hydrolysis' (DH), defined by Rutherfurd (2010) as *"the proportion of cleaved peptide bonds in a protein hydrolysate"*. Several methods are available for quantifying this parameter, each possessing distinct advantages and disadvantages (Rutherfurd, 2010) (**Table 1**). Considering all of this, it was concluded that the OPA assay offers the highest efficiency and accuracy for monitoring fermentation-induced proteolytic changes. Therefore, the pH-stat and TNBS methods will not be further discussed.

***Table 1**. Advantages and disadvantages of analytical methods for determining the degree of protein hydrolysis. Adapted based on Rutherfurd* (2010).

Method	Advantages	Disadvantages
pH-stat	Real-time monitoring No derivatization	Estimation of DH requires knowledge on *i.a.* the number of peptide bonds in the protein Underestimation of DH in the presence of exopeptidases
TNBS	Rapid derivatization	No real-time monitoring Reducing agents cannot be used Inaccurate for proline-rich proteins
OPA	Rapid derivatization Real-time monitoring Reducing agents can be used	Inaccurate for proline-rich proteins

Although the DH is a useful parameter, it also has limitations. As pointed out by Wouters et al. (2016), while the DH indicates the overall extent of hydrolysis of a sample, it does not allow specifying which proteins in a complex protein mixture are hydrolyzed, or which types of peptides are released. These factors can however significantly affect the nutritional and technological outcome of a proteolytic process. Therefore, as complementary approach to determining the DH, it is useful to investigate changes in the protein molecular mass distribution of a sample. The two main methods to do this are size exclusion high performance liquid chromatography (SE-HPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The major advantage of SE-HPLC analysis is that it provides quantitative information on protein extractability (based on the total area of the chromatograms) and protein apparent molecular weight distribution (based on well-defined protein molecular weight markers). However, it is important to note that the UV intensity at 214 nm depends on the number of peptide bonds in a protein. Thus, lower molecular weight peptides will generate a lower UV intensity than higher molecular weight proteins (Kuipers & Gruppen, 2007). This means that proteolytic changes in the molecular mass distribution of a protein sample cannot be directly compared (Kuipers & Gruppen, 2007). Alternatively, aromatic amino acids (tryptophan, tyrosine and phenylalanine) can be detected by UV at 280 nm. Peptides lacking these amino acids will however not be detected. SDS-PAGE is a widely used low-cost technique for separating and analyzing proteins based on their molecular weight. However, it also has several drawbacks, as it provides only qualitative information on the protein apparent molecular weight distribution and it is more time-consuming than SE-HPLC analysis. Therefore, SDS-PAGE will not be further discussed.

In what follows, an approach will be discussed that combines determination of free amino groups via the OPA assay and apparent molecular mass distribution analysis via SE-HPLC, to establish an integrated view on the effect of food fermentation induced proteolysis on oat and wheat proteins.

2.2 Sample preparation

Food fermentation can be performed by inoculating oat or wheat whole meal suspensions (e.g. in a 1:5 w:v ratio) with 10^7 CFU/g dm followed by an incubation period. These fermented suspensions can then be diluted (e.g. to a 1:10 w:v ratio) and centrifuged (e.g. at 10,000 *g* for 10 min) to obtain an aqueous extract. It is important to prevent continued protein structural changes after the desired fermentation timeframe, by inactivating proteases in the system. This can be achieved in several ways. First, a heat shock (e.g. 10 min at 95 °C) can be applied prior to determining the DH and protein apparent molecular weight distribution. If such heat treatment results in the formation of insoluble protein aggregates, an additional centrifugation step is required for separating the insoluble and soluble protein material. If the extent to which the heat shock causes protein aggregation and precipitation is deemed too large for specific purposes, the heat shock should be omitted. In addition, when a sufficiently long fermentation process is applied, it is possible that no substantial changes in protein molecular structure are expected after the fermentation timeframe. In such case, the heat shock can be omitted as well. Alternatively, if the below described analyses can be performed immediately after the fermentation process, proteases can be inactivated by the addition of SDS-containing medium (see below).

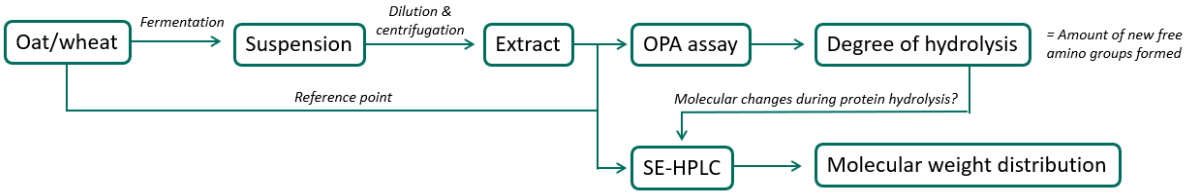

***Figure 2**. Schematic overview of the experimental set-up.*

2.3 Analysis of the degree of protein hydrolysis via the ortho-phthaldialdehyde assay

2.3.1 General principle

The DH reflects the percentage of peptide bonds cleaved during a food fermentation. Peptide bonds cleavage liberates terminal NH_2 groups (**Figure 1**) which, in the presence of a reducing agent, react with ortho-phthaldialdehyde (OPA), thereby forming a fluorophore compound that absorbs UV light at 340 nm (**Figure 3**). Complete protein solubilization is achieved by using media containing SDS and DTT to break non-covalent interactions and covalent disulfide bonds, respectively. Quantification of free NH_2-groups is performed by means of a calibration curve produced with a purified amino acid with one free amino group (e.g. L-leucine, L- lysine).

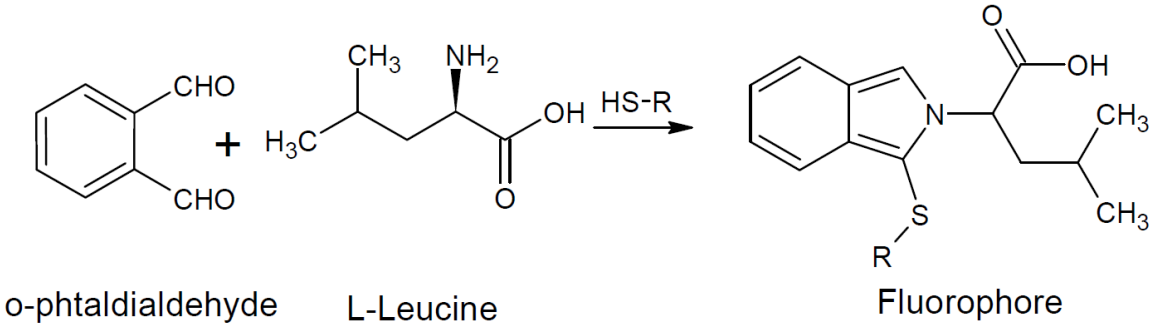

Figure 3. *Reaction of o-phtaldialdehyde with L-leucine forming a fluorophore compound.*

2.3.2 Reagents

- The OPA reagent should be prepared fresh:
    - Prepare a 0.10 M disodium borate (Borax) solution containing 1.0% (w/v) SDS in deionized water. Use only 90% of the total volume.
    - Heat the solution at approximately 60 °C until everything is dissolved.
    - When cooled down to room temperature, add 0.080% (w/w) OPA (dissolved in aqueous ethanol so that 2% (v/v) ethanol is achieved in the final volume) and 0.088% (w/w) DTT (dissolved in deionized water at 2% (v/v) of the final volume).
    - Add deionized water until the desired volume is reached and flush with nitrogen gas.
- Prepare a sodium phosphate buffer (0.050 M, pH 6.8) containing 1.0% w/v SDS.
- Prepare a L-leucine stock solution (2mM) in sodium phosphate buffer (0.050 M, pH 6.8) containing 1.0% w/v SDS.

2.3.3 Sample preparation

Samples

- Weigh in triplicate around 2-3 mg of protein (on dry matter basis) and add 2 mL 0.050 M sodium phosphate buffer containing 1.0% w/v SDS.
    - For liquid samples with a known protein concentration, pipet a volume containing 2-3 mg protein in triplicate. Increase the molarity of the sodium phosphate buffer and the SDS concentration to achieve the same final concentrations as when analyzing dry samples.
- Vortex extensively and centrifuge (10 min at 10,000 *g*).
- Transfer 400 µL of the supernatant to a test tube.

Calibration curve

- Prepare a dilution series (from 0 mM to 2 mM, where 0 mM represents a blanc) of the L-Leucine stock solution in 0.050 M sodium phosphate buffer containing 1.0% w/v SDS.
- Transfer 400 µL of each L-Leucine standard solution to a test tube (in triplicate).

2.3.4 Sample analysis

- Add 3 mL OPA reagent to each test tube and incubate for 20 min at room temperature.
- Immediately after exactly 20 min, measure the absorbance at 340 nm.

2.3.5 Calculations

Based on the calibration curve (absorbance at 340 nm against the concentration of L-Leucine), the amount of free NH_2 groups in a sample can be calculated as mmol NH_2-groups/g protein. The increase in the amount of free NH_2 groups by fermentation can be obtained if a non-fermented sample is included as reference. The degree of hydrolysis is given by:

$$DH\ (\%) = \frac{h}{h_{tot}} * 100$$

With h the calculated hydrolysis-equivalents (mmol/g protein) and h_{tot} the total theoretical number of peptide bonds present in a sample (mmol/g protein).

Adler-Nissen (1986) provided some numeric values for h_{tot} of relevant raw materials. If the amino acid composition of the starting material is known, h_{tot} equals the sum of mmols of the individual amino acids per gram of protein. It can also be determined with the OPA assay after complete acid hydrolysis of the protein. The h value is calculated as follows:

$$h = \frac{\triangle Abs * M * d}{\varepsilon * c}$$

With △Abs the absorbance of the fermented sample at 340 nm minus the absorbance of the non-fermented sample at 340 nm, M the average molecular mass of the test protein (Da), d the dilution factor, ε the extinction coefficient at 340 nm (6000 $mol^{-1}.cm^{-1}$) and c the protein concentration of the samples (g/L) (Adler-Nissen, 1977).

2.4 Analysis of the protein molecular weight distribution via size-exclusion chromatography

2.4.1 General principle

SE-HPLC allows separating proteins based on their hydrodynamic volume. The basic principle is that larger proteins are able to pass through a porous gel matrix more quickly than smaller proteins, which get trapped in the pores and take longer to elute. The separation efficiency is largely determined by the choice of the column, with specific pore size distribution and properties, and the elution rate. The absorbance of the eluted proteins via their peptide bonds is monitored with a UV detector at 214 nm (peptide bonds) and/or 280 nm (aromatic amino acids). Apparent molecular weights are estimated based on the elution times of molecular weight protein standards, analyzed under identical conditions. Wet extracts or dry powders will be diluted and dispersed, respectively, in a sodium phosphate buffer containing SDS, urea and DTT to disrupt all non-covalent interactions and covalent disulfide bonds and to analyze proteins in their molecular state. For wheat proteins, conducting SE-HPLC analysis without a reducing agent would enable differentiating proteolytic degradation patterns of gliadins and glutenins.

2.4.2 Reagents

- Prepare a sodium phosphate buffer (0.050 M, pH 6.8) containing 1.0% w/v SDS.
- Prepare a sodium phosphate buffer (0.050 M, pH 6.8) containing 0.10% w/v SDS.
- Prepare a sodium phosphate buffer (0.10 M, pH 6.8) containing 2.0% w/v SDS.

2.4.3 Sample preparation

- **Dry powders:**
  - Weigh accurately 1.0 mg protein (in triplicate) in an Eppendorf tube and write down the weight.
  - Add 0.050 M sodium phosphate buffer containing 1.0% w/v SDS to obtain a solution with a concentration of 1.0 mg protein/ml.

- **Wet extracts**:
  - Dilute the extract (in triplicate) with deionized water to a concentration of 2.0 mg protein/mL.
  - Dilute an aliquot of the extract (1:1 v:v) in 0.10 M sodium phosphate buffer containing 2.0% w/v SDS.

- **Blanks:**
  - Dry powders: prepare an Eppendorf tube containing 0.050 M sodium phosphate buffer with 1.0% w/v SDS.
  - Wet extracts: prepare an Eppendorf tube containing equal volumes of deionized water and 0.10 M sodium phosphate buffer containing 2.0% w/v SDS.

- **Molecular weight protein standards**

- Potential candidates are phosphorylase B (97 kDa), bovine serum albumin (66kDa), chicken egg ovalbumin (45 kDa), carbonic anhydrase from bovine erythrocytes (30 kDa), trypsin inhibitor (20.1 kDa), alpha-lactalbumin (14.2 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa), insulin B chain (3.5 kDa), angiotensin III (931 Da), $(Ala)_5$ (373 Da) and Ala-Gln (217 Da).
- Weigh 1.0 mg protein in an Eppendorf tube.
- Add 1.0 mL 0.050 M sodium phosphate buffer containing 1.0% w/v SDS.

- When working under reducing conditions, always work under nitrogen atmosphere and ensure that the samples are analyzed within 24 h after preparation.
- Vortex the Eppendorf tubes extensively.
- Shake the Eppendorf tubes at room temperature for 60 min at 150 rpm.
- Centrifuge the Eppendorf tubes for 10 min at 10,000 *g* to remove insoluble material.
- Filter the supernatant over a 0.45 µm filter (polyether sulfone membrane Millex-HP, Merck Millipore, Carrigtwohill, Ireland) and transfer an aliquot into 2.0 mL HPLC vials.

2.4.4 Sample analysis

- Analyze an aliquot (*ca.* 10 µL) of the sample with an SE-HPLC system containing a BioSep Sec-S2000 column (300 x 7.8 mm, Phenomenex, Torrance, US) with a separation range from 0.2 to 75 kDa in 0.50% w/v SDS solution.
- Set the flow rate at 0.50 mL/min and the oven temperature at 30 °C.
- Monitor the UV signal at both 214 nm (peptide bonds) and 280 nm (aromatic amino acids).

2.4.5 Calculations

- Establish a calibration curve by plotting the elution times of the molecular weight protein markers as a function of the logarithm of their corresponding molecular weight (**Figure 4**).
- Use the obtained equation to estimate the molecular weight of a given peak in the chromatogram of a sample based on the elution time.
- Identify peaks corresponding to specific proteins (e.g. 12S oat globulins) or to protein fractions with similar apparent molecular masses based on molecular mass values available in literature (see section 1.1).

- Determine the total area under each profile using the HPLC software or an external program (e.g. Matlab or OriginLab).

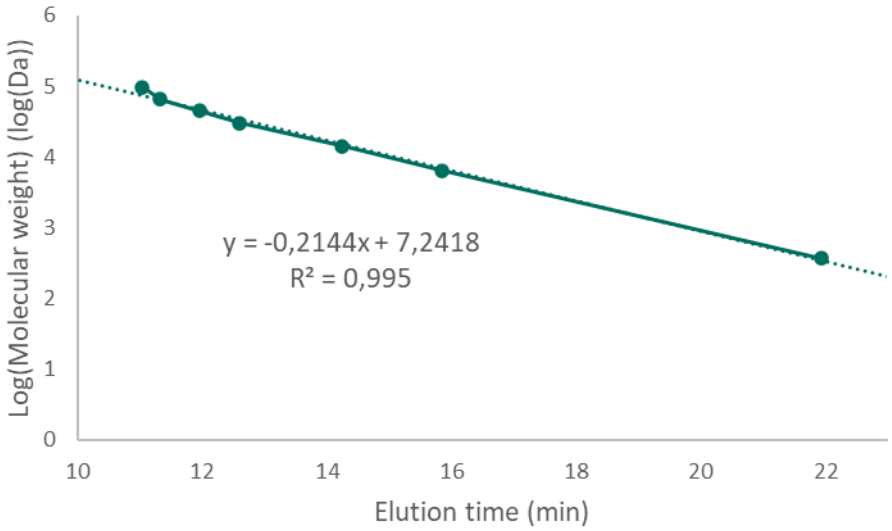

Figure 4. Correlation between the logarithm of the molecular mass and respective elution time of phosphorylase B (97 kDa), bovine serum albumin (66kDa), chicken egg ovalbumin (45 kDa), carbonic anhydrase from bovine erythrocytes (30 kDa), alpha-lactalbumin (14.2 kDa), aprotinin (6.5 kDa) and $(Ala)_5$ (373 Da) protein standards.

3. Others

A similar approach as outlined above can likely be applied for other raw materials (e.g. legumes), but will require optimization. To determine the degree of hydrolysis with the OPA assay, the amount of analyzed sample should be optimized first. The required amount of sample should result in an absorbance value within the range of the calibration curve. Also the incubation time for the OPA reagent can be altered, according to the type of sample. Therefor it can be useful to follow up the absorbance in function of the time. Choose an incubation time where the absorbance is rather stable and does not decrease again, but makes it also feasible to measure multiple samples in one batch.
For SE-HPLC, the specifications of the separation column have to be optimized according to the molecular mass distribution of the analyzed proteins. If needed, multiple columns can be combined to obtain a better peak separation in certain molecular weight ranges.

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Chapter 4
Starch

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Summary

- **Raw material**: Wheat, oat, yellow pea, faba bean
- **Relevant applications:** Bread, plant-based yogurts, tempeh
- **Fermentation-induced changes**: Starch hydrolysis by microbial and endogenous α-amylases, altered starch gelatinisation and retrogradation behaviour, modified starch molecular structure.
- **Analytical methods**:
  - Megazyme kits:
    - Total starch
    - Resistant starch
    - Damaged starch
  - HP-SEC
    - Molecular size and Chain length distribution
    - Amylose and Amylopectin ratio
  - Microscopy
    - Transmitting light microscopy
    - Polarized light microscopy
    - SEM
  - Differential scanning calorimetry (DSC)
    - Crystal melting enthalpy
  - Gelatinisation temperaturePasting properties
    - Rapid visco analyser (RVA)
  - Starch isolation

1. Introduction

1.1 What is starch

Storage starch is the main carbohydrate in many human diets and is stored in semi-crystalline granules in seeds, stems, and tubers (Pfister and Zeeman 2016). Both cereals and legumes are classed as high starch foods with reported starch contents ranging between 60-75% for wheat, 40-60% for oat, 46-47% for yellow pea and 40-44% for faba bean (Lu et al. 2021; Johansson et al. 2022). The consumption of starchy foods that induce high glycaemic responses (e.g. white bread) are linked to an increased risk of obesity, cardiovascular disease, metabolic syndrome, and type-2 diabetes (Svihus and Hervik 2016). Therefore, strategies to reduce the glycaemic index of starchy foods by modulating their starch

digestibility are being extensively studied in the literature. Food fermentation technology is regarded as such a strategy (Scazzina et al. 2009).

Starch is a glucose polymer consisting of the two components; amylose and amylopectin (see (**Fig 1A**)), with amylopectin typically being the more predominant/abundant constituent. Amylose is an essentially linear polymer composed primarily of α-1,4 glucosidic bonds (and very few α-1,6 D-glucopyranosyl residues) . The long amylose chains form a helix and have a molecular weight ranging from about 243,000 to 972,000, but typically the MW is < 0.5 million (Thomas and Atwell 1999). Highly branched amylopectin, is a much larger polymer consisting of both linear α-1-4-glucosidic bonds and α-1-6-glucosidic bonds at the branching points (Belitz, Grosch, and Schieberle 2009). The molecular weight of amylopectin ranges between 10 to 500 million, making it one of the largest polymers found in nature (Thomas and Atwell 1999). The starch granule consists of alternating layers of amorphous (amylose and branched segments of amylopectin) and semi-crystalline (double helices of amylopectin) as depicted in figure 1B. Starch is classified into three different polymorphs (A, B and C-type) characterised by their crystalline pattern (Dome et al. 2020). The A-types are usually found in cereals whereas the B-types are more predominant in tubers and stems. Legumes and most roots have a mixture of packing arrangements for A and B types, which are referred to as C-types (K. Wang, Henry, and Gilbert 2014). The thermal transition temperatures is higher for A-type starches than B, therefore the proportion of the two is a critical factor for legume starches (K. Wang, Henry, and Gilbert 2014). The degree of crystallinity and the polymorphic patter can be determined using X-ray diffraction scattering (XRD) techniques (Sarko and Wu 1978).

Milling can cause mechanical damage to the starch granules,(Donald 2004) which increases the susceptibility of these disrupted strucutres to hydrolysis by endogenous amylases which is important to provide sufficient amounts of fermentable sugars during e.g. breadmaking (Q. Wang, Li, and Zheng 2020). The amount of damaged starch in wheat and oat flours are in the range of 5-15 % and 1-10 %, respectively (Barak, Mudgil, and Khatkar 2014; Hüttner, Bello, and Arendt 2010)

Starch granules are in general insoluble in cold water, but when heated the granules swell as water is absorbed (Vamadevan and Bertoft 2015). Gelatinization is an important property of starch occurring at the initial stages of a pasting curve which corresponding to when the starch irreversibly goes from an ordered to a disordered state. The starch must be fully gelatinized before pasting can commence, which is when a starch paste is developed resulting in an increase in viscosity during progressive heating because of continual granular swelling and leaching out of starch matter (Delcour and Hoseney 2010) . Upon cooling gelation occurs as the leached amylose aggregates forming a network followed by short-term retrogradation as the amylose recrystallize. During storage the long term retrogradation occurs as the amylopectin branches recrystallize (e.g. setting of crumb structure during cooling of a bread loaf) (Yan et al. 2021).

Starch digestion starts in the mouth with mechanical processing through mastication and exposure to enzymatic α-amylase. The starch is further enzymatically digested in the small intestine by pancreatic amylases and glucosidases (Brownlee et al. 2018).Resistant starch is starch that cannot be digested, thus functioning as a fibre. When the resistant starch reaches the colon, it becomes fermented by the gut microbes. Resistant starch has documented benefits for human health such as a prebiotic effect, controlling blood sugar, and preventing colonic cancer (Bede and Zaixiang 2021). Resistant starch is classified into five categories: physically inaccessible starch (RS1), native starch (RS2), retrograded starch (RS3), chemically modified starch (RS4) and V-complex starch (RS5). Legumes tend to contain more resistant starch than cereals, Nilsson et al (2022) concluded that starch isolates from fava bean hull and cotyledon contained 13.1 % and 6.7% resistant starch, respectively (Nilsson et al. 2022). Which is considerably more compared to the wheat starch that only contained 0.5% resistant starch.

The resistant starch content of a product may be altered because of several factors, including variety, preparation or cooking method, storage temperature and duration, and serving temperature (Patterson, Maiya, and Stewart 2020).

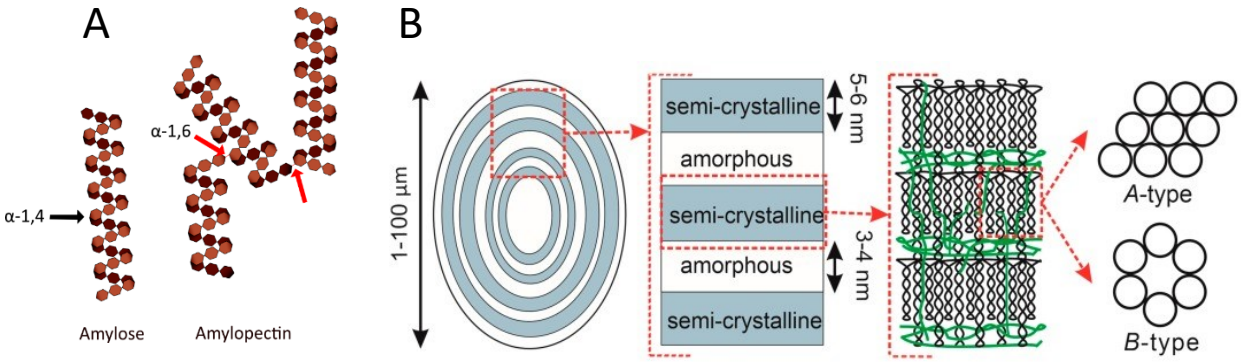

***Figure 1**. A) General structure of amylose (left) and amylopectin (right). α-1,4 linkage and α-1,6 linkages are indicated by a black and red arrow, respectively. B) Semi-crystalline organisation of a starch granule (Dome et al. 2020)*

1.2 How can fermentation affect starch?

Starch plays a crucial role in grain-based fermentations. Microorganisms require fermentable sugars such as glucose and maltose to produce organic acids, alcohol, and CO_2 as main metabolites. However, the content of fermentable sugars in grain-based products is often low and additional hydrolysis of starch is required to maintain microbial activity (Gobbetti and Gänzle 2013).

Fermentable sugars are supplied via the hydrolysis of damaged starch by both endogenous and microbial amylases (Leman et al. 2006)). For instance, *L. amylovorus, L. plantarum* and *L. fermentum* (Padmavathi et al. 2018) have been reported to exhibit amylase activity. Other species such as *Aspergillus sp*. and *Rhizopus sp*. are capable of converting native starch to glucose without the need for prior gelatinisation(Sun et al. 2010). The degree of starch hydrolysis during fermentation of grain-based flours is expected to impact the molecular structure of starch such as the molecular weight distribution, amylose/amylopectin ratio and relative crystallinity (Zhao et al. 2019). Such changes might have implications for starch gelatinisation and retrogradation behaviour during and after a heat-treatment (e.g. baking).

Besides starch hydrolysis, fermentation-induced changes in the food matrix will affect starch physicochemical properties. For instance, lactic acid is believed to alter gelatinisation and retrogradation behaviour of starch, which could impact its susceptibility to pancreatic amylase (Östman et al. 2002). Several studies have suggested that the interaction of starch and lactic acid is responsible for the reduced digestibility of starch in sourdough bread (Poutanen, Flander, and Katina 2009). However, underlying mechanisms remain to be elucidated.

To be able to produce protein-rich plant-based yogurts from fava bean the starch needs to be removed or gelatinised and pretreated with α-amylase before the introduction of lactic bacteria (Jiang et al. 2020). This will prevent the starch to gelatinase during the formation of the protein-based emulation gel.

Traditional tempeh is made from dehulled soybean, which is soaked, boiled, and dried before introducing *Rhizopus* ssp. sporangiospores to induce fermentation (Ahnan-Winarno et al. 2021). These pre-processing steps by soaking and boiling the bean before will partly gelatinize the starch granule and make the amylose more accessible for the fungal α-amylase.

There are a few studies of tempeh produced from other raw materials than soy such as peas, oat and fava bean (Ashenafi and Busse 1991; Cai et al. 2014; Eklund-Jonsson, Sandberg, and Larsson Alminger 2006; Nassar, Mubarak, and El-Beltagy 2008; Polanowska et al. 2020). However, the changes in starch composition during the pre-treatments and after fermentation were not well characterised. Van der Riet et al., (1986) reported a reduction in total starch of soybean after fermentation with *Rhizopus*

oligospours; the starch reduction benefited of prolonged incubation with the spores (van der Riet et al. 1987), a similar reduction in starch has also been reported for tempeh made from spelt wheat (Starzyńska-Janiszewska et al. 2019).
Flour made from soy-based tempeh has been reported to improve wheat bread quality made with regular baking yeast. (Huang et al. 2019). The addition of tempeh flour generated an increased volume and viscoelastic properties of the dough compared to the control bread made only with wheat or the addition of non-fermented soy protein. The addition of tempeh flour also leads to a reduction in moister migration and water loss in the breadcrumbs. Huang et al. (2019) suggested that the increase in bread volume was due to fungal amylases present in the tempeh flour that catalysed the hydrolysis of starch to low molecular weight components such as fermentable sugars that were used by the yeast to generate a larger amount of gas. Fungal amylase activity was also the reason for the increased softness of the baked product due to less retrogradation of the starch.

2. Method(s) of analysis

2.1 Sample preparation

Based on the type of sample and the analytical method different sample preparations are required. Figure 3 provides a schematic overview of the type of sample preparations and experimental methods for determining content, structure and relevant physiochemical properties of starch.

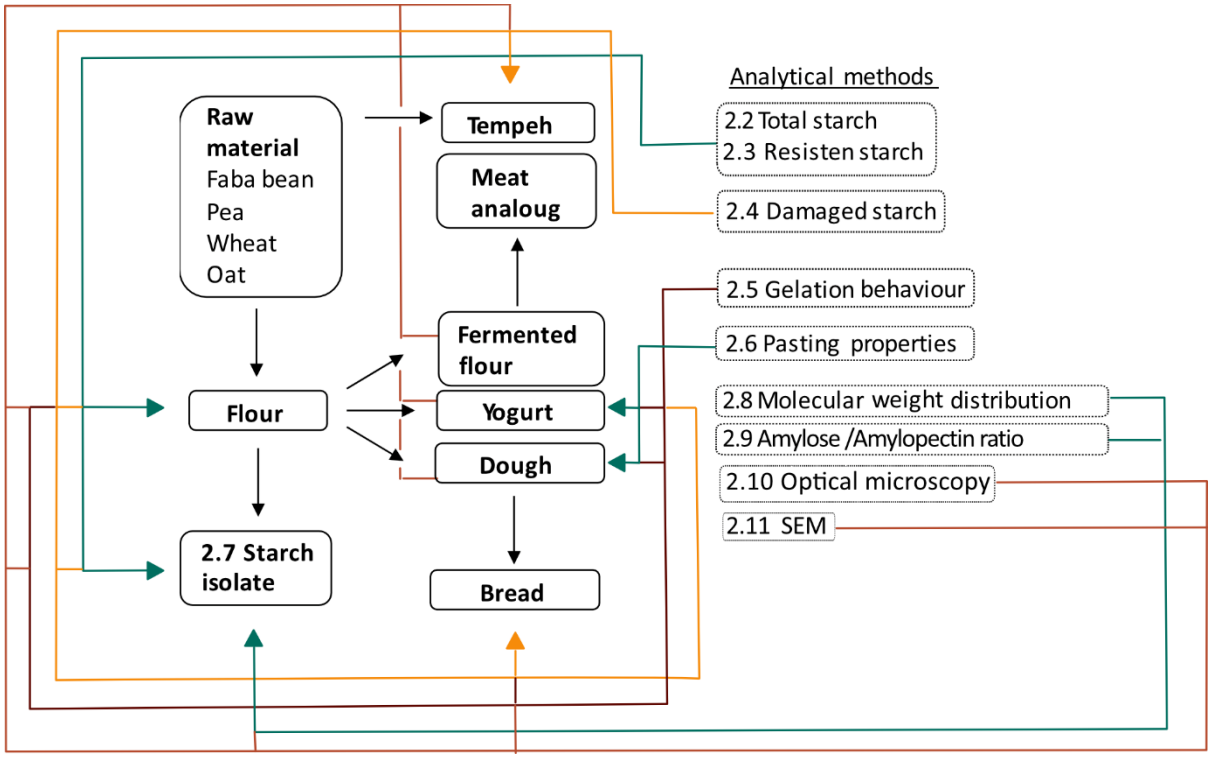

***Figure 2**: Schematic overview of the sample preparation and analytical methods required for analysing content, structure and physicochemical properties of starch.*

2.2 Total starch content

2.2.1 General principle

The samples are boiled to completely solubilise the starch. Afterwards, a thermostable α-amylase is added which converts starch into glucose, maltose and α-limit dextrins. These hydrolysis products are further converted to glucose by amyloglucosidase which is quantified with GOPOD-analysis.

2.2.2 Method

The procedure is based on the Megazyme protocol K-TSTA-50A / K-TSTA-100 (procedure **A**) with following modifications:
https://www.megazyme.com/documents/Assay_Protocol/K-TSTA-100A_DATA.pdf

- Use small GC test tubes at the start of the method and downscale the procedure to 50 mg samples (instead of 100 mg) and 5 mL of Sodium acetate buffer instead of 10 mL.
- Place round bottom screw cap tubes in a heating block at 110°C instead of using the boiling water bath.
- Before transferring the tubes to the 50°C water bath, the tubes should be cooled down under streaming water. Next pre-incubate the tubes for 10, rather than 5, minutes in the 50°C water bath.

2.3 Analysis of resistant starch content

This kit for resistant starch can be used in calculating total starch content of samples, particularly if the resistant starch content is high.

2.3.1 General principle

The method is based on the hydrolysis of non-resistant starch using pancreatic amylase and determining the residual starch content recovered in the pellet after centrifugation.

2.3.2 Method

The complete method is described in the K-RSTAR procedure of Megazyme.
https://www.megazyme.com/documents/Assay_Protocol/K-RSTAR_DATA.pdf

2.4 Analysis of damaged starch content

2.4.1 General principle

Measuring damaged starch is based on the use of a fungal α-amylase which has a high specificity for damaged starch only. Afterwards, the produced maltose and α-limit-dextrins are further hydrolysed into D-glucose by amyloglucosidase which can be quantified with GOPOD analysis.

2.4.2 Method

The complete procedure is described in the K-SDAM procedure of Megazyme.
https://www.megazyme.com/documents/Assay_Protocol/K-SDAM_DATA.pdf

2.5 Analysis of starch gelatinisation and retrogradation behaviour (DSC)

2.5.1 General principle

Differential Scanning Calorimetry uses a temperature-time profile to measure the gelatinisation enthalpy (i.e. melting of crystalline structures) of starch in a sample. The difference in heat required to heat a sample pan and an empty reference pan results in an endotherm (heat flow in function of temperature). This graph can be used to define gelatinisation temperature and total gelatinisation enthalpy (in J/g dry matter). After the first measurement, the gelatinised sample can be measured again to study melting transition of retrograded starch.

2.5.2 Method

2.5.2.1 Solid samples

- Weigh empty pan
- Weigh sample (between 2.5 and 4 mg, depending on moisture content of sample)

- Add water if needed such that the dry:matter ratio is 1:3 (dry matter mass x 4 – sample mass = µL water to be added)
- Place cover on pan with sample and seal hermetically with DSC press
- Leave the sealed pans overnight at room temperature to achieve uniform distribution of water before DSC analysis.
- DSC settings
    - Temperature range: 0°C-130°C
    - Rate: 10°C/min
    - Nitrogen flow: same as purge rate during measurement (50 mL/min)

2.5.2.2 Liquid samples

- Weigh empty pan
- Carefully dispose a small drop of the liquid sample into the DSC pan
- Weigh drop
- Close pan with DSC press
- After the measurement, the pan can be opened and dried overnight to calculate the exact dry matter weight.
- Gelatinisation enthalpy is expressed as J/g dry matter

2.5.3 Interpretation of results

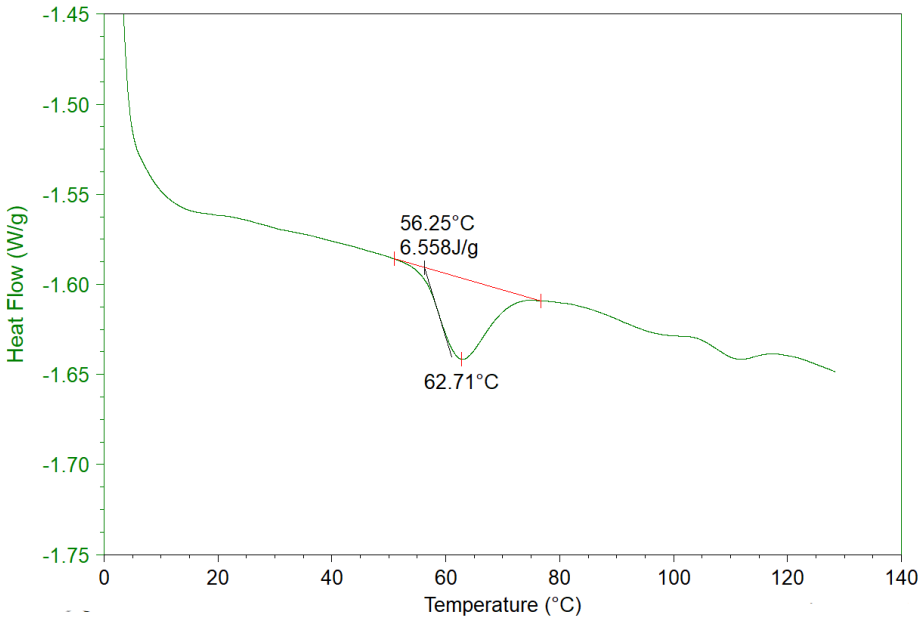

Figure 3: Example of a DSC endotherm of wholemeal oat flour (1:6 flour to water ratio) showing starch gelatinisation Peak temperature is 62.7°C. Metling enthalpy is 6.6 J/g.

2.6 Analysis of pasting properties

2.6.1 General principle

With a pasting curve is it possible to analyse the changes that occurs when the starch is exposed for water and heat (Delcour and Hoseney 2010). The traditional instrument to measure pasting properties has been a Brabender viscoamylograph, but in later years this instrument has been replaced more and more with rapid visco analyzers (RVA) due to faster measuring time and the need for smaller sample volumes (Deffenbaugh and Walker 1989). A rheometer with a Peltier

pressure cell can also be used in a similar fashion to analyse the changes in the viscosity of starch depending on the heating rate and temperature ranges. Nilsson et al., (2022) showed that RVA consistently measures higher viscosity than the rheometer at similar parameters (see figure 4). This was probably due to that, the RVA is not a closed system as the Peltier pressure cell, which leads to water evaporation during the heating cycle. However, the not closed RVA system probably mimics to a lager extent how most food products are produced. An advantage to use the fully sealed Peltier pressure cell is that the samples can be heated to temperatures surpassing 100 °C, which may be necessary for the samples to achieve peak viscosities.

2.6.2 Method

2.6.2.1 RVA

For the exact quantities of solid sample to water for the RVA analysis please consult the instrumental procedure instructions.

- An appropriate dry matter: water ratio should be chosen (A ratio of 1:5 to 1:6 usually works well for flour-based samples)
- Weight the sample into a RVA cup. The total weight of the suspension should be around 25 g.
- Examples of suitable RVA settings are :
    - heating rate at 12 °C/min or 1.5 °C/min with a paddle rotation to 160 rpm
    - Equilibrate the sample at 50 °C for 1 min, with the rotational paddel speed for the initial 10 seconds being 960 RPM, before reducing the rotational speed and heating the sample at contant heating rate to 95 °C.
    - For heating rate 12 °C/min keep at maximum (95 °C) for 2.5 min before cooling to 50 °C stay at the final temperature for 2 min.
    - For heating rate 1.5 °C/min keep at maximum (95 °C) for 5 min before cooling to 50 °C stay at final temperature for 2 min.

2.6.2.2 Rheometer

If using the rheometer (HR-3, Discovery Hybrid Rheometer, New Castle, DE, USA) equipped with Peltier pressure cell and steel starch paddle the minimal water content to be added to the pressure cell is 26 mL, the maximal heating rate is 13 °C/min (although some research groups have found having heating rate higher than 5 °C/ min produced erratic results) and maximal heating temperature is 150 °C (TA Instruments, 2017).
The sample should be prepared as for the RVA with appropriate adaptations in the method (I.e. sample quantity/ratio, heating rate and ranges) for the specific rheometer.

2.6.3 Interpretation of results

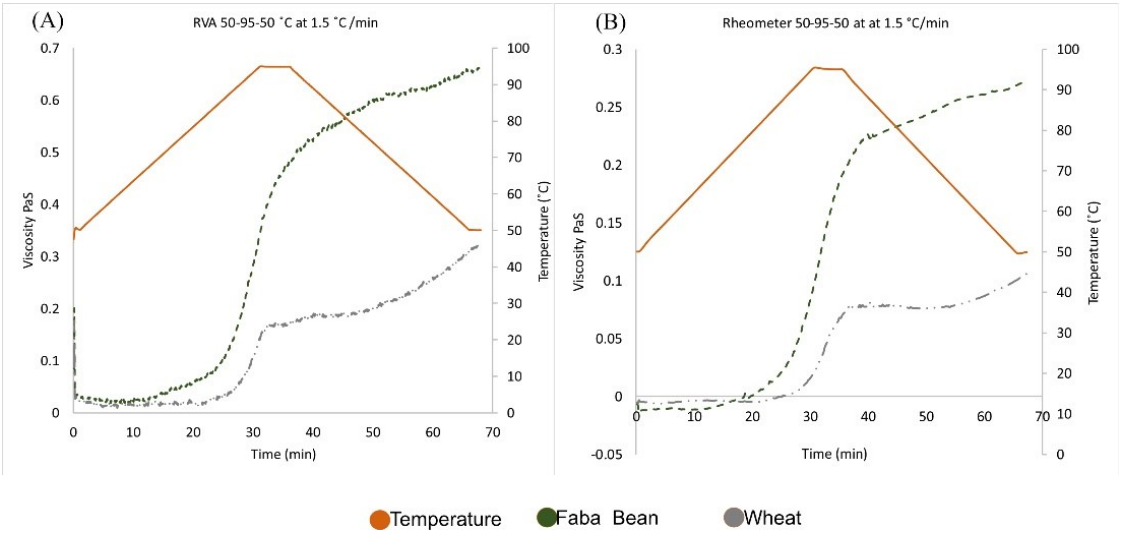

Figure 4: Pasting curves of faba bean starch and wheat starch; a Rapid viscosity analysis RVA heating profile 50–95–50 at heating rate 1.5 °C/min; c rheometer heating profile 50–95–50 at heating rate 1.5 °C/min; figure adapted from Nilsson et al. 2022.

2.7 Starch isolation

2.7.1 General principle

For **methods 2.8-2.9** listed below starch must be isolated before analysis. Depending on the type of raw material (i.e. pulses, wheat and oat) a slightly different isolation method is required.

2.7.2 Method

2.7.2.1 Wheat

- Weigh 250 g flour (on 14% moisture basis)
- Mix flour to a dough in a kitchen aid bowl with deionized water (150 mL)
- Allow the dough to rest for 8 min
- Slowly add 250 mL of tap water and mix for 25 min (position 1 on mixer, use flat beater)
- Add 1000 mL of water and mix for 35 min (position 1 on mixer, use flat beater)
- Pour the batter on a system of vibrating sieves (400 µm, 250 µm, 125 µm, 38 µm)
- Sift for 5 min at c.a. 70 Hz vibration speed
- Wash three times for approximately 2 min with 1000 mL of tap water.
- Recover the gluten fraction from each sieve, put them on metal plates and freeze them in liquid nitrogen and lyophilize
- Collect all wash water (containing starch) and centrifuge at 2,000 x *g* for 10 min.
- Wash pellet with ethanol
- Discard pellet on filter paper and dry overnight

2.7.2.2 Oat (Hoover and Vasanthan 1992)

Compared to other cereals oat extraction is rather complicated due to the strong association with proteins and β-glycans.

- Steep oat grain in water at 50 °C for 3 h.
- Mix soaked oat grain with distilled water at a ratio of 1:3 mix for 3 min in a blender at low speed.

- Pass the slurry through a cheesecloth.
- Centrifuge the filtrate at 5000 g for 15 min.
- Remove the supernatant and suspend the pellet in excess of 0.02 % NaOH, let stand for 1h and remove the supernatant. Repeat this procedure six times.
- Suspend the sediment in distilled water and vacuum filtrate the solution with a Buchner funnel with a 70 and 20 µm polypropylene mesh.
- Wash the sediment thoroughly on the mesh with distillate water.
- Dry the filtrate overnight at 30 °C.

2.7.2.3 Pulses (Li et al. 2020) (Nilsson et al. 2022)

- Dissolve flour in 0.02% sodium hydroxide (NaOH) at a ratio of 1:10 for 24h with stirring at room temperature then an additional 24 h without stirring at 4 °C.
- Centrifuge the slurry at 3700 x *g* for 5 min.
- Remove the supernatant and replace it with distilled water and centrifuge once more at 3700 x *g* for 5 min. Repeat the process until the sample has a pH of 7.
- Collect the pellet after the final wash and mix the pellet with distilled water in a kitchen blender
- Filtrate the homogenized solution with a Buchner funnel with a 70 µm nylon filter.
- To maximize starch extraction mix the filtercake once more with distilled water in the kitchen blender and filter as earlier.
- For optimal starch recovery leave the filtrate standing without agitation overnight at 4 °C.
- Scape away the brown top layer of the starch and let it dry at 40 °C for 48 h.

2.8 Analysis of molecular weight distribution

2.8.1 General principle

Isolated starch granules are suspended in a buffer containing dimethylsulfoxide (DMSO) to completely solubilise the starch. Afterwards, the amylose and amylopectin are separated based on their molecular size (SE-HPLC).

2.8.2 Method

- Weigh 20.0 mg of isolated starch granules in a 10.0 mL glass tube with round bottom screw cap tube.
- Add 5.0 mL DMSO containing 0.5% (*w/w*) Lithium bromide (LiBr) and vortex.
- Heat the starch suspension overnight at 80 °C in a water bath while shaking at 150 strokes/min.
- After cooling to 30 °C in a water bath, centrifuge the starch suspension for 10 min at 4,000 *g* and 30 °C (Note! It is important to that the sample don't go below 30 °C, otherwise another heating step might be necessary before analysis).
- Transfer 1 mL of the supernatant to an HPLC vial.
- Column specifications: GRAM 3000 and GRAM 30
- Elution solvent: DMSO containing 0.5% (*w/w*) LiBr in a water bath at 40°C
- Injection volume: 100 µL
- Detector temperature: 40°C
- Oven temperature: 80°C
- Flow rate: 0.3 mL/min

2.9 Analysis of amylose/amylopectin ratio

2.9.1 General principle

Starch amylopectin is debranched by isoamylase. The debrached amylopectin chains can be distinguished from the longer amylose chains during SE-HPLC analysis.

2.9.2 Method

- Weigh 30.0 mg of isolated starch granules in a 10.0 mL glass tube with red screw cap
- Add 4.5 mL milli Q and vortex
- Heat the samples for at least 60 min at 100°C and vortex every 5 min. *Make sure that the starch is fully gelatinized and that no lumps remain.*
- After cooling to 40°C in a water bath, add 500 µL sodium acetate buffer (0.1 M, pH 4.0)
- Add 60 µL isoamylase solution (Megazyme, Ireland) and incubate the sample for 24h at 40°C
- Add another 60 µL of isoamylase solution and incubate the sample for 24h at 40°C
- Neutralize the suspension by the addition of 480 µL 0.1 M NaOH
- Heat the samples at 80°C for 2h in a water bath
- Centrifuge at 4,000 *x g* for 10 min at 30°C.
- Transfer the supernatant to a large falcon tube (50 mL), freeze with liquid nitrogen and freeze-dry.
- Continue the procedure according to 2.8.2

2.10 Analysis of starch granules with optical microscopy

2.10.1 General principle

Transmitting light microscopy is a quick technique that can be used to study the starch granules shape and size ratio and distribution within the sample. The contrast can be enhanced by staining the sample with Lugol´s iodine stain*. The crystalline region of the starch granules shows birefringence under polarized light, which can be seen as a Maltese Cross (Chakraborty et al. 2020). This technique is a quick method to study if the fermentation process has induced any changes in the internal structure of the granule.

*NOTE! Lugol´s iodine stain has traditionally been used to detect amylose due to iodin's ability to form a complex with the amylose helix (Chen and Bergman 2007). When the dye binds it forms a deep blue colour. However, longer branches of amylopectin (DP<60) also form complexes with iodin which lead to an overestimation of amylose. Phospholipids and free fatty acids also tend to bind to the helical structure of amylose competing with the iodine dye. Therefore Lugol´s staining is considered to be a method to measure amylose equivalent or apparent amylose.

2.10.2 Methods

2.10.2.1 Transmitting light microscopy

- Prepper the concentrated Logulus solution by mixing one part iodine and two parts potassium iodide and store the mixture in light proof bottle in the fridge.
- For sample analysis create a diluted Logulus/water solution (50 % v/v)
- **For powder samples:** Suspend the sample in 50 % (v/v) glycerol/water and pipet the slurry on a glass slide.
- **For complete dry food product;** such a tempeh, piece of bread cut a small slither of the food sample and place it on a glass slide.
- **For wet food** simply place a drop of the sample on the glass slide.

- To emphasize the contrast and granular structure stain the starch with diluted Logulus/water solution before sealing with a cover slip.
- The starch granules should instantly change colour the intensity of colour depends on the amount of dye used and starch composition. The amylose dyes blue/purple and amylopectin pink/brown.
- Analyse the sample under bright field light.

2.10.2.2 Polarized Light microscopy, and Differential interference contrast (DIC)

- **For powder samples:** Suspend the sample in 50 % (v/v) glycerol/water and pipet the slurry on a glass slide.
- **For complete dry food product**; such a tempeh, piece of bread cut a small slither of the food sample and place it on a glass slide.
- **For wet food** simply place a drop of the sample on the glass slide.
- Analyse under polarised light, to see the "Maltese cross" caused by the birefringent properties of the starch granules.
- Analyse under Differential Interference Contrast (DIC) to emphasise the contrast and study cell structures of unstained samples

2.11 Analysis of starch granule with Scanning Electron Microscopy (SEM)

2.11.1 General principle

With SEM microscopy it is possible to get an images of the starch granules other surface with much higher magnification compared to optical microscopy (Govindaraju et al. 2021). SEM can be used for instance to study/monitor the degradation effects of α-amylase on the granule surface (Chakraborty et al. 2020) To perform SEM, samples must be solid and they must fit into the microscope chamber.

2.11.2 Methods

- Place the sample on carbon tape, mount it on a STEM stub, and sputter coat with gold.
- Analyse at 15 kW at an image size of 2560x1920 pixels.

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Chapter 5
Mono-, di-, and oligosaccharides, FODMAPs and raffinose family oligosaccharides

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Summary

- **Raw material**: Cereal- and legume-derived flours, protein isolates, and starch isolates
- **Relevant applications:** Solid (breads and other baked products), semi-solid (yogurt-type, creamy-filling, etc.), and liquid (milk-type) foods and their preparations
- **Fermentation-induced changes**: Utilization of carbohydrates for metabolism of lactic acid bacteria and yeasts, reduction in FODMAP and raffinose family oligosaccharides content
- **Analytical methods**:
    - HPLC-RI:
        - Quantification of mono- and disaccharides
    - Enzymatic digestion of FODMAPs
        - Quantification of fructans
        - Quantification of raffinose family oligosaccharides

1. Introduction

1.1 Carbohydrates

Carbohydrates are a group of molecules with chemical, physical, and physiological properties that can serve as a substrate during metabolic processes, including colonic fermentation, with consequent health implications. They are chemically classified based on the molecular size determined by the degree of polymerization (DP), linkage type, and individual monomers as detailed in Table 1 (Cummings and Stephen, 2007). However, Englyst and Englyst (2005) suggested an alternate classification of dietary carbohydrates into glycaemic (hydrolysed and absorbed in the small intestine) and non-glycaemic (enter the large intestine) carbohydrates based on their digestion in the intestine.

Table 1. Classification of carbohydrates

Class	Degree of polymerization	Subgroup	Examples
Sugars	1–2	Monosaccharides	Glucose, fructose, galactose
		Disaccharides	Sucrose, lactose, maltose, trehalose

		Polyols (sugar alcohols)	Sorbitol, mannitol, lactitol, xylitol, erythritol, isomalt, maltitol
Oligosaccharides (or short-chain carbohydrates)	3–9	Malto-oligosaccharides (α-glucans)	Maltodextrins
		Non-α-glucan oligosaccharides	Raffinose, stachyose, fructo- and galacto-oligosaccharides, polydextrose, inulin
Polysaccharides	⩾10	Starch (α-glucans)	Amylose, amylopectin, modified starches
		Non-starch polysaccharides (NSPs)	Cellulose, hemicellulose, pectin, arabinoxylans, β-glucan, glucomannans, plant gums, and mucilage, hydrocolloids

Generally, the dietary carbohydrates are converted to monosaccharides (mainly glucose, fructose, and galactose) prior to absorption in the small intestine by the enzymatic action of salivary and pancreatic amylases along with brush border disaccharidases (Wright et al., 2003). These enzymes hydrolyse α-1,4 and/or α-1,6 glycosidic linkages in di- and oligosaccharides, including starch, to release monosaccharides for absorption, and hence, are rendered digestible. However, some oligo- and polysaccharides are resistant to digestion in the small intestine, for e.g., cellulose, resistant starch, fructans, etc. These non-digestible carbohydrates (NDCs) originate due to the configuration of anomeric C atom (C1 or C2) of monosaccharide units that resists the hydrolysis of some glycosidic bonds by the digestive enzymes (Roberfroid & Slavin, 2000). These NDCs are therefore subjected to colonic fermentation by the gut microbiota, mainly bifidobacteria and lactobacilli, in the large intestine (Jandhyala et al., 2015). Moreover, some of the NDCs are also regarded as a source of dietary fibres as they promote the growth of beneficial intestinal bacteria releasing metabolites, such as short-chain fatty acids (Holscher, 2017). A higher DP and branching are associated with prolonged fermentation time in the large intestine (Hernot et al., 2009).

While the absorption of carbohydrates in the small intestine is linked to their complexity (e.g., DP, branching, etc.), it is also influenced by the type of monosaccharides. For instance, fructose absorption is slow in the presence of excess of glucose in the small intestinal lumen and polyols are only passively absorbed (Gibson and Halmos, 2020). This prolonged presence may lead to an increased water content due to the osmotic load in the small intestine that may be transferred for the colonic fermentation in the large intestine. Similar behavior is observed for the disaccharides, like lactose, in case of limited lactase activity in some individuals (Forsgård, 2019). The accumulation of carbohydrates in the large intestine may lead to physiological effects causing gastrointestinal symptoms, such as increased gas formation, distention, bloating, cramping, and diarrhea. Such carbohydrates are collectively termed as fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs). FODMAPs include slowly absorbed monosaccharides (e.g., fructose, polyols, etc.), fructans, raffinose family oligosaccharides (RFOs), and other di- and oligosaccharides not digested effectively by the brush border hydrolases. A low-FODMAP diet has been shown to exhibit a therapeutic potential for the treatment of digestive disorders, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Bellini et al., 2020; Simões et al., 2022). RFOs are galactosyl derivatives of sucrose which includes raffinose, stachyose, verbascose, and ajugose, belonging to tri-,

tetra-, penta-, and hexasaccharide groups, respectively (Vanhaecke et al., 2010). The RFOs are present abundantly in legumes and possess antinutritional properties causing indigestibility in humans upon consumption (Elango et al., 2022). Therefore, efforts are being made continuously to reduce the content of indigestible carbohydrates and accelerate the metabolism of other carbohydrates by the virtue of enzymatic digestion using enzymes secreted by beneficial microorganisms during fermentation.

1.2 Effect of fermentation on carbohydrates

Carbohydrates act as a substrate for the growth of microorganisms (e.g., lactic acid bacteria and yeasts) during the fermentation process. These microorganisms metabolize the carbohydrates (pentoses and hexoses) through different metabolic pathways that can change the physiological and functional properties of the fermented foods (Gänzle, 2015). Foods rich in starch content (e.g., cereals and legumes) have demonstrated an increase in the glucose concentrations due to the starch-hydrolysing effect of microbial maltase and α-amylase during fermentation (Osman, 2011). The microbial enzymes secreted during the fermentation process have been shown to degrade FODMAPs, including fructans and RFOs (Gänzle, 2014). Besides, the addition of sourdough during the preparation of baked goods degraded fructans and mannitol, consequently reducing the FODMAP content, by the metabolic activity of sourdough microbiota (Loponen and Gänzle., 2018). Legumes and the flours or isolates derived from them are abundant in α-galactosides (or RFOs) that are reduced by the action of α-galactosidase secreted by lactic acid bacteria or other endogenous enzymes during fermentation (Curiel et al., 2015; Nkhata et al., 2018; Galli et al., 2019). Interestingly, *in vitro* fermentation of raffinose demonstrated the prebiotic potential of this antinutritional factor by increasing the abundance of beneficial gut bacteria, including *Bifidobacterium* and *Lactobacillus* sp. (Amorim et al., 2020). Morever, the metabolic availability of NDCs to the gut microbiota has been shown to be enhanced as a result of fermentation of whole grains (Smith et al., 2020). Hence, quantification of carbohydrates plays a crucial role in determining the nutritional and functional values of the fermented foods.

1.3 Quantitative determination of carbohydrates

Simple/soluble carbohydrates, including glucose, fructose and sucrose, can be determined using refractometric or colorimetric methods. High-performance liquid chromatography (HPLC) is the most common technique used for analysing individual carbohydrates (Ma et al., 2014). Complex carbohydrates (DP > 2) can also be separated and determined using HPLC coupled with suitable columns and detectors, including refractive index detector (RID), evaporative light scattering detector (ELSD) and pulsed amperometric detector (PAD) (Biesiekierski et al., 2011). Furthermore, carbohydrates are present in foods as free and/or complex sugars along with other nutritional components, e.g., proteins, fibres, fats, etc., that requires an extraction step, such as enzymatic hydrolysis, before analysing the carbohydrates (Shi et al., 2012). Furthermore, enzymatic methods, such as hydrolysis of sucrose or phosphorylation of glucose and fructose, are also used for the quantification of specific sugars by measuring the change in pH or absorbance (Al-Mhanna, 2011). Quantification of fructans requires sophisticated analytical techniques, including HPLC, gas chromatography (GC), and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Ispiryan et al., 2019). However, a lack of suitable standards leads to semi-quantitative measurements requiring alternate methods (Muir et al., 2007). Therefore, enzymatic hydrolysis is the preferred method of choice for quantification of FODMAPs. Additionally, combination of enzymes, such as invertase, inulinase, and α-galactosidase, have been investigated for their potential to reduce the total FODMAP content in food ingredients (Atzler et al., 2020). Cereals and baked products are being continuously examined for their FODMAP profile for providing alternate solutions to IBS patients as dietary therapy (Biesiekierski et al., 2011; Ispiryan et al., 2020; Ispiryan et al., 2022). Commercial kits are now available with optimized protocols based on enzymatic digestion

for the quantification of mono-, di, and oligosaccharides as well as specific FODMAPs (fructans) and RFOs (raffinose/α-galactose) (Megazyme, Ireland).

2. Method(s) of analysis

2.1 Quantification of monosaccharides using HPLC

2.1.1 General principle

HPLC is commonly used to determine the composition of carbohydrates in diverse samples. Because of the lack of chromophores in their molecular structure, monosaccharides and oligosaccharides cannot be detected directly by absorption, limiting the modalities of detection by HPLC (Bai et al., 2015).The refractive index detector is well-suited to analyse carbohydrates but with less sensitive compared to other detection methods. As the separated components of a sample pass through the refractometer flow cell, the composition of the sample solution in the flow cell changes, the refractive index of the solution changes and consequently the light beam passing through the solution is refracted. The refractometer detects the position of the refracted light beam, generating a signal that varies from the baseline signal.

2.1.2 Reagents

- Perchloric acid
- Acetonitrile
- Mono- and disaccharides

2.1.3 Steps

2.1.3.1 Extraction

- Transform raw and fermented samples into powder using a freeze drier (Epsilon 2-6D LSC plus freeze-drier, Martin Christ, Osterode am Harz, Germany).
- Extract 2 g of samples with 20 mL of water/perichloric acid (95:5, v:v) and sonicate (amplitude 60) with a macro-probe for 1 min (2 cycles, 30 s/cycle, 5 min interval between cycles) in an ice-bath.
- Put the samples under stirring conditions at room temperature for 1 h.
- Keep the suspension at 4 °C overnight.
- Centrifuge for 10 min at 10,000 rpm.
- Filter the extracts using 0.22 µm filters and store at −20 °C.

2.1.3.2 HPLC analysis

- Run the extracts through HPLC analysis equipped with a Spherisorb column (Waters, Millford, USA) and a Perkin Elmer 200a refractive index detector.
- Set the flow rate at 1 mL min^{-1}, using acetonitrile 80% as mobile phase.
- Set the column temperature at 32 ^{o}C.
- Generate a standard curve using solutions of known mono- and disaccharides concentrations (0.1–15 g/L).
- Calculate the mono- and disaccharides concentrations using the respective standard curves, taking into account any dilution of the sample.

2.2 Quantification of fructans

2.2.1 Principle

Fructans vary in molecular structure and molecular weight and can be classified into three main types: the inulin, levan, and branched groups. The inulin group consists of carbohydrates with mostly or exclusively 2→1 fructosyl–fructose linkage as opposed to levan group with mostly or exclusively 2→6 fructosyl–fructose linkage. The branched group has both 2→1 and 2→6 fructosyl–fructose linkages in significant amounts. The method used for the quantification of fructans is optimized using multiple enzymes releasing monosaccharides that can be measured colorimentrically (K-FRUC, Megazyme, Ireland). Sucrose is first hydrolyzed by the action of sucrase (Eq. 1).

$$Sucrose + H_2O \xrightarrow{sucrase\ (pH\ 6.5,\ 30\ °C)} D\text{-}glucose + D\text{-}fructose \quad \text{(Eq. 1)}$$

Starch and maltodextrins are hydrolyzed to maltose and maltotriose by pullulanase and β-amylase followed by their hydrolysis to D-glucose by maltase (Eq. 2).

$$Starch + maltodextrins \xrightarrow[(pH\ 6.5,\ 30\ °C)]{pullulanase,\ \beta\text{-}amylase} maltose + maltotriose \xrightarrow{maltase} D\text{-}glucose \quad \text{(Eq. 2)}$$

Furthermore, D-glucose and D-fructose are reduced by sodium borohydride to the corresponding sugar alcohols, i.e., D-sorbitol and D-mannitol, respectively (Eq. 3). It is important to note that during this reaction, the D-fructosyl residue at the reducing end of FOS, in hydrolysed inulin preparations, is also reduced to the sugar alcohol (Eq. 4).

$$D\text{-}glucose + D\text{-}fructose \xrightarrow[40\ °C]{sodium\ borohydride} D\text{-}sorbitol + D\text{-}mannitol + reduced\ FOS \quad \text{(Eq. 3)}$$

$$Fructan + H_2O \xrightarrow[(pH\ 4.5,\ 40\ °C)]{exo\text{-}\ and\ endo\text{-}inulinase,\ endo\text{-}levanase} D\text{-}glucose + D\text{-}fructose \quad \text{(Eq. 4)}$$

The reduced FOS are then hydrolyzed by exo- and endo-inulinase and endo-levanase to D-glucose and D-fructose that can be measured using p-hydroxybenzoic acid hydrazide (PAHBAH) reducing sugar method (Eq. 5).

$$D\text{-}glucose + D\text{-}fructose \xrightarrow{PAHBAH\ reagent\ (100\ °C,\ 6\ min)} PAHBAH\ color\ complex \quad \text{(Eq. 5)}$$

This enzymatic assay is specific for all types of fructans, including inulin and levan groups. However, the samples containing RFOs (e.g., raffinose, verbascose, and stachyose) are removed by hydrolyzing with α-galactosidase to avoid the overestimation of fructans, and RFOs are therefore measured separately.

2.2.2 Reagents

- *Sodium maleate buffer (100 mM, pH 6.5)*: Dissolve 11.6 g of maleic acid (Sigma-Aldrich, Cat No. M0375) in 900 mL of distilled water. Adjust the pH to 6.5 using 2 M NaOH solution and bring the volume to 1 L. The buffer is stable for >3 months at 4 °C.
- *Sodium acetate buffer (100 mM, pH 4.5)*: Add 5.8 mL of glacial acetic acid to 900 mL of distilled water. Adjust the pH to 4.5 using 1 M NaOH, and then bring the volume to 1 L. The buffer is stable for >3 months at 4 °C.
- *Enzyme solution A*: Prepare a solution containing 50 mg of bovine serum albumin in 100 mL of sodium maleate buffer. Add 22 mL of this solution to the mix of enzymes provided as a freeze-dried powder containing sucrase, β-amylase, pullulanase, and maltase. The enzyme solution should be stored at –20 °C.

- *Enzyme solution B*: Add 22 mL of sodium acetate buffer to the vial containing freeze-dried fructanase powder, which is a mix of recombinant exo- and endo-inulinases and endo-levanase. The enzyme solution should be stored at –20 °C.
- *Alkaline borohydride (10 mg/mL)*: Dissolve 50 mg of sodium borohydride (Sigma-Aldrich, Cat No. 213462-100G) in 50 mM NaOH. This solution is stable only for 4–5 h at room temperature and hence should be prepared freshly on the day of the experiment.
- *PAHBAH reducing sugar assay reagent*: Prepare solutions A and B as described below.
  Solution A: Dissolve 10 g of PAHBAH (Sigma-Aldrich, Cat. No. H9882-100G) in 60 mL of distilled water in a 250-mL beaker using a magnetic stirrer. Then, add 10 mL of conc. HCl to it and bring the volume to 200 mL with distilled water. This solution is stable for approx. 2 years at room temperature.
  Solution B: Dissolve 24.9 g of trisodium citrate dihydrate in 500 mL of distilled water. Then add 2.2 g of calcium chloride dihydrate followed by the addition of 40 g of NaOH. The solution may be milky while mixing but will clarify when the volume is adjusted to 2 L with distilled water. This solution is stable for approx. 2 years at room temperature.
  PAHBAH working reagent: The working solution is freshly prepared by mixing 20 mL of solution A with 180 mL of solution B. The working reagent is stable up to 4 h on ice.

2.2.3 Sample preparation

Baked products are subjected to grinding to obtain a powdered form. Weigh 400 mg (for samples containing 0–10% fructans) or 100 mg (for samples containing 0–10% fructans) in a 50-mL falcon tube and add 25 mL of distilled water to it. Loosen the cap and place the tube in a boiling water bath for 10 min. After 5 min, tighten the tube cap and mix the contents by vortexing. Repeat the boiling step for 5 min and mix the contents again. Transfer 2 mL of the mix to a 2-mL microcentrifuge tube and centrifuge at maximum speed (approx. 12,000 rpm) for 5 min. The supernatant (or fructan extract) is further used for the quantification of fructans.

2.2.4 Preparation of controls for the experiment

- With each set of quantifications, reagent blanks and D-fructose controls should be included and analysed concurrently.
  - The reagent blank consists of 300 µL of 100 mM sodium acetate buffer with 5.0 mL of PAHBAH working reagent.
  - D-fructose controls are prepared by adding 200 µL of D-fructose standard solution (1.5 mg/mL) to 900 µL of 100 mM sodium acetate buffer. Dispense 200 µL of this solution, in quadruplicate, into glass test tubes. Add 100 µL of 100 mM sodium acetate buffer and 5 mL of PAHBAH working reagent to each tube (immediately before incubation in the boiling water bath).
  - With each set of determinations an inulin/cellulose control powder and/or a levan/cellulose control powder is included. The fructan content of these powders are given on the vial label.
- An inulin (16.2%) and levan (10.7%) control powders are included as positive controls and fructans are extracted in the same manner as for the samples.
- A sucrose/cellulose control powder is included as a positive control. If the sucrase treatment is effective, the fructan value for the control will be approx. 0.2% (w/w). An ineffective sucrose digestion will yield 10% (w/w) fructan value for the control.
- D-Fructose controls (quadruplicates) and reagent blank solutions (duplicates) are are incubated in the boiling water bath together with the samples.
- The effectiveness of borohydride reduction is checked using D-fructose standard solution (200 µL, 1.5 mg/mL).

2.2.5 Procedure

- Dispense 200 µL of fructan extract at the bottom of a glass test tube.
- Add 200 µL of enzyme solution A to it and incubate at 30 °C for 30 min.
- Then, add 200 µL of of alkaline borohydride to the tube, vortex vigorously, and incubate at 40 °C for 30 min to allow the reduction of sugars to the corresponding sugar alcohols.
- Add 500 µL of 200 mM acetic acid to the tube and vortex vigorously. An efferevescence is observed at this step, which is further used for the hydrolysis of fructans.
- Transfer 200 µL of solution from the previous step to three glass test tubes.
- Add 100 µL of enzyme solution B to two of the above glass tubes (samples) and 100 µL of 100 mM sodium acetate buffer to the third tube (sample blank).
- Cover the tubes with the caps and incubate for at 40 °C for 30 min.
- Add 5 mL of PAHBAH working reagent to all the tubes (samples, sample blanks, D-fructose standard, reagent blank, and inulin and levan control extracts).
- Incubate the tubes in the boiling water bath for exactly 6 min.
- After the incubation, immediately place the tubes in cold water (approx. 20 °C) for 5 min.
- Measure the absorbance for all the sample and control tubes at 410 nm against the reagent blank.

2.2.6 Sensitivity of the assay

The minimum absorbance value can be 0.01 units that corresponds to 12.8 µg/mL of D-glucose plus D-fructose in a sample. The absorbance values should be measured immediately as the PAHBAH color complex fades with time.

2.2.7 Calculations

Following equation is used for calculating the percentage of fructans:

$$Fructan\ (\%w/w) = \Delta A \times F \times 5 \times 25 \times \frac{1.1}{0.2} \times \frac{100}{W} \times \frac{1}{1000} \times \frac{162}{180} \times D$$

Where,

ΔA = sample absorbance – sample blank absorbance

F = factor to convert absorbance values to µg of D-fructose

= (54.5 µg D-fructose)/(absorbance for 54.5 µg D-fructose)

5 = factor to convert from 200 µL as assayed to 1 mL

25 = volume (mL) of extractant used

$\frac{1.1}{0.2}$ = 0.2 mL was taken from 1.1 mL of enzyme digest for analysis

W = weight (mg) of sample extracted

$\frac{1}{1000}$ = factor to convert from µg to mg

$\frac{162}{180}$ = factor to convert from free D-fructose to anhydrofructose

D = Dilution of the sample extract

2.3 Quantification of RFOs

2.3.1 Principle

The quantification of RFOs is based on the principle of stepwise enzymatic action of multiple enzymes (Eq. 6–8) that converts α-galactosides, including raffinose, stachyose, verbascose, and galactinol, into D-galactose with the release of NADH. The released NADH is measured by the increase in absorbance at 340 nm using a commercial kit (K-RAFGA, Megazyme, Ireland). Raffinose is the most abundant carbohydrate among all the RFOs in cereal and legume flours. While this procedure uses a commercial kit designed for the quantification of raffinose, the calculations consider all the α-galactosides (RFOs) present in the sample being analyzed.

$$raffinose + stachyose + verbascose + H_2O \xrightarrow{\alpha\text{-galactosidase}} D\text{-galactose} + sucrose \quad \text{(Eq. 6)}$$

$$\alpha\text{-D-galactose} \xrightarrow{galactose\ mutarotase} \beta\text{-D-galactose} \quad \text{(Eq. 7)}$$

$$\beta\text{-D-galactose} \xrightarrow{\beta\text{-D-galactose dehydrogenase}} D\text{-galactonic acid} + NADH + H^+ \quad \text{(Eq. 8)}$$

The formation of NADH is stoichiometric with the amount of D-galactose released from the hydrolysis of RFOs.

2.3.2 Reagents

- *Solution I*: Buffer containing sodium azide (0.02% w/v) at pH 8.6.
- Dissolve NAD^+ powder provided with the kit in 12.2 mL of distilled water and store at –20 °C until further use.
- A combined suspension of D-galactose dehydrogenase and galactose mutarotase stored at 4 °C.
- Dissolve the lyophilized powder of α-galactosidase in 12 mL of distilled water and store at –20 °C until further use. It should be used at a temperature of 25–30 °C.
- Galactose standard at a concentration of 0.4 mg/mL in 0.02% (w/v) of sodium azide.
- Weigh 0.5 g of raffinose control powder (positive control) provided at 4% (w/w) concentration and dissolve in 25 mL of distilled water. Divide the aliquots (10 mL each) and store at –20 °C until further use.

2.3.3 Sample preparation

Weigh 0.5 g of ground sample (freeze-dried ground sample in case of dough) into a 50-mL falcon tube and add 5 mL of ethanol (95% v/v). Incubate at 84–88 °C for 5 min to inactivate the endogenous enzymes. Bring the volume to 50 mL with 50 mM sodium acetate buffer (pH 4.5) and extract by shaking (200 rpm) at room temperature for 15 min. Then, transfer 5 mL of this mix to a 15-mL falcon tube, and add 2 mL of chloroform to it. Mix vigorously using a vortex for 15 s followed by centrifugation at 1500 × *g* for 10 min. This step aids in the transfer of lipids from the aqueous phase to the chloroform. The upper phase is used for the analysis of RFOs.

2.3.4 Procedure

- The assay is modified to adapt to 96-well plate format for the ease of analysis of multiple samples at once.
- A sample may contain free-galactose; therefore, for each sample, 4 different wells are considered, including RFOs with and without free-galactose along with the blank for each condition.
- Note that the raffinose provided with the kit is processed during the analysis as a sample as it is considered as a positive control.
- Add the sample and enzyme solutions as mentioned in the table below.

	Blank + RFOs + free D-galactose	RFOs + free D-galactose sample	Blank free D-galactose	free D-galactose sample
Sample solution	-	20 µL	-	20 µL
Distilled water	20 µL	-	30 µL	10 µL
α-galactosidase solution	10 µL	10 µL	-	-

- Incubate the samples for 20 min at room temperature (approx. 25 °C).
- Add 20 µL of solution I (buffer), 200 µL of distilled water, and 10 µL of NAD^+ to each of the sample and blank wells.
- Incubate the samples for 3 min at room temperature and read the absorbance (A_1) at 340 nm.
- Then, add 2 µL of the mix of D-galactose dehydrogenase and galactose mutarotase to each of the wells.
- Incubate at 25 °C for 40 min and measure the absorbance (A_2) at 340 nm.

2.3.5 Calculations

- Subtract the blank values from the samples for the respective conditions (RFOs + free D-galactose and free D-galactose) (Eq. 9).

$$\Delta A = (A_2 - A_1)_{sample} - (A2 - A1)_{blank} \quad \text{(Eq. 9)}$$

- The values of ΔA should be minimum 0.1 units to achieve efficient results.
- The concentrations of individual RFOs and free D-galactose can be calculated using the following formula (Eq. 10):

$$C = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \quad \text{(Eq. 10)}$$

Where,

V = final volume (mL)
MW = molecular weight of substance assayed (individual RFOs or D-galactose) (g/mol)
ε = extinction coefficient of NADH at 340 nm
= 6300 ($l \times mol^{-1} \times cm^{-1}$)
d = light path (cm)
v = sample volume (mL)

- The sample should be multiplied by the dilution factor, if diluted after the extraction for the assay.
- The concentration calculated for each RFO or free galactose using the above formula for solid samples should also consider the weight of the sample as follows:

$$\text{Content of RFO/free galactose (g/100 g)} = \left(\frac{\text{C [g/L sample solution]}}{\text{weight of sample [g/L sample solution]}} \times 100\right.$$

- The concentration of raffinose control powder should be in the range of 0.2 and 1.25 g/L.

2.3.6 Sensitivity of the assay

The assay can quantify beginning from an absorbance value of 0.01 units, which corresponds to 10.5 mg of D-galactose per liter of sample solution in 200 µL of sample volume. The assay is linear over the range of 4–83 µg of D-galactose per assay.

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Chapter 6
Arabinoxylan: analysis of the content, extractability and fine structure

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Summary

- **Raw material**: Cereals (wheat & oat) (wholemeal, bran)
- **Relevant applications:** dough, bread, meat and dairy alternatives, digesta
- **Fermentation-induced changes**: solubilization, debranching, release of antioxidants (e.g. ferulic acid), …
- **Analytical methods**:
  - GC-FID:
    - Total AX content and extractability
    - Degree of arabinose substitution
    - Number average degree of AX polymerization
  - SE-HPLC-RI
    - AX molecular mass distribution
  - ^{1}H-NMR
    - AX substitution pattern

1. Introduction

1.1 What is arabinoxylan?

Arabinoxylan (AX) is one of the major non-starch polysaccharides of cereals and represent an important source of dietary fiber (DF) in the human diet. AX constitutes 4.0-9.0% and 2.2-4.1% of wheat and oat kernels, respectively, and is more abundantly present in the outer layers of the kernel (i.e. the bran). The AX content of wheat and oat bran varies between 13.2-33.0% and 3.5-13.2%, respectively (Maina *et al.*, 2021). The high variability originates mainly from differences in milling methods. AX consists of a backbone of β-1,4-linked D-xylopyranosyl residues substituted with α-L-arabinofuranosyl residues at the O-2 and/or O-3 positions (Error! Reference source not found.). Other rare substitutions can be present depending on the cereal and tissue of the kernel and include the linkage of glucuronic acids, uronic acids, D-galactose, D-glucose and acetyl residues to the xylose backbone at the O-2 and/or O-3 position. Furthermore, ferulic acid can form ester linkages with the 5-OH group of terminal arabinoses and can form cross-links by formation of diferulic acid bridges (Wang *et al.*, 2020). A distinction is generally made between AX molecules which are water-extractable (WE-AX) and those which are water-unextractable (WU-AX). WU-AX are not extractable in water because they have very high molecular weights due to their involvement in covalent linkages as well as in non-covalent interactions with proteins, phenolic acids, lignin or cellulose (Iiyama *et al.* 1994). Both WE-AX and WU-AX populations have a great structural heterogeneity, with molecules varying

considerably in molecular weight, degree of substitution (as assessed by the arabinose-to-xylose ratio), and substitution pattern (Dervilly *et al.* 2000).

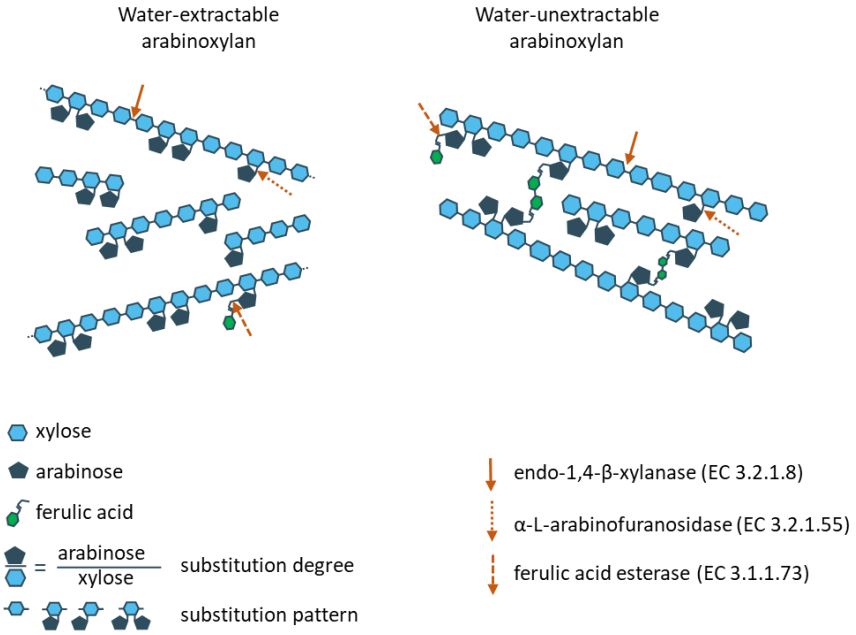

***Figure 1**. The general structure of cereal arabinoxylan and the cleavage sites for the main degrading enzymes.*

1.2 How can fermentation affect arabinoxylan?

Fermentation can affect the content, extractability and fine structure of AX. The effects are in the first place induced by enzymes, which can originate from endogeneous grain enzyme activity, enzyme activity from micro-organisms populating the outer grain kernel layers or enzyme activity from starter cultures added for fermentation (Dornez, 2009). Furthermore, microorganisms can use arabinose and xylose released by enzymes as a carbon source for their metabolism, which would result in a decrease of the AX content.

Due to the complex chemical nature of arabinoxylan, its breakdown requires several hydrolytic enzymes. Xylanase (endo-1,4-β-xylanase (EC 3.2.1.8)) is the key enzyme for depolymerization of the xylose backbone as it cleaves the internal β1-4-D-xylosidic bonds. The bonds selected for hydrolysis depends on the chain length, degree of branching and presence of substituents. Many microorganisms produce xylanases and individual microorganisms can express a multiplicity of xylanases with different characteristics. Another backbone hydrolyzing enzyme is β-xylosidase (1,4-ß-D-xyloside xylohydrolase; EC 3.2.1.37) which does not act on AX, but hydrolyzes xylooligosaccharides and xylobiose. Furthermore, different debranching enzymes exist that eliminate the side-groups. For AX originating from cereals, the most important are α-L-arabinofuranosidases (EC 3.2.1.55), which eliminate terminal arabinose residues and ferulic acid esterases (EC 3.1.1.73) which cleave between arabinose and ferulic acid side-groups (Ergün, 2016 & Bajpai, 2014 & Poutanen 1997)

The main evidence of structure changes of AX during cereal fermentations can be found in the context of breadmaking. It was shown that during breadmaking 5-20% of wheat flour AX can be solubilized. This was attributed either to the mechanical work input during mixing, the temperature increase during fermentation or the impact of endogenous and grain-associated microbial xylanases present in wheat flour (Maina, 2021). It was also shown that during sourdough fermentation, AX is solubilized and depending on the fermentation type, molecular weight (MW) of AX can decrease or remain unaffected (Maina, 2021). Furthermore, fermentation of wheat bran also increased extractability and released ferulic acid.

1.3 Implications of arabinoxylan structure on functional and bioactive properties

The fine structure of AX is very important for its functional and bioactive properties. An important **structure-function relation** is the effect of the molecular weight and degree of branching on the viscosity of AX. This is important for example in bakery products where the structure of AX affects dough rheology and therefore also bread volume, but also in liquid food systems (film forming?). Furthermore, the properties of hydrogels are directly proportional to molecular mass and ferulic acid content (He, 2021, REF).

The **bioactive** properties of AX also largely depends on its structure. AX is a dietary fibre and it is shown that the extent and rate of colonic fermentation is higher for soluble fibers than for insoluble (Verspreet, 2016). Example (REF). Furthermore, the antioxidant activity of AX is related to the substitution of ferulic acid. Finally it has been shown that the structure of AX is related to immune activity and glucose metabolism. Example (REF) (He, 2021, Chen 2019, REF).

2. Method(s) of analysis

2.1 Sample preparation

Figure 2 provides a schematic overview of the sample preparation and experimental methods needed for analyzing the content and fine structure of AX. The determination of the content, degrees of substitution and degree of polymerization can be done on dry or liquid (fermented) samples as such and on their extracts to analyse WE-AX. It might be important to inactivate cereal endogenous and micro-organism-associated xylanases to avoid enzymatic activity during the analytical procedures, but the need for this inactivation procedure should be evaluated case by case. Enzyme inactivation for dry samples could be done with a reflux in 80% v/v ethanol, while for liquid samples a heat treatment should be more efficient.

For SE-HPLC and ^{1}H-NMR analyses of WE-AX, high purity is required. To achieve this, the supernatant obtained by extraction of (fermented) wholemeal/bran is typically incubated with an α-amylase, cooled down to room temperature and centrifuged. The obtained supernatant is then diluted to 65% v/v ethanol and WE-AX is allowed to precipitate overnight and recovered by centrifugation. The reader is referred to De Man et al. (2021) for a detailed description of the method for making WE-AX isolates.

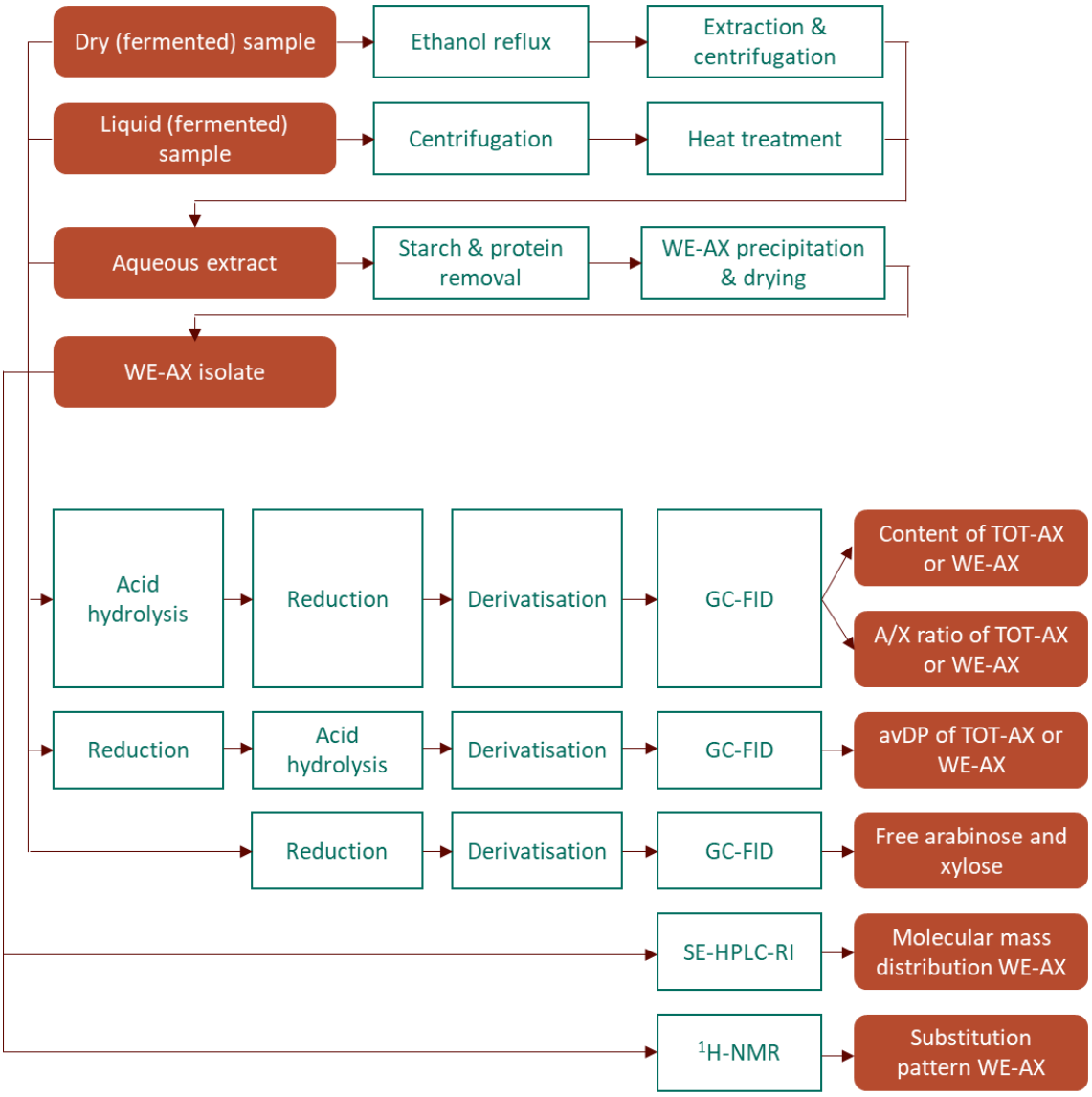

Figure 2. *Schematic overview of the sample preparation and analytical methods required for analyzing the content and fine structure of cereal arabinoxylan.*

2.2 Analysis of total, water-extractable and water-unextractable arabinoxylan content and their degrees of substitution and polymerization

2.2.1 General principle

The gas chromatography method for quantifying the total monosaccharides (MS) is based on the protocol described by Englyst and Cumming (1984). First, AX is degraded into MS through **acid hydrolysis**. These MS are then **reduced** to alditols with $NaBH_4$ under alkaline conditions. Following reduction, the samples are acidified with acetic acid to stop the reduction reaction and the resulting alditols are **derivatized** to alditol peracetates with acetic anhydride using a catalyst (1-methylimidazole) (Englyst and Cummings, 1984). The volatile alditol peracetates are transferred to a test tube, dried to remove residual water and subsequently analyzed with GC. The obtained chromatograms allow calculating the levels of WE-AX and TOT-AX as well as their degree of substitution (i.e. the arabinose/xylose ratio). Subtracting the WE-AX values from the TOT-AX values allows calculating the level and degree of substitution of WU-AX.

Reducing end xylose levels are determined as in Courtin et al. (2000) after reduction to alditols, acid hydrolysis and conversion to alditol peracetates. Doing so allows distinguishing alditol peracetates originating from reducing end monosaccharides from those derived from non-reducing monosaccharide residues, which in turn allows calculating the number average degree of polymerization (avDP) of WE-AX and TOT-AX.
To quantify free sugars, the acid hydrolysis step is omitted.

2.2.2 Reagents

- 50% (v/v) saturated benzoic acid (purity >99.5%) solution
- Internal standard solution: dissolve 100 mg allose (purity>99%) in 100 mL of the benzoic acid solution
- Calibration solution: dissolve 40 mg L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose in 100 mL of the benzoic acid solution
- 0.04% (w/v) bromophenol blue solution in dH2O.
- NH_3 (purity >25%).
- 2.0 M NH_3 (purity >25%) in dH2O.
- $NaBH_4$ (purity >96%).
- 7.5 M KOH (purity >86%) in dH2O.
- 2.0 N and 4.0 N TFA (purity >99%) in dH2O.
- 2-Octanol (purity >98%).
- Glacial acetic acid (purity >99%).
- 1-Methylimidazole (purity >99%).
- Acetic anhydride (purity >99.0%)
- Ethanol absolute (purity >99.8%).
- Ethyl acetate (purity >99.5%).
- Anhydrous Na_2SO_4 (purity >99%).

2.2.3 Acid hydrolysis (total monosaccharide protocol)

2.2.3.1 TOT-AX

- Transfer 40 to 50 mg of accurately weighed sample to a 12 ml glass centrifuge tube with a screwcap (in triplicate). In the case of WE-AX isolates, use around 15 mg.
- Add 5.0 ml TFA 2N to the samples.
- Add 2.5 mL TFA 4N to 2.5 ml of the calibration solution (see below).
- For the blanks, add 5.0 ml TFA 2N.
- Incubate the tubes for 60 min at 110 °C in a heating block and vortex the tubes before, in between and afterwards.
- Cool the samples down to room temperature.
- Filter the samples over paper.

2.2.3.2 WE-AX

- Transfer 2.5 ml aqueous extract (see section 2.2.1) or calibration solution to a 12 ml glass centrifuge tube with a screwcap (in triplicate).
- Add 2.5 ml TFA 4N to the supernatant and calibration solution.
- Add 5.0 ml TFA 2N to 12 ml glass centrifuge tube with a screwcap (in triplicate) as a blank.
- Incubate the tubes for 60 min at 110 °C in a heating block and vortex the tubes before, in between and afterwards.
- Cool the samples down to room temperature.
- Filter the samples over paper.

2.2.4 Reduction (first step in the case of reducing monosaccharide protocol!)

- **Total sugars:** transfer 3.00 ml of cooled hydrolysate into a 12 ml glass tube with screw cap, add 1.0 ml internal standard solution and vortex
- **Reducing** and **free sugars**:
    - Aqueous extracts**:** Add 2.5 ml extract (in triplo) or calibration solution into a 12 ml glass tube with screw cap, add 500 µl internal standard solution and vortex
    - Dry samples**:** weigh 40 to 50 mg sample in into a 12 ml glass tube with screw cap (in triplo), add 2.5 ml dH2O and 500 µl internal standard and vortex
    - Blanks**:** use 3.0 ml of deionised water
- Place the tubes in ice water, add 1.0 mL (**total sugars**) or 50 µl (**reducing and free sugars**) 25% NH_3 and vortex. Verify if the pH is alkaline with a pH indicator strip. If this would not be the case, add more NH_3. That more NH_3 is added in the total sugar protocol is due to the acidity of the hydrolysate.
- Add 2 droplets (**total sugars**) or 8 droplets (**reducing and free sugars**) of 2-octanol to avoid foaming in the following steps.
- Add 200 µl sodium borohydride solution (= 200 mg sodium borohydride in 1 ml 2M NH_3) and vortex.
    - Add 2M NH_3 just before using the solution
    - Sodium borohydride is very toxic and explosive
- Close the tubes but do not tighten the screws completely
- Incubate for 30 min at 40 °C in water bath.
- Gradually add 400 µl glacial acetic acid, vortex and wait at least 15 min before continuing.
    - Be careful, the reaction is exothermic and the solution can effervesce.
- The procedure can be paused here.

2.2.5 Acid hydrolysis (reducing monosaccharide protocol)

- Transfer 2.5 ml of the reduction solution to a 12 ml glass tube with screw cap.
- Add 0.5 ml concentrated TFA
- Incubate the tubes for 60 min at 110 °C in a heating block and vortex the tubes before, in between and afterwards.
- Cool the samples down to room temperature
- Filter the samples over paper

2.2.6 Peracetylation (all protocols)

- Transfer 500 µl into a 50 ml glass tube with screw cap.
- Add 500 µl 1-methylimidazole, 5.00 ml acetic anhydride and vortex. Wait at least 10 min.
- Add 1,000 µl absolute ethanol and vortex. Wait at least 5 min.
- Add 10 ml deionized water and vortex. Wait at least 5 min
- Add 500 µl 0.04% w/v bromophenol blue solution. Put the tubes in ice water.
- Add 5.0 ml 7.5 M KOH, wait a few min, and add again 5.0 ml 7.5M KOH.
- Mix the tubes by turning them upside down. Wait for at least 30 min.
- Add a small amount of anhydride sodium sulfate to a small test tube and carefully transfer the upper organic phase. Ensure that no blue phase is present.
- Transfer the solution to a GC vial.

2.2.7 Gas chromatography

- The alditol acetates can be separated on a Supelco SP-2380 polar column (30 m length, 0.32 mm internal diameter, 0.2 µm film thickness) in an Agilent 6890 Series Chromatograph equipped with autosampler and splitter injection port (injection volume: 1.0 µL; split ratio: 1:20). Detection is with a flame ionization detector. The carrier gas is helium. Separation is at 225°C, while injection and detection are at 270°C. Table 1 lists the different gasses and the

corresponding flows and inlet pressures for the analytical conditions and system configuration described above.

Table 1. Gebruers et al.

Table 1. Summary of the different gasses used for GC analysis of non-cellulosic carbohydrates with inlet pressures and flow rates.

	Gas	Inlet pressure (bar)	Flow (mL/min)
Carrier gas	He	0.73	26
Fuel gas	H_2	4	30
Oxidant gas	Dry air	6	400
Make-up gas	N_2	4	25

2.2.8 Calculations

- For each type of MS a correction factor (CF) is used to account for losses during acid hydrolysis under the conditions used. A CF is determined as ratio of the normalized peak area of a MS of a non-hydrolyzed calibration solution over the normalized peak area of a MS of an equal amount of hydrolyzed calibration solution:

$$CF = (\text{peak area } MS_{\text{non-hydrolysed}}/\text{peak area IS})/(\text{peak area } MS_{\text{hydrolysed}}/\text{peak area IS})$$

- For each type of monosaccharides (MS), a response factor (RF) is calculated based on the analysis of a calibration solution, which contains a known amount of arabinose, xylose, allose, mannose, galactose and glucose. Allose is used as internal standard (IS) to normalize all GC chromatogram peak areas. A RF is calculated as follows:

$$RF = [(\text{mg MS}/\text{mg IS})/(\text{peak area MS}/\text{peak area IS})] \times CF$$

- The MS concentrations in a sample are calculated from their normalized GC peak areas and corresponding RF as follows:

$$[MS]\ (\%) = (\text{peak area MS}/\text{peak area IS}) \times RF \times (\text{mg IS}/\text{mg dry sample}) \times 100$$

- The levels of WE-AX, TOT-AX and WU-AX and their A/X ratios and number average degrees of polymerization are calculated as follows:

$$[WE\text{-}AX]\ (\%) = ([ara]_{WE} + [xyl]_{WE} - B) \times 0.88$$
$$A/X_{WE\text{-}AX} = ([ara]_{WE} - B)/[xyl]_{WE}$$
$$[TOT\text{-}AX]\ (\%) = ([ara]_{TOT} + [xyl]_{TOT} - B) \times 0.88$$
$$A/X_{TOT\text{-}AX} = ([ara]_{TOT} - B)/[xyl]_{TOT}$$
$$[WU\text{-}AX]\ (\%) = [TOT\text{-}AX] - [WE\text{-}AX]$$
$$A/X_{WU\text{-}AX} = ([ara]_{TOT} - [ara]_{WE})/([xyl]_{TOT} - [xyl]_{WE})$$
$$avDP\ TOT\text{-}AX = ([ara] + [xyl] - B) / [xyl]_{\text{reducing end}}$$
$$avDP\ WE\text{-}AX = ([ara]_{WE} + [xyl]_{WE} - B) / [xyl]_{WE,\ \text{reducing end}}$$
$$avDP\ WU\text{-}AX = avDP\ TOT\text{-}AX - avDP\ WE\text{-}AX$$

- with $B = 0.7 \times [Gal]_{WE}$, a correction factor for the arabinose which originates from arabinogalactan peptide, which is water extractable. This correction can be done for flours from spring and winter wheat, durum wheat, barley, rye, dicoccum, monococcum and spelt. No correction is used for flour from oat, bran and whole meal samples because in these samples the galactose present cannot be solely attributed to arabinogalactan peptide.

2.3 Analysis of apparent molecular mass distribution of water-extractable arabinoxylan and its substitution pattern

2.3.1 General principle

The apparent molecular weight distribution of WE-AX is determined with High Performance Size Exclusion Chromatography (HPSEC). A sample is injected onto a column, containing fine and porous beads, that is flushed with a mobile phase. Small molecules will migrate into the pores resulting in larger retention times, while large particles do not enter the beads and therefore reach the column end faster.

Liquid-state ^{1}H nuclear magnetic resonance (NMR) spectroscopy allows characterizing branching patterns in AX by assessing H-1 peaks of xylose and arabinose residues (Rainer A. Hoffmann et al., 1992). Spectroscopic measurements are based on the interactions between atomic nuclei and an external magnetic field. These allow to obtain detailed information on molecular structure of target constituents (De Man, 2022). Hoffmann, Kamerling, et al. (1992) provided a detailed identification of 1H resonances in wheat endosperm WE-AX by combining 1H NMR spectroscopy with methylation analysis and monosaccharide identification (Figure 3).

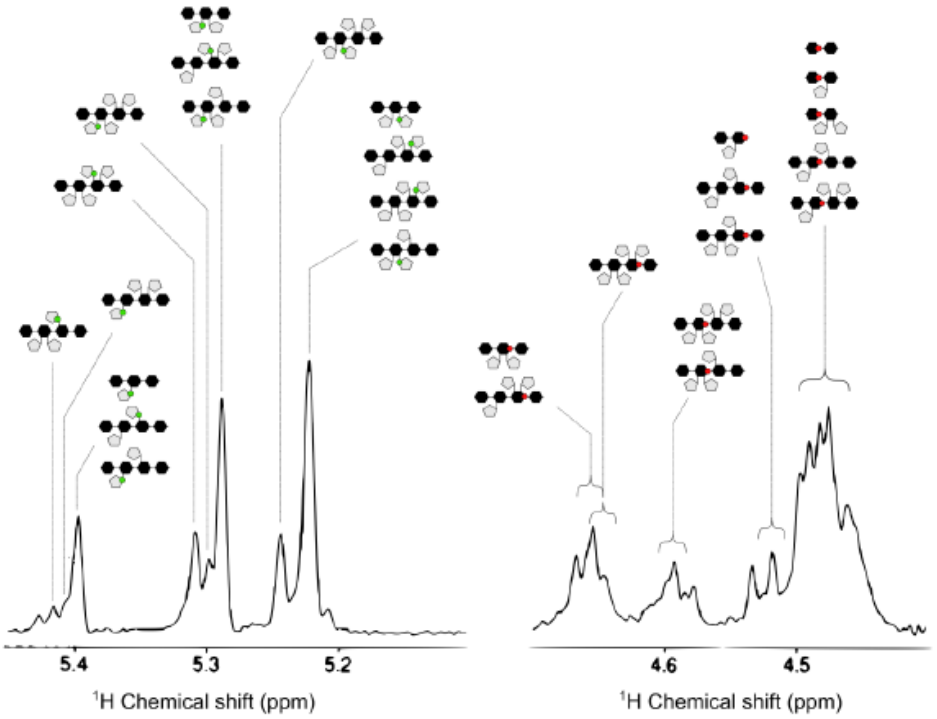

Figure 3. *Detailed regions of ^{1}H NMR spectrum of wheat endosperm water-extractable arabinoxylan, illustrating the H-1 spectral resonances of arabinofuranosyl (δ 5.2 – 5.4 ppm; ; green dots) and xylopyranosyl (δ 4.4 – 4.7 ppm; ; red dots) residues [adapted from De Man (2022) and Hoffmann, Kamerling et al. (1992)].*

2.3.2 High Performance Size Exclusion Chromatography (HPSEC)

50.0 µl samples containing 5.0 mg AX in 1.0 ml 0.3% NaCl were injected (SIL-HTc Auto sampler, Shimadzu, Kyoto, Japan) on a Shodex (Showa Denko KK, Tokyo, Japan) SB-806 HQ column with a Shodex SB-G guard column (50 mm x 6 mm). Elution (0.5 ml/min) with 0.3% NaCl was with a modular Shimadzu SIL-HTC unit equipped with a LC-20AT pump, a DGU-20A5 degasser, an RID-10A detector operating at 40 °C and a column oven (CTO-20A, Shimadzu) at 30 °C. Shodex P82 pullulan standards (MWs 342, 1.32 x 10^3, 6.2 x 10^3, 10 x 10^3, 23 x 10^3, 48.8 x 10^3, 133 x 10^3, 200 x 10^3, 348 x 10^3, 805 x 10^3, and 1,600 x 103 g/mole; 1.0 mg/ml in 0.3% w/v NaCl) were injected for calibration purposes.

2.3.3 ^{1}H-NMR

- A sample containing 10.0 mg of AX was exchanged twice with 3.0 mL of deuterium oxide (D_2O), followed by intermediate and final freeze-drying.
- Redissolve the freeze-dried sample in 1.0 mL D_2O.
- Record ^{1}H NMR spectrum in a nonspinning 5 mm NMR tube. An 800 MHz Bruker spectrometer (Bruker Biospin, Ettlingen, Germany) at 333 K operated by an AVANCE NEO console can be used. The spectrometer, located in a temperature-controlled room (22 °C, maximum fluctuation 0.1 °C over a 24 h time span) with a vibration-resistant floor, was equipped with a 5 mm multinuclear Bruker BBO probe (1H/2D/X) with active shielding and a maximum gradient strength of 4.45 G/mm. 1H NMR spectra were referenced at 0 ppm, using sodium trimethylsilylpropanesulfonate (DSS) (0.0173 ppm) as a secondary reference. All 1D spectra were obtained with 64 scans, 20 ppm sweep width, 24 192 complex data points, and active suppression of residual solvent at 4.363 ppm (HDO shift at 333 K). Exponential line broadening (0.3 Hz), automatic phase correction, baseline correction, and integration were performed using Bruker Topspin 4.0.6 software.
- The spectral ranges should be assigned to arabinose O-3 mono-substitution (Mono), O-2 di-substitution (O2di), and O-3 di-substitution (O3di). This can be done by ^{1}H-^{1}H COSY measurements as described by De Man *et al.* 2021.

2.3.4 Calculations

The ratio of di-substituted to mono-substituted xylose (D/M) can be calculated as:

$$\mathrm{D/M} = \frac{0.5 \text{ x } (\mathrm{O2di} + \mathrm{O3di})}{Mono} \times 100$$

Combining the this calculation with the A/X of a given sample allowed calculating the relative proportions of unsubstituted, mono-substituted and di-substituted xylose (uXyl, mXyl, dXyl) as follows:

$$mXyl\ (\%) = \frac{A/X}{1 + (2\ X\ D/M)} \times 100$$

$$dXyl\ (\%) = mXyl \times D/M$$

$$uXyl\ (\%) = 100 - mXyl - dXyl$$

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HealthFerm

Chapter 7
Cereal β-D-Glucan: quantification, extractability, fine structure, molecular weight distribution

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Summary

- **Raw material:** mostly oat and barley, minor in rye, very little in wheat.
- **Relevant applications:** dough, bread, dairy- and meat-alternatives, digesta
- **Fermentation-induced changes:** molecular weight reduction, solubilization
- **Analytical methods:**
  - Enzymatic assay kit + colorimetry:
    - Total cereal β-glucan content
    - Extractability
  - Lichenase treatment + HPAEC-PAD:
    - DP3/DP4 ratio
    - For total vs. water-extractable β-glucan
  - BG isolation + SEC-RID (& light scattering)
    - Weight-average (M_w) & number-average molecular weight (M_n), dispersity ($Đ = M_w/M_n$)

1. Introduction

1.1 Cereal β-glucan (BG): structural features, functionalities, and extraction

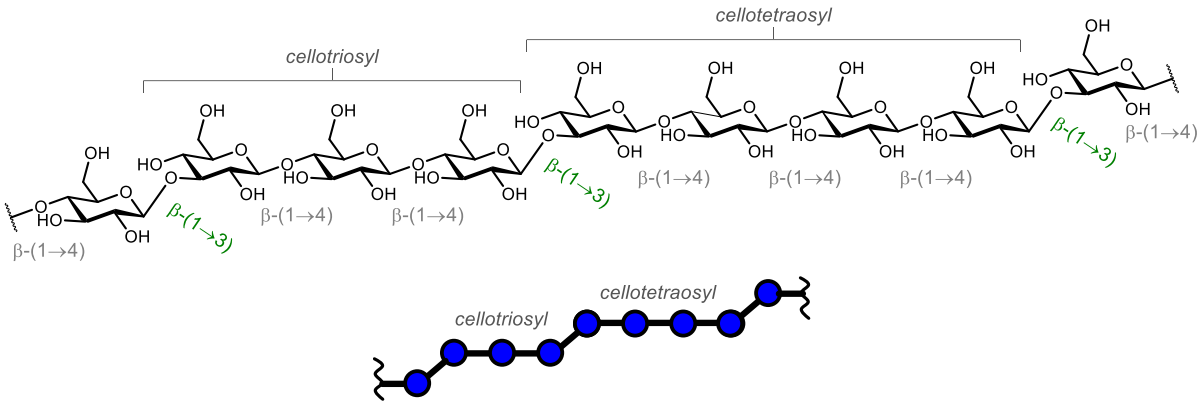

Figure 1. *Chemical structure of mixed-linkage cereal β-glucan (BG) comprised mainly of cellotriosyl and cellotetraosyl units β-(1→3)-linked as indicated in the figure above. The bottom figure is the symbolic representation of the same BG polysaccharide, with β-(1→4)- and β-(1→3)-linkages drawn as horizontal and angled bonds, respectively. The fine structure of BG, defined as the cellotriosyl to cellotetraosyl ratio (DP3/DP4) depends on the source of BG. Blue symbols represent Glc units.*

Cereals are the main source of dietary fibers in the human diet (EFSA, 2010). The two major cereal dietary fibers are arabinoxylans (see **Chapter 8**) and mixed-linkage (1→3,1→4)-β-D-glucans (BG) (Torbica et al., 2022). BG is a high molecular weight (Mw), partially water-soluble, linear homopolysaccharide composed mainly of cellotriosyl (degree of polymerization 3 (DP3)) and cellotetraosyl (DP4) units, linked through β-(1→3)-bonds (**Figure 1**). This fiber is most abundantly found in oat and barley (3–11% content, see **Table 1**), and in lower levels (≤2%) in other cereals, such as rye and wheat (Schmidt, 2022). Depending on the cereal source, the chemical structure of BG can vary in terms of DP3/DP4 ratio. For example, the DP3/DP4 ratio is lower in oat (1.5–2.4) than in barley BG (2.3–3.4; **Table 1**) (Burton & Fincher, 2012; Cui & Wood, 2000; Schmidt, 2022). This difference in DP3/DP4 ratio influences the gelation characteristics of BG: the higher the proportion of DP3 (cellotriosyl) units, the faster the gelation (Wood, 2007). Therefore, gelation velocity is in the order of wheat BG > barley BG > oat BG, when comparing materials of the same Mw and at the same concentration (Lazaridou et al., 2004).

Table 1: *Mixed-linkage β-glucan (BG) contents, molecular weight (Mw), and fine structure (molar DP3/DP4 ratio) for BG from the most relevant cereal sources. Reproduced with adaptations from a review by Schmidt* (2022).

Cereal	β-Glucan content [%]	Mw [kDa]	DP3/DP4 ratio
Barley	5.0–11.0	400–2500	2.3–3.4
Oat	3.0–7.0	400–2500	1.5–2.3
Rye	~2.0	up to 1130	2.4–2.7
Wheat	~0.5	210–420	3.0–4.5

Another important characteristic of BG is its capability to form viscous solutions due to its high M_w (for Mw ranges, see **Table 1**) (Regand et al., 2009; Wolever et al., 2010). The BG chemical structure and its ability to form viscous solutions has been linked to various technological and health promoting

properties (Daou & Zhang, 2012; Lazaridou & Biliaderis, 2007). Among various health promoting properties, the most recognizable ones are:

1) reduction of blood cholesterol levels in hypercholesteremic individuals and attenuation of blood glucose (EFSA, 2010, 2011; FDA, 2009);
2) positive effects on satiety (Barone Lumaga et al., 2012; Pentikäinen et al., 2014);
3) and immunomodulatory properties (Estrada et al., 1997), with indications that it acts favorably against certain cancers (Choromanska et al., 2015) of the digestive system (Daou & Zhang, 2012).

In addition, it has been shown that regular intake of food products containing cereal BG is among one of the most effective natural ways to reduce the risk for cardiovascular diseases and diabetes II (Patel, 2015). It should be noted that the positive effects of BG on digestion are less dependent on its viscosity/ high Mw. In this regard, BG can display pre- and probiotic effects (Schmidt, 2022). BG can be a fermentable substrate to the gut microbiota, resulting in the release of short chain fatty acids (SCFA) (Bai et al., 2021; Sayar et al., 2007), which play an important role in the gut and metabolic health, being positively involved in the control of obesity, insulin resistance, and type 2 diabetes (Blaak et al., 2020; Shoukat & Sorrentino, 2021).

The solubilization of BG from cereals is important to optimize their use in food applications. The extractability of native BG depends on the extraction conditions (e.g. solvent, temperature, pH, time, particle size, and fat content of the sample) (Maheshwari et al., 2017; Schmidt, 2022). The most commonly used extraction type with minimal depolymerization is based on the solubility of BG in hot water, with or without concurrent presence of enzymes to digest starch. When performing extractions under physiological conditions (*in vitro* digestion at 37°C) as reported by Beer et al. (1997), the extractability of BG was low and varied between 13–28% for three tested oat brans (previously heat-treated for enzyme deactivation). Hot water extractions at 90°C/2 h in the presence of termamyl, on the other hand, lead to 51–64% extractable BG on the same oat bran raw materials – a marked increase compared to the *in vitro* digestion at 37°C. In that study, both extractions methods lead to BG of similar Mw (~1–2 MDa).

Cereal BG exist as high Mw polymers in the cereal cell walls, tangled together physically or linked chemically to other components (Lazaridou & Biliaderis, 2007). This might limit its potential functional contribution in liquid and semi-solid plant-based foods. In addition, it is worth noting that cereals, in particular oats, are often heat-treated (or kilned) (Maina et al., 2021), inactivating endogenous β-D-glucanases (Ames et al., 2015; Andersson et al., 2004; Rieder et al., 2015), which would otherwise assist in increasing water-extractability by partial hydrolysis, releasing more BG, though potentially of lower Mw as a consequence. Besides heat, the pH of the extraction also determines the activity of endogenous β-D-glucanases (Gangopadhyay et al., 2015). Extraction of BG from barley at pH 8.0 led to inactivation of β-D-glucanases, and consequent extraction of high Mw BG polymers.

1.2 Influence of fermentation on the molecular structure of cereal BG

Fermentation processes used to develop new cereal-based fermented foods containing BG can lead to modification of the physicochemical and structural properties of native BG. Some studies have investigated the influence of fermentation on the molecular properties of BG (Angelov et al., 2006; Degutyte-Fomins et al., 2002; Lu et al., 2019). For example, Lu et al. (2019) investigated the effect of fermentation (0 up to 12 h, 30 °C), using lactic acid bacteria (LAB; *Lactobacillus plantarum*), on the physicochemical properties of BG in oat sourdough. These authors verified that the total BG content decreased (by around ~13%) after 12 h of fermentation, which was assigned to be caused by LAB growth, since oat endogenous β-glucanases were deactivated by heating before fermentation. Regarding soluble BG, it varied to some degree throughout the fermentation process, fluctuating between around 40% water-soluble BG (extractability relative to total BG at t = 0 h). In the first 4 h of fermentation, the soluble BG content did not change significantly (~38% extractability). From 4–8 h fermentation, the soluble BG increased (statistically significantly to ~45%), likely due to solubilization

of insoluble BG by β-glucosidases produced by LAB, while the LAB population stayed stable (lag phase). After 8–12 h of fermentation, the soluble BG amounts decreased again down to ~40% BG extractability, due to the consumption of BG by LAB for their own propagation. The observed Mw profile was in accordance with the hypothesized behavior of LAB, i.e. the proportion of high Mw BG (> 100 kDa) decreasing from initially ~44% (t = 0 h) down to ~31% (t = 12 h). It is evident that there are two processes during fermentation that influence the amount of extractable/soluble BG and its Mw, namely on one hand the release of relatively high Mw BG from the matrix, e.g. through partial enzymatic hydrolysis of insoluble BG, increasing the soluble BG content and keeping Mw high, and on the other hand, the degradation and consumption of already soluble BG as food for the microorganisms, reducing both the Mw as well as the contents of soluble and total BG. Depending on the relative rate of these two processes, soluble BG contents and Mw can be increasing or decreasing during fermentation.

In another sourdough fermentation study also with a *Lactobacillus plantarum* strain, Rieder et al. (2012) observed the Mw reduction as a function of sourdough fermentation time, going from initially ~550 kDa for soluble BG in various barley flours down to 115–452 kDa and 50–112 kDa after 30 min and 18 h sourdough fermentation, respectively. With (heat treated) oat bran, the observed Mw of the soluble BG fraction went from 630 kDa in the bran to 546 kDa and 488 kDa after 30 min and 18 h sourdough fermentation, respectively. This maximally 23% relative reduction in Mw during sourdough fermentation of oat bran can be attributed to the fermentation process itself, in contrast to the untreated barley flours with assumed high endogenous β-glucanase activity, where it is harder to distinguish what effect the endogenous enzymes had vs. the added LAB. BG extractability was not determined after fermentation in this study, but is assumed by the authors to be also increasing, as this would explain some of their observations.

An important factor determining fermentation and the influence of fermentation on the BG structure is the type of microorganisms involved during fermentation (Bai et al., 2021; He et al., 2022). He et al. (2022) studied the effect of five different LABs (*Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus bulgaricus* and *Streptococcus thermophilus*) on oat fermentation, verifying that the highest content in BG was retained when *S. thermophilus* was used in the fermentation process.

All in all, this shows that endogenous (when not previously deactivated) and microbial enzymes are key in modifying cereal BG properties (i.e. Mw, solubility, and total content) during fermentation. These molecular changes might alter the functional, nutritional and sensorial properties of foods (Singh et al., 2012). To date, to the best of our knowledge, no studies have investigated the influence of fermentation on the DP3/DP4 ratio of BG after fermentation. Although it is expected that this ratio will not be significantly affected by such enzymatic hydrolysis, depending on which parts of BG are solubilized via enzymes, the DP3/DP4 ratio of the water-extractable fraction might change. Hence, analytical procedures for characterizing fermentation-induced changes in the molecular structure of BG are described here, including proposed modifications of established methods.

2. Method(s) of analysis

The main methods and sample preparations described in this chapter are (see **Figure 2** for the overview):

- The quantification of total cereal BG content in fermented samples via the Megazyme (K-BGLU) McCleary Assay Kit, with the appropriate adjustments of the original methods for the low Mw BG that can be observed in enzymatically treated/fermented samples. This kit is also used for the determination of BG extractability.
- Analysis of the DP3/DP4 ratio of BG after lichenase treatment, via HPAEC-PAD.
- Isolation of BG (→ BG extract) & molecular weight distribution analysis by SEC-RID (& light scattering/viscosity detectors).

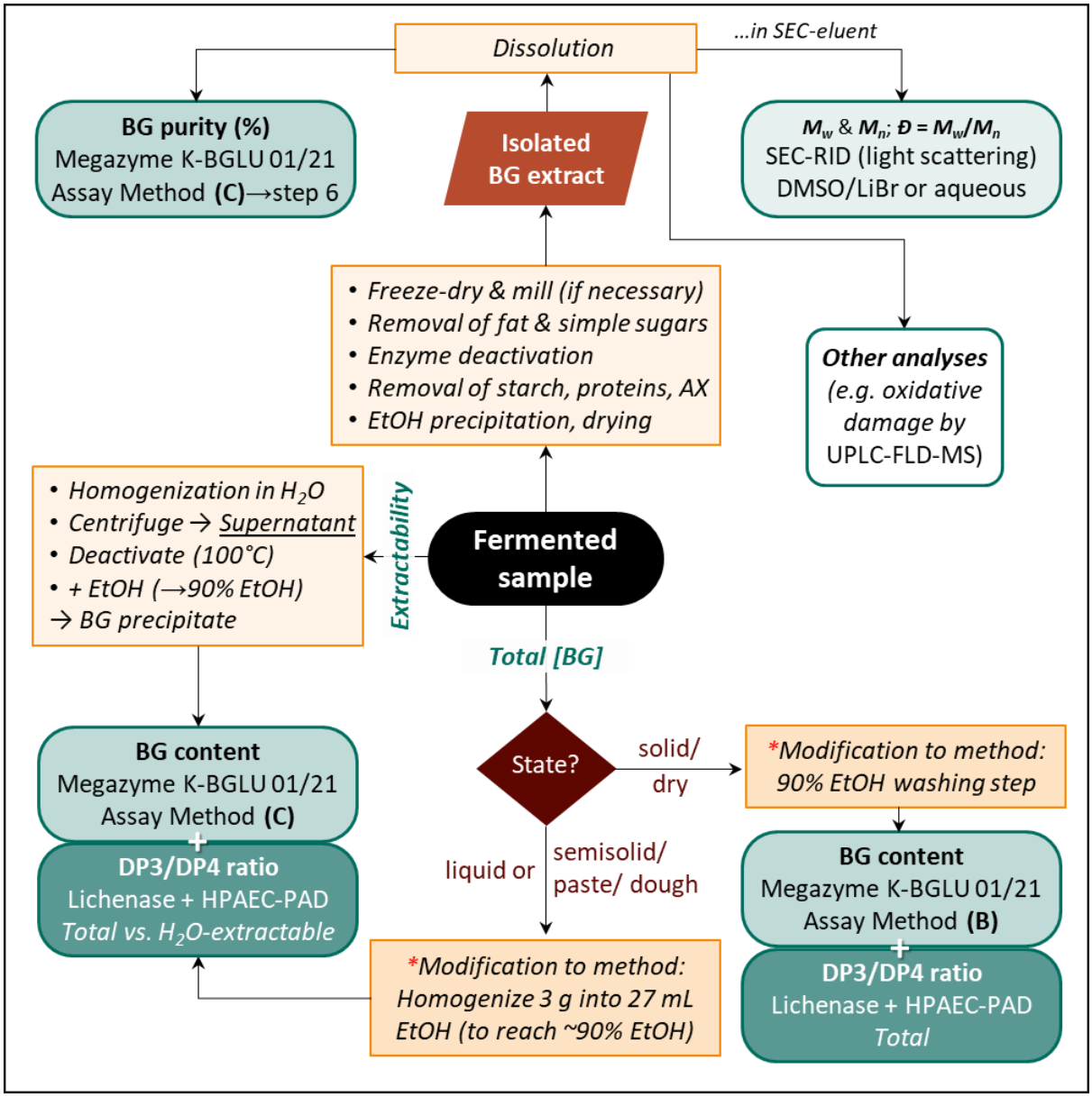

Figure 2. *Schematic overview of sample preparation steps and analytical methods for cereal β-D-glucan (BG), starting with the fermented sample in the middle. *, Modifications to the standard Megazyme K-BGLU method (Version 01/21) for BG quantification, introduced to minimize loss of small-MW BG, while still allowing removal of glucose that could interfere in the assay.*

Two of the main methods presented, the quantification of BG and the fine structure (DP3/DP4) analysis, rely on the specific action of the enzyme lichenase to produce BG oligosaccharides with characteristic β-(1→3)-linked reducing end units (see **Figure 3**). These are then fully hydrolyzed to glucose with β-glucosidase in the BG quantification method.

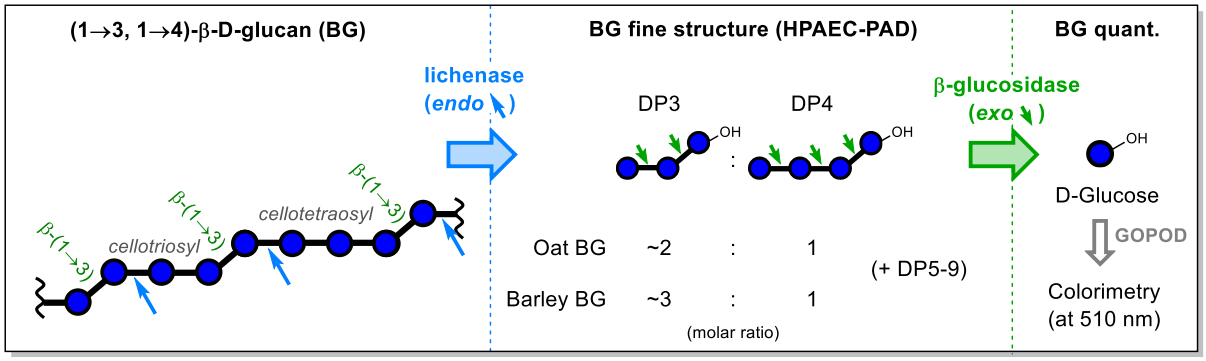

Figure 3. *Symbolic representation of the BG polysaccharide (on the left), most commonly found in oat and barley, as well as the impact the enzyme lichenase has on it, important for BG quantification and fine structure analysis. Lichenase (step i) hydrolyzes BG specifically at the β-(1→4)-linkage of a β-(1→3)-linked glucose unit (indicated with), releasing oligosaccharides with degree of polymerization (DP) 3 and 4 predominantly (plus small amounts of DP5–9). The DP3/DP4 ratio is the so-called fine structure of BG, which is characteristic depending on the source. The action of β-glucosidase (step ii) fully hydrolyzes these oligosaccharides to glucose, which can be quantified by means of an assay using glucose oxidase + peroxidase and 4-aminoantipyrine (GOPOD reagent; step iii).*

Equipment

- Analytical scale (0.1 mg accuracy)
- Adjustable volume pipettes (20–200 µL, 100–1000 µL, 0.1–5.0 mL, 1.0–10.0 mL)
- Shaking water bath/ shaking dry incubator
- Heating plates with large beakers for boiling water baths
- Laboratory oven
- Vortex mixer
- Centrifuge
- Spectrophotometer set at 510 nm
- HPAEC-PAD with analytical Dionex CarboPac PA1 (4 mm × 250 mm) with a CarboPac PA1 guard column (3 mm × 50 mm)

2.1 Quantification of BG – the Megazyme McCleary Method (Colorimetric)

The colorimetric McCleary method using the Megazyme Assay Kit K-BGLU – established for the reliable and specific quantification of mixed-linkage [(1-3),(1-4)]-β-D-glucan in oat, barley, malt, wort, and beer – is a recommended standard method (AOAC Official Method 995.16, AACC (Method 32-23), ICC (Method No. 168)). It can also be used for fermented samples. The kit's documentation (K-BGLU version 01/21) has several methods described depending on the sample, with the most relevant ones being (for suggested adaptations, see section **2.1.2**):

- Method **(A)** for "barley/ oat flours and fiber samples" (standard method).
- Method **(B)** for "cooked, toasted, or extruded cereal products" – most suitable for fermented solid samples, and includes an aqueous ethanol washing step to remove free sugars and fat.
- Method **(C)** for "milkshake, yogurt and other liquid samples", including an alcohol precipitation step to remove free sugars and fat – most suitable for plant-based fermented semi-solid samples.

2.1.1 Method principle & explanation of the various steps:

2.1.1.1 Total BG quantification

The method is based on the selective hydrolysis of cereal BG through the highly purified enzyme lichenase, which hydrolyzes BG specifically at the β-(1⟶4)-linkage of the C3-substituted glucose units in the chain (**Figure 3** step *i*). Hence, cellulose, for instance, which is also a type of β-glucan, but perfectly linear with only β-(1⟶4)-linkages, is not hydrolyzed, allowing the selective release of BG-oligomers from samples that also contain cellulose and other polysaccharides. This enzyme also helps in releasing cereal BG quantitatively from the milled sample matrix, including the water-insoluble portion of cereal BG that otherwise would stay in the solid matrix. These enzymatically released BG-oligomers (mostly DP3 and DP4, with 5–10% DP5–9) are water-soluble. Hence, a centrifugation step allows removing water-insoluble constituents, including among others cellulose, starch, and arabinoxylan. This is critical, as the next step is using the supernatant to treat with β-glucosidase to hydrolyze the BG-oligomers (DP3–9) to glucose (**Figure 3** step *ii*), and this enzyme is not selective for cereal BG anymore but would also slowly degrade β-linked polymers other than cereal β-glucan such as cellulose (structurally also a β-glucan). In the last step, the resulting glucose is treated by the GOPOD reagent, which is a reagent mixture that enzymatically oxidizes glucose, producing gluconic acid and H_2O_2, with the latter reacting with 4-aminoantipyrine to produce the purple/pink color of the assay, allowing spectrophotometric quantification at 510 nm.

For this assay to allow for accurate quantification of cereal BG, the initial content of glucose (or disaccharides/ oligosaccharides that can produce glucose by the action of β-glucosidase) has to be rather low. Although a sample reaction blank is also measured after the lichenase step when working with the supernatant, which allows for correction of smaller residual amounts of already present glucose, some enzymatically pretreated and fermented products may contain even more free glucose than BG. Especially for these types of samples, an aqueous ethanol washing step at the very beginning (as in Megazyme's Method **(B)** & **(C)**) may be necessary to remove free sugars, and hence, for reasons of simplicity, comparability, and uniformity, is suggested here to generally be applied for all fermented samples and their controls.

2.1.1.2 BG Extractability

The water-extractability of cereal β-glucan is here defined as the percentage of total BG contained in the same sample that can be solubilized when mixed in a 1:20 ratio of solids to water, during 2 hours at 40°C under shaking. Ideally, extractability of cereal BG is determined directly in a sample "as is", and not after freeze-drying, as freeze-drying can change the properties of BG including extractability.

However, if all samples are treated the same way, determination of BG extractability from freeze-dried samples can still allow comparisons between samples.
While the time for extraction in this extractability determination is kept constant (2 h at 40°C; the same as for preparing BG extract isolates described in section **2.3.2**), what has to be taken into account experimentally when comparing the effect of fermentation on BG solubilization, is the fact that fermentations typically take place for several hours, leading to solubilized BG just due to this contact time with water, irrespective of the fermentation. Hence, a proper control, to be able to assess what effect the fermentation alone has on the BG solubilization (and not just the time in contact with water), would be to incubate the material under the same conditions as the fermentation but without starter cultures, and instead, adding antibiotics to inhibit spontaneous fermentation. A suitable recipe for this purpose would be chloramphenicol (as antibacterial agent) and cycloheximide (as antimycotic) both at 0.01% (w/w) (Arte et al., 2015).

2.1.2 Summary of adaptations for Megazyme K-BGLU McCleary methods

The main modifications of the original Megazyme methods (A), (B), and (C) are summarized here, largely to minimize the loss of low Mw BG in the case of enzymatically pre-treated/fermented samples:

- For samples of high BG content (oat bran concentrate, isolated BG extracts): longer dispersion/heating times and double the amount of lichenase (Method **(A')** in section **2.1.3**).
- For fermented solid samples and their controls: use of higher ethanol concentrations, namely 90% (v/v) aq. EtOH instead of only 50% in Method **(B)** (section **2.1.4**).
- For semi-solid samples: adding pure EtOH to reach ~90% (v/v) EtOH instead of only reaching ~70% for the precipitation in Method **(C)**, as well as washing with 90% aq. EtOH (section **2.1.5**).
- Modification of the respective procedure to allow for BG extractability determination, namely including a centrifugation step and working with the supernatant (sections **2.1.4** and **2.1.5**).

2.1.3 Complete procedure for the standard total BG Method (A)

Here, the complete standard procedure for total mixed-linkage BG content for oat or barley flours is presented step by step, including some additional details that go beyond Method **(A)** described in Megazyme's K-BGLU (01/21) booklet, and some minor adjustments. Some alterations for samples with high BG contents (oat bran concentrate, BG extracts) based on Zielke et al. (2017) are also presented as Method **(A')**.
Controls: Megazyme control flours from oat and barley that are part of the K-BGLU kit, with indicated BG content on the bottle. Measure at least one of them with each batch.
Reagents, buffers, solutions: Prepare according to the booklet of the Megazyme K-BGLU kit.

Method (A): *For unfermented, dry, milled samples with max. 10% BG content:*

(1) If necessary, mill sample to pass through a 0.5 mm screen.

(2) Accurately weigh milled sample (~100 mg), e.g. in Falcon tubes (15 mL size) in triplicate.

(3) Add 0.4 mL 50% (v/v) aqueous EtOH to wet the sample and aid dispersion. Then, add 5.0 mL phosphate buffer (20 mM, pH 6.5) in a fast stream pointing at the sample for instant mixing,

cap tube immediately, and vortex mix until fully dispersed (if necessary, shake manually until all lumps disappear).[1]

(4) Then, place the tube with dispersed sample right away in a boiling water bath for 60 s, vortex mix vigorously, and repeat two times with 2 min incubations for a total of 5 min boiling time (for glass tubes, a total of 1 min + 2 min = 3 min suffices).[2]

(5) Place tubes in a shaking water bath at 50°C, allow to equilibrate for 5 min.

(6) Add 200 µL lichenase solution (50 U/mL in 20 mM phosphate buffer; 10 U), cap and vortex mix. Incubate for 1 h at 50°C in shaking water bath, with regular vigorous vortex mixing every 15–20 min (or continuous stirring if such a device is available).

(7) Add 5.0 mL sodium acetate buffer (200 mM, pH 4.0), vortex mix vigorously.

(8) Keep tubes at room temperature for 5 min, then centrifuge (10 min at >3000 x g). For each replicate sample tube, carefully dispense 100 µL aliquots of the supernatant into the bottom of three new Falcon tubes (15 mL size; tubes #1, #2, and #3).

(9)
a. Samples:
- The reaction: to tubes #1 and #2, add 100 µL β-glucosidase solution (2 U/mL in 50 mM sodium acetate buffer pH 4.0; 0.2 U).
- The reaction blank: to tube #3, add 100 µL sodium acetate buffer (50 mM, pH 4.0).

b. Calibration standard: in new Falcon tubes (15 mL size), mix (in quadruplicate) 100 µL D-glucose standard (1.0 mg/mL) + 100 µL sodium acetate buffer (50 mM, pH 4.0).

c. Reagent blank: in a new Falcon tube (15 mL size), mix 100 µL water + 100 µL sodium acetate buffer (50 mM, pH 4.0).

(10) If some drops of solution happen to be on the tube walls instead of the tube bottoms, centrifuge tubes briefly to bring everything down. Then, incubate all these tubes at 50°C in a shaking water bath for 10 min.

(11) Add 3.0 mL GOPOD Reagent to each tube (including standards and blanks) and incubate at 50°C for a further 20 min.

(12) Take tubes out of the water bath and measure the absorbance at 510 nm within 1 h (zero spectrophotometer using the prepared reagent blank).

To calculate the β-glucan content from the absorbance readings, use the "Mega Calc" spreadsheet available on the Megazyme website (https://secure.megazyme.com/Beta-Glucan-Assay-Kit). Enter the measured absorbances (three values for each replicate: the reaction from tubes #1 & 2, and the reaction blank from #3), the exact Sample weights (~100 mg), Sample volume = 0.1 mL, Dilution (-fold) = 1, and Extract volume = 10.6 mL for all samples.

[1] Original Megazyme method uses 0.2 mL aq. EtOH and 4 mL phosphate buffer, however, experience showed that 0.2 mL is rarely enough to fully wet the flour sample (→ 0.4 mL), and using slightly more phosphate buffer (5 mL instead of 4 mL) aids in the dispersion, especially in cases of high BG contents.

[2] If there is waiting time in between steps, ensure that the tubes are regularly vortex mixed to avoid clumping.

Method (A'): *For oat bran concentrates or BG extracts with >10% BG content,* the following modifications apply to Method **(A)** for the indicated steps (based on Zielke et al. (2017))[3]

- In Step (2): use only ~35 mg (for 10–35% BG) or ~25 mg (for ≥35% BG).
- In Step (3): additionally, add a magnetic stirring bar to each tube.
- New Step (4): heat in boiling water bath under stirring for 2 h, with regular manual vigorous shaking and vortex mixing every ½ h. For BG extracts, the sample has to fully dissolve before continuing.
- In Step (6): double the added lichenase amount and incubation time at 50°C (→ 400 µL, 2 h).
- New Step (7): Add 5.0 mL sodium acetate buffer (200 mM, pH 4.0), vortex mix vigorously. Then, transfer whole content, including any solids, to a 50 mL Falcon tube with the help of 30 mL sodium acetate buffer (50 mM, pH 4.0), stir for 10 min. Then continue with the regular Step (8) from Method **(A)**.

For the calculation of BG content with the "Mega Calc" spreadsheet, use the exact Sample weights (in mg), Sample volume = 0.1 mL, Dilution (-fold) = 1, and Extract volume = 40.8 mL.

[3] For isolated BG extracts that are precious, where one does not want to use 3x 25 mg = 75 mg just for the BG purity determination, one could either, if available, use a scale with 0.01 mg accuracy and use ~5.00 mg per replicate instead, where then for Step (7), the original Method **(A)** Step (7) can be applied – or use the procedure described in the BG extraction section for BG quantification in pure solutions (section **2.3.3**).

2.1.4 Total and extractable BG for fermented solid samples & controls

Herein, the suggested procedures are presented to determine total and extractable BG of fermented solid (e.g. freeze-dried) samples and their unfermented controls. Extractable BG for solid, milled raw materials such as oat flour, oat bran etc. may also be determined using this method.

For total BG content – *adapted Method* ***(B)***	***For extractable BG*** *(solid/dry samples)*
(1) Accurately weigh ~100 mg sample in Falcon tubes (15 mL size) in triplicates.	(1) Accurately weigh ~200 mg sample in Falcon tubes (15 mL size) in triplicates.
(2) Next, enzymes are deactivated (and free glucose amounts reduced) by adding 5.0 mL aqueous ethanol (90% v/v), vortex mixing, and incubation in a boiling water bath for 5 min. Cool to room temperature (e.g. under cold running water) before opening the tubes, then add 5.0 mL more of 90% (v/v) aq. EtOH and vortex mix. Centrifuge for 10 min at >3000 x g. Discard the supernatant. (3) Again, resuspend the pellet in 5.0 mL of 90% (v/v) aq. EtOH, vortex mix, then add 5.0 mL more, mix, then centrifuge for 10 min at >3000 x g. Discard the supernatant.	
(4) Add 5.0 mL phosphate buffer (20 mM, pH 6.5) in a fast stream pointing at the pellet for instant mixing, cap tube immediately, and vortex mix until fully dispersed. (5) Continue with Method **(A)** Step 6 (section **2.1.3**). Calculate **total BG content (g/100 g "as is")** with the Megazyme excel file, entering the used exact Sample weights (~100 mg), Sample volume = 0.1 mL, Dilution (-fold) = 1, and Extract volume = 10.4 mL.	(4) Add 4.0 mL water to the pellet (→ 1:20 solids/water ratio) in a fast stream pointing at the pellet for instant mixing, cap tube immediately, and vortex mix until fully dispersed. (5) Incubate at 40°C for 2 h in a shaking water bath, mix manually every ½ h. (6) Centrifuge for 15 min at >3000 x g. (7) Take aliquot of supernatant (1.5 mL) and transfer to a new 15 mL Falcon tube. (8) Add 1.5 mL sodium phosphate buffer (40 mM, pH 6.5) (to result in 20 mM). (9) Add 150 µL lichenase (50 U/mL), vortex mix. (10) Incubate for 1 h in a shaking water bath at 50°C, with regular vigorous vortex mixing every 15–20 min. (11) Add 4.0 mL sodium acetate buffer (200 mM, pH 4.0), vortex mix thoroughly. (12) Continue with Method **(A)** Step (8) (section **2.1.3**). Calculate **extractable BG content (g/100 g "as is")** with the Megazyme excel file using the used Sample weights (~200 mg), Sample volume = 0.1 mL, Dilution (-fold) = 2.8, Extract volume = 7.15 mL.[4]

[4] Extract volume of 7.15 mL = (1.5+1.5+0.15+4.0) mL, and dilution factor of 2.8 comes from 4.2 mL/1.5 mL, namely taking a 1.5 mL aliquot from the ~200 mg sample dispersed in 4.0 mL water + 0.2 mL (assumed) residual 90% EtOH stuck to solid sample from the washing step = 4.2 mL.

2.1.5 Total and extractable BG for fermented semi-solid/liquid samples & controls

Herein, the suggested procedure is presented to determine total and extractable BG of fermented semi-solid/liquid samples (such as plant-based yogurt products) and their unfermented controls.

For total BG content *– adapted Method* ***(C)***	***For extractable BG*** *(semi-solid/liquid samples)*
(1) Record the weight of empty Falcon tubes (50 mL size), to then accurately weigh ~3.0 g sample in them in triplicates. (2) Next, enzymes are deactivated (and free glucose amounts reduced) by adding 5.0 mL absolute ethanol, vortex mixing, and incubation in a boiling water bath for 5 min. Cool to room temperature (e.g. under cold running water) before opening the tubes, then add 20 mL more of abs. EtOH and vigorously vortex mix (→ ~90% EtOH). Centrifuge for 10 min at >3000 x g. Discard the supernatant. (3) Resuspend the pellet in 8.0 mL of 90% (v/v) aq. EtOH, vortex mix, then centrifuge for 10 min at >3000 x g. Discard the supernatant. (4) Resuspend the pellet in sodium phosphate buffer (20 mM, pH 6.5) adjusting the volume to 5.0 mL (by weight), from the known weight of the empty tube. Incubate the tube at 50°C for 5 min. (5) Continue with Method **(A)** Step 6 (section **2.1.3**). Calculate **total BG content (g/100 g "as is")** with the Megazyme excel file, entering the used exact Sample weights (~3000 mg), Sample volume = 0.1 mL, Dilution (-fold) = 1, and Extract volume = 10.2 mL.	(1) Accurately weigh ~3.0 g sample in Falcon tubes (15 mL size) in triplicates. (2) Add the appropriate amount of water to each replicate to reach a solids/water ratio of 1:20 – in this case example with 15% (m/m) dry matter and 1.0% BG "as is" content, it is 6.45 mL (adjust amounts accordingly for different dry matter contents).[5] Mix until properly dispersed. (3) Incubate at 40°C for 2 h in a shaking water bath, mix manually every ½ h. (4) Centrifuge for 30 min at >3000 x g.[6] (5) Take aliquot of supernatant (1.5 mL) and transfer to a new 15 mL Falcon tube.[7] (6) Add 13.5 mL absolute ethanol to reach ~90% (v/v) EtOH. Vortex mix. Centrifuge (>3000 x g, 15 min), and decant supernatant. (7) Resuspend the pellet in 3 mL sodium phosphate buffer (20 mM, pH 6.5). Incubate the tube at 50°C for 5 min. (8) Add 150 µL lichenase (50 U/mL in 20 mM phosphate buffer pH 6.5; 7.5 U), vortex mix. (9) Incubate 1 h in a shaking water bath at 50°C. (10) Add 4 mL sodium acetate buffer (200 mM, pH 4.0), vortex mix, then wait 5 min. (11) Proceed according to Method **(A)** described in section **2.1.3** from Step (8) onwards. Calculate **extractable BG content (g/100 g "as is")** with the Megazyme excel file, entering the used exact Sample weights (~3000 mg), Sample volume = 0.1 mL, Dilution (-fold) = 6.3,[8] and Extract volume = 7.15 mL.

[5] Calculation: for sample with 15% (m/m) solids, the sample contains 3.00 g × 0.15 = 0.45 g solids, hence 20 × 0.45 g = 9.00 g = desired total amount of water, and the amount of water that needs to be added = 9.00 g – (3.00 g – 0.45 g) = 6.45 g.

[6] If e.g. ≥5000 x g is available, centrifugation time may be reduced to 15 min.

[7] If this solution should be frozen/stored, the enzymes need to be deactivated, e.g. by incubating in boiling water bath for 10 min. This is not necessary, if the aliquots are used right away in the next step (precipitation with EtOH).

[8] Dilution factor of 6.3 comes from 9.45 mL/1.5 mL, namely taking a 1.5 mL aliquot from sample diluted to 3 g + 6.45 g.

2.1.6 Calculations

$$\text{BG extractability } [\%] = \frac{extractable\ BG\ content\ \left[\frac{g}{100g}\right]"as\ is"}{total\ BG\ content\ \left[\frac{g}{100g}\right]"as\ is"} \times 100\%.$$

However, since the total BG content may also change during fermentation (consumption of BG through microorganisms), for reasons of comparability between samples and controls, it may also make sense to calculate the BG extractability as the extractable BG content relative to the initial total BG content (at t = 0 h of the fermentation) for all samples and controls, instead of relative to each sample's actually detected total BG content.

2.2 Fine structure analysis – DP3/DP4 by HPAEC-PAD

2.2.1 Method principle & explanation of the various steps

The fine structure – or molar DP3/DP4 ratio, corresponding to the cellotriosyl to cellotetraosyl ratio in the BG chain – can be determined representing various populations of the total BG in the sample. We propose to focus on the two most straight-forward ones, namely:

i. Total population (incl. water-insoluble BG),
ii. Water-extractable population.

Same principles apply as for the Megazyme McCleary assay for BG quantification with regard to the use of the enzyme lichenase (see subchapter **2.1.1**). To measure the former, total population, one takes advantage of the fact that lichenase is able to hydrolyze also insoluble BG, releasing the soluble BG-oligosaccharides just as in the total BG quantification assay – simply without any β-glucosidase addition. The water-extractable population, on the other hand, is proposed to be measured under the same conditions as BG-extractability (see sections **2.1.4** and **2.1.5**), namely after a simple water-extraction of the sample material and working with the supernatant after centrifugation.

2.2.2 Procedure for DP3/DP4 ratio for total BG

(1) Depending on the sample, follow the first four steps of the same procedure used for total BG quantification of that sample:
   - Method **(A)** in section **2.1.3** for control flours/raw materials.
   - Adapted Method **(B)** in section **2.1.4** for solid fermented samples and their controls.
   - Adapted Method **(C)** in section **2.1.5** for semi-solid/liquid fermented samples and their controls.

(2) Add 200 µL lichenase solution (50 U/mL in 20 mM phosphate buffer; 10 U), cap and vortex mix. Incubate for 2 h at 50°C in shaking water bath, with regular vigorous vortex mixing every 15–20 min (or continuous stirring if such a device is available).

(3) Deactivate lichenase by incubation in boiling water bath for 10 min (5 min if glass tubes are used).

(4) Centrifuge tubes (15 min, >3000 x g), take supernatant and dilute it 20x with water, filter (0.45 µm), and transfer 1000 µL into HPLC vials (1.5 mL).

(5) Analyze by HPAEC-PAD continuing with section **2.2.4**.

2.2.3 Procedure for DP3/DP4 ratio for water-extractable BG

(1) Follow the first 8 steps of the procedure for the **Case 2b** BG extractability method above, up to and including step 8 (lichenase treatment of diluted supernatant).

(2) Deactivate lichenase by incubation in boiling water bath for 10 min (5 min if glass tubes are used).

(3) Centrifuge tubes (15 min, >3000 x g), take supernatant and dilute it 20x with water, filter (0.45 µm), and transfer 1000 µL into HPLC vials (1.5 mL).

(4) Analyze by HPAEC-PAD, continuing with section **2.2.4**.

2.2.4 HPAEC-PAD conditions

Two general procedures are commonly used for high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), depending on the available specific column setup:

Column setup	**Eluents**	**Reference**
CarboPac PA1 analytical (4x250 mm) plus guard (3x25 mm) columns from Dionex (Sunnyvale, CA, USA)	A: 0.15 M NaOH B: 0.5 M NaOAc in 0.15 M NaOH	Johansson et al. (2004)
CarboPac PA-100 analytical (4×250 mm) plus guard (4×50 mm) columns from (Dionex, Sunnyvale, CA, USA)	A: 1.0 M NaOAc in 0.1 M NaOH B: 0.1 M NaOH	Rantanen et al. (2007) Laitinen et al. (2023)

For the gradients, detector pulse potentials and durations, see the respective references above. Glucotriose (O-BGTRIB) and glucotetraose (O-BGTETB) are recommended to be used as external standards to quantify cereal BG's DP3 and DP4 (up to 100 µM and 50 µM, respectively), and e.g. xylotriose (O-XTR) as an internal standard[9] (added e.g. at 25 µM level to all samples and standards; all oligosaccharides are from Megazyme, Ireland).

[9] Check first with injection of samples before xylotriose addition that the samples do not have a peak co-eluting with the internal standard.

2.3 Isolation of BG for SEC

The following procedure for the extraction and isolation of cereal BG necessary for SEC-RID for M_w-distribution analysis is based on the publication of Laitinen et al. (2023), which in turn is based on procedures published by Mäkelä et al. (2021) and Lazaridou et al. (2004). Modifications were made to allow for the handling of several samples in parallel, and to account for the expected potentially lower molecular weight for samples that underwent enzymatic pretreatments/ fermentation, such as freeze-dried plant-based yogurt (gurt) samples.
Note that this procedure extracts BG at 40°C in water and includes a centrifugation step (i.e. removal of the solids) before the digestion of starch, proteins, and AX in the supernatant, to isolate the water-extractable BG population. This contrasts with other methods that use extractions in the presence of sample solids and termamyl at 90-95°C, where the high temperature presumably allows for the extraction of also higher M_w BG and hence potentially produces an extract that is slightly more representative of the total BG. However, such a high-temperature extract does not represent anymore the water-extractable population of BG that can be expected to be solubilized during human digestion and hence is of special interest due to its immediate availability, e.g. for beneficial bacteria in the gut.

2.3.1 Method principle & explanation of the various steps

The chosen, adapted method starts with deactivation of enzymes and concomitant removal of lipids and sugars through the treatment with 80% EtOH(aq.) at 80°C. After decantation of the supernatant, water is added and the solubilization of BG started with an extraction temperature of 40°C, which is specifically chosen to be slightly above room temperature to help with the dissolution, but well below the gelatinization temperature of starch. The next steps include enzymatic treatments, taking advantage of Termamyl's high activity in a wide pH-range, such that no pH-adjustments are necessary, while the other two enzymes are added dissolved in their appropriate buffer, which proved to lead to adequate pH levels for each enzyme without having to measure and adjust the pH manually by dropwise addition of an acid/base for each step, making this a streamlined, efficient method allowing for parallel BG extractions on several samples at once – e.g. 10 samples per person/ 3 days.

2.3.2 Detailed procedure for BG isolation

Preparation of samples, buffers, enzymes, solvents, solutions

- If necessary, freeze-dry and mill solid samples, e.g., using a blender to have material that passes a 0.5 mm screen.
- Sodium acetate buffer (0.5 M NaOAc, pH 4.0): Add 7.25 mL glacial acetic acid to 200 mL water. Adjust to pH 4.0 by the addition of 4 M sodium hydroxide (160 g NaOH/L; needs ~27 mL). Adjust the volume to 250 mL with water.
- Sodium Bicarbonate (0.15 M $NaHCO_3$): 315 mg $NaHCO_3$ in 25 mL water.
- Termamyl 300L (α-amylase from *Bacillus licheniformis* from Sigma, A4862-250ML): ready as is.
- Pancreatin solution: 18.75 mg/mL in 0.15 M $NaHCO_3$
  To have enough for 10 samples, dissolve 131.3 mg Pancreatin from porcine pancreas (8 × USP specification, Sigma P7545) in 7 mL 0.15 M $NaHCO_3$ (from above).
- Xylanase solution: 50 U/mL in 0.5 M NaOAc (pH 4.0)
  To have enough for 10 samples, dissolve 220 mg of xylanase from *Thermomyces lanuginosus* (Sigma X2753, 2500 U/g) in 11 mL 0.5 M sodium acetate buffer (pH 4; from above).
- Milli-Q water (0.5 L for 10 samples)
- Ethanol abs. (EtOH) (1.5 L for 10 samples)
- ~80% EtOH (0.5 L for 10 samples; 400 mL EtOH abs. + 100 mL water)
- ~95% EtOH (0.5 L for 10 samples; 475 mL EtOH abs. + 25 mL water)

Suggested needed vessels for 10 sample extractions

- 10 x 4 = 40 Falcon tubes (size 50 mL)
- 10 Nalgene centrifugation bottles or equivalent vessels (size 250 mL)
- 10 Falcon tubes (size 15 mL) (**tared**! To be able to determine the final isolated amount)

DAY 1: DEFATTING, WASHING, AND AQUEOUS EXTRACTION

(1) Weigh 5.0 g solid, milled sample in 50 mL falcon tube. Add 40 mL 80% EtOH.

(2) Shake at 80°C for 2 h in a shaking water bath (200 rpm), with manual shaking every 1/2 hour.

(3) Centrifuge for 15 min at >3000 x g.

(4) Decant and discard supernatant.

(5) Resuspend in 30-40 mL 95% EtOH (fill tube to 40 mL mark), mix well using spatula.

(6) Centrifuge for 15 min at >3000 x g.

(7) Decant and discard supernatant.

(8) Resuspend in 30-40 mL 100% EtOH (fill tube to 40 mL mark), mix well using spatula.

(9) Centrifuge for 15 min at >3000 x g.

(10) Decant and discard supernatant. Remove as much liquid from the tube as possible, i.e. by carefully inverting tubes while holding kitchen towel over the opening.

(11) Evaporate some of the remaining ethanol in an oven at 60°C for 1 h.

(12) Fill the tube with Milli-Q water up to the 50 mL mark. Mix well to ensure even suspension.

(13) Place the tubes horizontally in shaking/rotating incubator (avoids settling of solids, for better mass transfer/ BG extraction), and incubate at 40°C for 2 h, regularly turn/shake the tubes to enhance extraction of BG.

(14) Centrifuge for 15 min at >3000 x g.

(15) Collect supernatant in fresh 50 mL falcon tube.

(16) Store in fridge until next day.

DAY 2: DIGESTION OF STARCH, PROTEINS, AND ARABINOXYLAN; PRECIPITATION OF BG

(17) Get samples from fridge. Add 0.5 mL Termamyl 300L to each sample.

(18) Incubate at 80°C for 1 h in shaking water bath.

(19) Heat in boiling water bath (covered with aluminum foil) for 30 min to precipitate proteins.

(20) Cool the tubes by placing them in cold water.

(21) Centrifuge for 15 min at >3000 x g.

(22) Collect supernatant in fresh 50 mL falcon tube (discard pellet).

(23) Add pancreatin solution (18.75 mg/mL in 0.15 M $NaHCO_3$) to reach 1.5% (v/v) (by this point, it is usually ~35 mL sample solution à add 0.525 mL).

(24) Incubate for 1 h at 40°C in shaking water bath at 120 rpm.

(25) Place tubes in boiling water bath, start timer for 10 min once inside temperature reaches 80°C.

(26) Cool the tubes by placing them in cold water.

(27) Centrifuge for 15 min at >3000 x g.

(28) Collect supernatant in fresh 50 mL falcon tube (discard pellet).

(29) Add 1 mL of xylanase solution (50 U/mL in 0.5 M NaOAc pH 4 buffer).

(30) Incubate at 55°C for 1 h in shaking water bath at 120 rpm.

(31) Place tubes for 10 min. in boiling water bath (see step 19), start timer once it reaches 80°C (which should be after ~4 min).

(32) Cool in cold water.

(33) Centrifuge for 15 min. at 4000 rpm.

(34) Collect supernatant in 250 mL Nalgene centrifugation bottle.

(35) Add 5.7 volumes of absolute EtOH portion-wise while swirling to precipitate BG, to reach H_2O: EtOH (v/v) = ~15:85 (for the typically obtained ~35 mL supernatant from the previous step, add 200 mL EtOH).

(36) Store in the fridge at 4-5°C until the next day.

DAY 3: COLLECTION OF β-GLUCAN

(37) Centrifuge for 15 min at > 3000 x g.

(38) Transfer & wash pellet with ~15 mL EtOH abs. into tared 15 mL falcon tube (use 3 mL plastic pipettes; keep Nalgene bottle & pipette for 2nd washing round).

(39) Centrifuge for 5 min at > 3000 x g.

(40) Decant supernatant to waste.

(41) Repeat washing/centrifugation with ~10 mL of EtOH abs. (use pipette from before, put ethanol in Nalgene bottle first to recover last bits of precipitate before transferring to the Falcon tube containing the BG pellet; use thin spatula to help resuspend the pellet).

(42) Paste the BG along the walls of the falcon tube with the thin spatula (to increase surface area and facilitate drying).

(43) Dry in oven at 60°C for several hours until fully dried.

(44) Weigh the tubes and determine the isolated amount of BG extracts. Store BG extracts in desiccator in the dark.

2.3.3 Dissolution of BG extracts & purity determination

This procedure is designed to determine purity of BG extracts with minimal use of material, using a solution of BG extract in pure water that may also be used for analysis by SEC (after further dilution with SEC-eluent).[10]

(1) If necessary, ground BG extract into a powder using a mortar and pestle.

(2) Accurately weigh ~30 mg BG extract into a cryo tube (4 mL size) and wet with 0.1 mL 50% (v/v) aqueous EtOH. Then, add 2.9 mL water and immediately cap and vortex mix.

(3) Heat the tube in a boiling water bath for 30 min with occasional vigorous manual and/or vortex mixing, followed by, if necessary, another 1–2 h at 80–85°C under stirring until fully dissolved. As soon as the extract is fully dissolved, cover tube with aluminum foil and stir overnight at RT.

(4) Starting from this 10 mg/mL β-glucan extract solution, accurately weigh ~500 mg into Falcon tubes (15 mL size) in triplicates (record exact amounts). Keep the rest in the fridge (e.g. to be used for SEC-analyis later, see section **2.4**).

(5) Add 4.50 mL sodium phosphate buffer (20 mM, pH 6.5), vortex mix and shake until homogeneous.

(6) Continue with the Megazyme McCleary Assay Method **(A)** from Step (5) onwards (section **2.1.3**). For the calculation using the "Mega Calc" spreadsheet, use Sample weight = 500 mg (use exact amounts), Sample volume = 0.1 mL, Dilution (-fold) = 1, and Extract volume = 10.2 mL.

To calculate the purity of the dissolved BG extract, divide the resulting **BG content (g/100 g) "as is"** from the excel sheet by the extract concentration in the used 500 mg extract solutions (in this example 10 mg/mL = 1.0% extract = 1.0 g/100 g):

$$\text{BG purity } [\%] = \frac{BG\ content\left[\frac{g}{100g}\right]\text{"as is"}}{dissolved\ BG\ extract\ concentration\left[\frac{g}{100g}\right]} \times 100\%$$

Hence, for example, if the "as is" result is 0.702 g/100 g, then 0.702/1.0 × 100% = 70.2% BG purity.

[10] Hence, instead of e.g. 3 x 25 mg BG extract = 75 mg being used just for the content determination (using Method (**A'**) in section **2.1.3**), this procedure only needs 30 mg in total to prepare 3 mL 1.0% extract solution, of which only half is consumed for the BG assay (3x 500 mg), leaving the rest for SEC-analysis after dilution with the SEC-eluent.

2.4 Molecular weight distribution analysis

The high performance size exclusion chromatography (SEC) analysis for the determination of Mw distribution of cereal β-glucan can be accomplished with various setups, two of which based on different eluent systems are presented here in detail that need isolated BG extracts (from section **0**), to have other co-extracted substances removed as much as possible (proteins, AX, starch). This is due to the reliance on refractive index (RI) and light scattering detectors that would give a signal for most eluting substances. Using a simple water-extract of sample material instead, other co-extracted soluble fibers, starch and proteins would overlap in the elugram, not allowing the characterization of BG. The alternative calcofluor white (CW) method, which allows selective detection of cereal BG also in simple crude extract mixtures due to the specifically enhanced fluorescent signal (SEC-FLR) of the CW-BG complex, is not discussed in detail here. It may not always be suitable, i.e. when the Mw distribution curve crosses below ~40 kDa for some enzymatically treated/fermented samples, even when using sodium chloride in the eluent to minimize these effects, as below this threshold, the CW-BG signal starts to loose intensity, with practically no signal for CW-BG below 10 kDa (Foldager & Jørgensen, 1984; Manzanares et al., 1991).

2.4.1 DMSO/LiBr eluent system for BG extracts

2.4.1.1 Description

The β-glucans are dissolved in water as described in section **2.3.3**, but at a 6 mg/mL concentration. Aliquots of 300 µL are diluted with 700 µL DMSO containing 0.01 M LiBr, followed by additional heating at 85 ◦C for 1 h. The solutions are filtered (0.45 µm) and analyzed using high-performance size exclusion chromatography (HPSEC), following the procedure of Laitinen et al. (2023). A Viscotek triple detection system is used which consisted of a combined light scattering and viscometric detector (Viscotek 270, Malvern Panalytical, Malvern, UK) with two light scattering angles (7◦ and 90◦, λ_0 = 670 nm), and a refractive index (RI) detector (VE 3580, Viscotek). Separation occurred in two PLgel MIXEDA LS 20 µm analytical columns (7.5 × 300 mm; Agilent Technologies, Santa Clara, CA, USA) maintained at 40°C and a PLgel MIXED 20 µm guard column (7.5 × 50 mm). 0.01 M LiBr in DMSO is used as the eluent at a flow rate of 0.8 mL/min. The injection volume is 100 µL. The weight-average molar mass values (Mw) are calculated with OmniSEC 4.6 software (Viscotek) using a dn/dc value of 0.062 mL/g for cereal BG.

2.4.1.2 Advantage

- Complete molecular dissolution of BG extracts and reproducible results especially with high Mw BG (which, in part due to aggregation, exhibits losses when filtered through a 0.22 um syringe filter when in aqueous solution; reduced SEC-recovery), but also more accurate results for low Mw BG, which have a tendency for visible aggregation in the SEC elugram.

2.4.1.3 Limitation

- Needs BG extract (elimination of other soluble polysaccharides and proteins).
- Does not represent the behavior in aqueous solutions. Obtained intrinsic viscosity, Rg, Rh etc. all only apply to BG in the DMSO/LiBr eluent.

2.4.2 Aqueous eluent system for BG extracts

2.4.2.1 Description

For aqueous SEC-analysis, heat up the diluted BG solution (1 mg/mL) to 80–85°C for 1 h before filtering hot through a syringe filter (0.45 µm, nylon) directly into an HPLC-vial that is placed into the 60°C autosampler. Weight-average molecular weight (Mw) and dispersity (Đ) of cereal BG extracts diluted in SEC-eluent to 1 mg/mL are determined by high-performance size exclusion chromatography (HPSEC) (OMNISEC, Malvern Panalytical Ltd, Malvern, United Kingdom) according to Lupo et al. (2020).

The system consists of an OMNISEC RESOLVE chromatography compartment combined with a pump, an autosampler, and a column oven equipped with two A'6000M columns in series (8.0 mm X 300 mm, Viscotek, parent organization: Malvern Panalytical Ltd, Malvern, United Kingdom). OMNISEC RESOLVE detector compartment is equipped with a low and right-angle laser light scattering detector (LALS/RALS), a refractive index (RI), a UV detector and a viscometer. OMNISEC software version v.10.30 is used for data acquisition, analysis, and reporting. A solution of 0.1 M $NaNO_3$ with 0.02 % NaN_3 is used as mobile phase. The temperature of the autosampler is set to 60°C, and both columns are kept at 30°C, with a flow rate of 0.7 mL/min and an injection volume of 100 µL. For the absolute molecular weight determination, a calibration is performed using narrow molecular weight distribution polyethyleneoxide (PEO-24K) standard using the refractive index increment (dn/dc) value of 0.146 mL/g for cereal β-glucan.

2.4.2.2 Advantage

- Aqueous system – closer to physiologically relevant conditions than in DMSO/LiBr.
- Hydrodynamic radius, intrinsic viscosity and other determined characteristics directly apply to aqueous solutions.

2.4.2.3 Limitation

- Needs isolated BG extract (elimination of other soluble polysaccharides and proteins).
- Limited ability to measure high Mw BG quantitatively (loss of material in the 0.45 µm and 0.22 µm filters during syringe filtration or as part of the SEC-system) – lower SEC-recovery of the detected BG peak than expected by the BG purity.
- BG aggregation for small Mw BG, potentially leading to less accurate Mw distribution results → solution: filtering hot; using the autosampler at 60°C largely takes care of this problem.

3. Others (tips&tricks,…)

- The water content of the solid samples should be known if dry matter contents are desired for BG contents.
- Always analyze the control flours together with the samples.
- It is imperative that the lichenase enzyme preparation is not cross-contaminated with the β glucosidase preparation (the reverse is not a problem).
- To be more efficient, some mixtures may be used for more than one characterization – e.g. when determining total and extractable BG, after the lichenase step, the supernatant may be used also for DP3/DP4 analysis by HPAEC-PAD after additional dilution.

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Chapter 8
Exopolysaccharides

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Summary

- **Raw material**: Cereal- and legume-derived flours, protein isolates, and starch isolates
- **Relevant applications:** dough, bread, meat and dairy alternatives, digesta
- **Fermentation-induced changes**: Microorganisms as natural factories of exopolysaccharides
- **Analytical methods**:
  - Molecular eps genes characterization in LAB and yeasts
  - Qualitative characterization of exopolysaccharides
    - Screening approaches on solid and liquid media
    - Biofilm preparation
    - CLSM analysis
  - Quantitative characterization of exopolysaccharides
    - Phenol–sulfuric acid assay
    - HPAEC-PAD

1. Introduction

1.1 What are exopolysaccharides?

Polysaccharides, widespread in multiple organisms, are high molecular weight polymeric carbohydrate structures that exhibit chemical heterogeneity, physiological properties and biological activities that are structure and source related (Chen & Huang, 2018). Polysaccharides are made of monosaccharide units connected together by glycosidic linkage. When these polymers are adhered to the cell surface, or they are fully expelled to the surroundings, generating slime, they are referred as exopolysaccharides (EPS) (Angelin & Kavitha, 2020). EPS are extracellular macromolecules made up of carbohydrates (sugar residues), replaced with proteins, DNA, phospholipids and non-carbohydrate substituents such as acetate, glycerol, pyruvate, sulfate, carboxylate, succinate and phosphates (F. Freitas et al., 2011). In general, EPS might be homopolysaccharides (HoPS) or heteropolysaccharides (HePS). HoPS and HePS may differ not only in the fundamental subunits, but also in their structure, chain length, degree of branching, and sugar bonds. HoPS are mainly composed of glucose or fructose units, which commonly form glucans (dextran, mutan, alternan, and reuteran), and fructans (Ex. levan-type and inulin-type polysaccharides), respectively (Ryan et al., 2015). On the other hand, the constituent repeating units of HePS are usually D-glucose, D-galactose, and rare sugars, such as L-rhamnose, mannose, arabinose and fucose, and in some cases N-acetylglucosamine, N-acetylgalactosamine, or glucuronic acid (Torino et al., 2015). They are designated as gellan, xanthan

and kefiran. HoPS have a molecular weight greater than 10^6 Da whereas HePs range from 10^4 to 6.0 × 10^6 Da (Angelin & Kavitha, 2020). **Figure 1** shows a schematic classification of EPS.

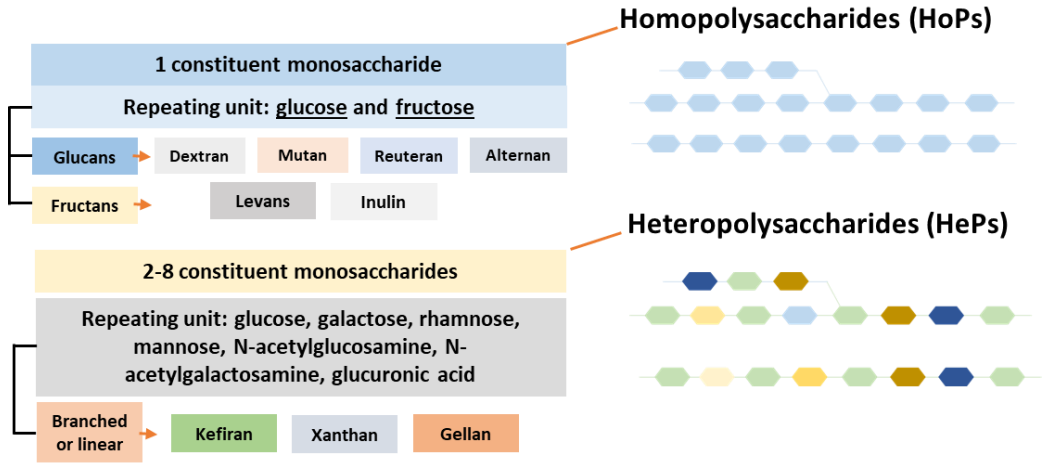

***Figure 1**. Schematic representation showing classification and characterization of exopolysaccharides.*

1.2 Natural factories of exopolysaccharides

Natural polymers versus synthetic polymer have garnered a lot of interest among scientific communities over the last few decades owing to their advantageous therapeutic potential. Remarkably, various microorganisms such as LAB and yeasts are well known to produce EPS with broad spectrum of structural diversity (Bernal & Llamas, 2012). Mounting evidence demonstrated the capability of *Fructilactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lactobacillus*, *Lactococcus*, *Latilactobacillus*, *Lentilactobacillus*, *Leuconostoc*, *Limosilactobacillus*, *Pediococcus*, *Streptococcus*, and *Weissella* species in biosynthesis of EPS (Angelin & Kavitha, 2020; Zheng et al., 2020). Among yeasts numerous genera of *Bullera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Lipomyces*, *Pichia*, *Hansenula*, *Aureobasidium*, *Pseudozyma*, *Rhodotorula*, *Kluyveromyces*, *Sporobolomyces*, *Kazachstania*, *Clavispora*, *Hanseniaspora*, and *Trichosporon* have been recognized as EPS producers (Rahbar Saadat et al., 2021). Generally, these organisms synthesize EPS to cope up with harsh and adverse growth circumstances such as dehydration, osmotic stress, and pathogenic microbes, not as an energy source. Indeed, EPS are implicated in the invasion of natural habitats by host bacteria enhancing biofilm formation and cell recognition (Karygianni et al., 2020). Biofilms are dominated by a population of microbial cells which are bonded to one another and to the solid surface. These microbial cells are encased in a slimy extracellular matrix made up of extracellular substances (exopolysaccharides, proteins and lipids). It is noteworthy that biofilm-forming microbial cells are more resistant to nutrient depletion, temperature, pH changes, reactive oxygen species, and antibiotics than individual microbial cells (Daba et al., 2021).

Although EPS is synthesized throughout the logarithmic and stationary phases, peak synthesis occurs only in the late logarithmic phase (F. Freitas et al., 2011). The level of EPS produced differs depending on the strains, medium composition, and culture conditions such as pH, temperature and carbon/nitrogen ratios. Besides, the EPS released by microorganisms varies in terms of monosaccharide composition, charge, linkage, presence of repetitive side-chains and substitutions (Angelin & Kavitha, 2020). All enzymes and proteins involved in the production of ESPs are regulated by specific genes (Badel, Bernardi, & Michaud, 2011). The genes encoding EPS biosynthesis are typically arranged in an operon cluster, with the genes oriented in the same direction and transcribed as a single mRNA. The process is driven by the promoter sequence in front of the first *eps* gene. These genes perform four distinctive tasks: (1) regulating EPS production, (2) determining chain length, (3) repeating unit biosynthesis, and (4) polymerization and export (Korcz & Varga, 2021). From a biochemical standpoint, EPS are synthesized in two ways, intra and extracellular. HoPS are typically produced by extracellular simple process because there are no active transportation phases in the synthetic pathway, other than from energy expenditure to release essential extracellular enzymes

such as glycosyl transferases and fructosyl transferases during the polymerization process (Korcz & Varga, 2021). With the aid of these enzymes, the sugar residues are split monomeric units outside the cell and assembled extracellularly into a polymer (Angelin & Kavitha, 2020). On the contrary, the mechanism of HePS synthesis is a more complex and energy intensive process which involves various enzymes, carriers and transporter proteins. The synthesis of HePs relies on distinct reactions, initiated with sugar transportation, followed by the synthesis of sugar nucleotide precursors from glucose-1-phosphate and fructose-6-phosphate, and the assembly of the monosaccharide repeating unit and its linkage to the glycan subunit carrier. Finally, the export of these carrier-bound polymers from the cytoplasm to the extracellular space is mediated by group of hydrophobic enzymes like flippase, permease or ABC transporters (De Vuyst et al., 2001).

1.3 Applications of exopolysaccharides

Nowadays, the contributions of EPS from microorganisms as biotechnological tools in food, pharmaceutical, and medical applications have been extensively documented. EPS are popular for their shelf-life extending and techno-functional enhancing properties in dairy and baking industries and cereal-based products. In the food industry, LAB-derived EPS generated during fermentation serve vital roles in food organoleptic properties. EPS such as xanthan, sphingan, and alginate are employed as emulsifiers, stabilizers, thickeners, gelling agents, as well as to retain moisture, influence rheology, firmness, and syneresis, and to improve texture, sensory, and mouthfeel characteristics (Daba et al., 2021). Challenges like low dough machinability, texture, volume, rheology, and shelf life are commonly encountered during bread making, which can be solved by EPS instead of expensive hydrocolloidal polysaccharides (Das et al., 2015). EPS have proven to be a reliable molecule for numerous drugs, and therapeutic and medical treatments in curing inflammation and other disorders. The EPS produced from natural sources have been reported as a good substitute to the synthetic anticancer drugs (Wang et al., 2014). In fact, these molecules are able to impede tumor cells proliferation by inhibiting the bacteria producing β-glucosidase and β-glucuronidase, which promote the conversion of pro-carcinogens to proximal carcinogens (Deepak et al., 2016). Besides, EPS can trigger a nonspecific reaction against viral and bacterial infections, and inflammation. Therefore, EPS have strong high potential to be used as immunomodulating drugs that activate the immune system to fight infectious diseases (Ubaidillah et al., 2015). In addition, the effect of the EPS treatment was relevant on antidiabetic activity through inhibiting α-glucosidase and α-amylase enzymes under in-vitro condition. On the other hand, in vivo experiments revealed that EPS extracted from kefir grains has good cholesterol-reducing effects, decreasing very-low-density lipoprotein cholesterol concentrations (Lim et al., 2017).

1.4 Techniques for characterization analysis of EPS

To evaluate the potential applications of natural-derived EPS, it is crucial to study their structure and composition. The primary step in EPS characterization is optimizing extraction and purification procedures. The features of the EPS and the desired outcome of the extraction should be considered while optimizing the methods (Barcelos et al., 2020). Extraction methods of EPS can be divided into chemical and physical methods. Alkaline, ethylenediamine tetraacetic acid (EDTA), and cation exchange resin are examples of chemical methods that might contaminate the extracted products (Comte et al., 2006). Alternatively, physical methods include centrifugation, ultrasonication, and heating, which mostly reduce contamination but achieve lower efficiencies when compared to chemical methods (Liang et al., 2010). Thereafter, a purification step is required since residues of DNA, proteins, and even chemicals may be present. EPS can be purified by re-precipitation from an aqueous solution, chemical deproteinization, and membrane techniques (ultrafiltration, diafiltration and gel filtration) (Barcelos et al., 2020). As next step, there are numerous approaches for identifying and analyzing natural-derived EPS. Biofilm membrane, electron microscopy (EM), and confocal laser scanning microscopy (CLSM) are all qualitative approaches for spotting EPS. To quantify and further analyze the composition and structure of EPS, colorimetric methods such as the phenol–sulfuric acid

method, high performance liquid chromatography (HPLC), gas chromatography (GC), size-exclusion (SEC) or ion-exclusion chromatography (IEC), Fourier transform infrared (FTIR) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy are commonly used (Loeffler et al., 2020). This book chapter is focused on description of main methods involved in molecular detection of EPS genes in LAB, followed by characterization of the evolution of microbial exopolysaccharides physiochemical properties and composition during cereals plant-based fermentation.

2. Method(s) of analysis

2.1 Molecular EPS genes characterization in LAB and yeasts

2.1.1 Principle

Screening of EPS production in microorganisms at the molecular level differs for HoPs and HePs due to the complex nature of HePs. The synthesis of HoPs, i.e., glucans and fructans, is catalyzed by glucantransferases and fructantransferases, respectively, which revealed a similar protein domain organization (van Hijum et al., 2006). Therefore, primers targeting the conserved catalytic domain encoding these enzymes have been developed previously (Kralj et al., 2003; Tieking et al., 2003; Tieking et al., 2005). The mechanism of HePs synthesis is more complex as the repeating unit is first produced intracellularly using sugar nucleotides as precursors, translocated across the cell membrane, polymerized, and then released into the medium (De Vuyst & Degeest, 1999). Each of these functions are encoded by gene clusters that are transcribed as a single mRNA (Jolly & Stingele, 2001). Therefore, several primer pairs have been designed targeting different regions (or genes) of these gene clusters.

2.1.2 PCR amplification of genes targeting HoPS and HePS syntheses

Based on the conserved regions for the catalytic domain of bacterial glucosyltransferases, specific primers can be purchased from Eurofins Genomics Srl (GmBH, Germany). The primer sequences and their corresponding conditions are mentioned in **Table 1**.

Table 1. *Primer sequence information used for the amplification of genes responsible for exopolysaccharide production in microorganisms.*

Primer	Sequence (5'–3')	Target gene	PCR conditions	Expected fragment length (bp)
DexreuV (forward)	GTGAAGGTAACTATGTTG	Glucosyltransferase (*gtf*)	Initial denaturation at 95 °C (5 min); 35 cycles of denaturation at 95 °C (30 s), annealing at 42 °C (45 s), and extension at 72 °C (1 min); final extension at 72 °C (10 min)	600
DexreuR (reverse)	ATCCGCATTAAAGAATGG			
epsD/E-F (forward)	TCATTTTATTCGTAAAACCTCAATTGAYGARYTNCC	Priming *gtf*	Initial denaturation at 94° C (9 min); 5 cycles of denaturation at 94 °C (30 s), annealing at 62 °C (30 s), and extension at 72 °C (30 s); 40 cycles of denaturation at 94°C (30 s), annealing at 52 °C (30 s), and extension at 72 °C (30 s); final extension at 72 °C (10 min)	220
epsD/E-R (reverse)	AATATTATTACGACCTSWNAYYTGCCA			

- Prepare the PCR reaction by mixing 1× PCR reaction buffer, 4 mM $MgCl_2$, 0·2 mM dNTPs, 0.5 µM each of forward and reverse primers, and 1·5 U of *Taq* polymerase.
- Add up to 40 ng of DNA template in the reaction mix of 25 µL.
- The amplified PCR products are then sent for amplicon sequencing (Eurofins Genomics, GmBH, Germany) to determine the nucleotide sequence.
- The sequenced amplicons can be identified using the Nucleotide BLAST tool available on the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
- The BLAST results showing the highest similarity (>98%) with previously reported gene sequences encoding the respective genes (as indicated in Table 1) indicate the EPS production.

2.2 Qualitative characterization of exopolysaccharides

2.2.1 Screening approaches on solid and liquid media

2.2.1.1 Principle

EPS production is strain-specific and frequently dependent on the composition of the surrounding media. Consequently, each microbe has its own requirements to produce EPS. The screening methods for evaluating the microbial ability to synthesize EPS are based on the cultivation of the microbes in a medium enriched with distinct sugars (glucose, fructose, sucrose, mannose, galactose, or lactose). The easiest way to assess EPS production is to visually observe its phenotypic traits on solid as well as liquid media. Generally, the terms “slimy” and “ropy,” are used for this visual characterization. The slimy phenotype is characterized by mucilaginous colonies, whereas the ropy phenotype is distinguished by the formation of long filaments when an inoculation loop is lifted from the colony surface. The lack of clear morphology definitions may lead to false negative strains, which are excluded. Therefore, novel interesting polymers might be undetected due to a missing obvious slime formation.

2.2.1.2 Media preparation

- Solid agar media: Suspend fructose 10 g, glucose 10 g, maltose 10 g, sucrose 10 g, bacteriological peptone 10 g, yeast extract 10 g, and agar 15 g in 1 L of distilled water.
- Liquid broth media: Suspend fructose 10 g, glucose 10 g, maltose 10 g, sucrose 10 g, bacteriological peptone 10 g, yeast extract 10 g in 1 L of distilled water.
- Under both conditions, sterilize the media in autoclave for 15 min at 121 ^{o}C.

2.2.1.3 Plating methods

Streaking method: is done using a sterile inoculation loop, which is dipped into the broth containing the 24 h old culture bacteria then streaked over the solid media. Afterwards, the plates are incubated at 30 °C from 3 to 5 days.

Spreading method: 200 µL of the broth containing the 24h old culture bacteria is pipetted onto the center of the surface of the agar plate. The sample is spread evenly over the surface of agar using the sterile L-shaped spreader, rotating the Petri dish underneath at the same time. Likewise, the plates are incubated at 30 °C from 3 to 5 days.

Broth method: 400 µL of the broth containing the 24h old culture bacteria is transferred to 10 mL liquid broth tube. Cultures are incubated at 30 °C for 24h.

2.2.1.4 EPS production appearance

EPS can also be seen in broth; even though agar methods allow for easier comparison. These methods are useful to detect the ropiness (a) or other mucoid (b) phenotypes by touching the microbial colonies

with a sterilized inoculation loop or sterilized toothpicks **(Figure 2).** The touched colony "extends" like a rope. The mucoid and slimy colony-producing strains are considered EPS positive.

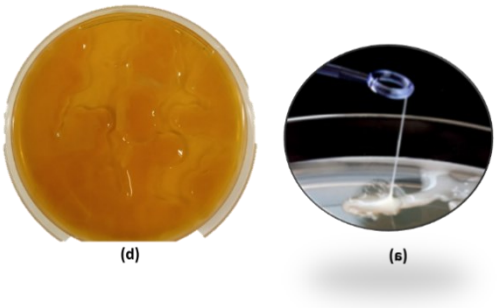

***Figure 2**. Morphological characteristics of exopolysaccharides.*

2.2.1.5 Biofilm preparation

- Dilute overnight cultures to a turbidity (600 nm) of 0.20 in 50 mM of sterile potassium phosphate buffer (pH 7.0).
- Add one 5-µl drop of diluted culture to inoculate individual sterile, black, polycarbonate membrane filters (diameter, 2.5 cm; pore size, 0.2 lm; Whatman, Maidstone, UK) resting on solid agar medium.
- Sterilize the membranes by UV exposure (15 min per side) before inoculation.
- To allow the biofilm growth, incubate the plates at 30 ^{o}C for 3 to 5 days.
- Inverted after inoculum and transfer the membrane-supported biofilms to fresh culture medium every 10– 12 h.

2.2.2 CLSM analysis

The use of CLSM in combination with different staining protocols allows the visualization of EPS.

2.2.2.1 Principle

Lectins are carbohydrate-binding proteins and several of their fluorescent conjugates are commercially available. Their binding specificity allows direct visualization of EPS in structures such as microbial biofilms, where EPS is one of the main component of the biofilm extracellular polymeric matrix. For instance, texas red-labelled Concanavalin A (ConA, 595 nm excitation maximum, 615 nm emission maximum) is widely used to label and visualize EPS. However, several other fluorescent conjugates (characterized by different spectral characteristics) can be used (e.g. alexa fluor, fluorescein, etc). ConA can be combined with fluorescent labels specific for diffrent microbial taxa. For instance, SYTO 9, is a blue-excited (485 nm excitation maximum) green-fluorescent (498 nm DNA, 501 nm RNA emission maxima) nucleic acid stain widely used to label bacterial cells. Such kind of combinations allow the simultaous visualization of both EPS and bactrial cells within the biofilm. The CLSM is an efficient approach to qualitatively investigate the presence and distribution of EPS in microbial biofilm by using the fluorescence emitted after the samples preparation through a staining procedure, producing 3-dimention models as output.

2.2.2.2 Reagents

- Phosphate buffered saline solution (PBS)
- Sodium bicarbonate
- SYTO9 nucleic acid stain
- Texas red-labelled ConA

2.2.2.3 Procedure

- Fix membrane-supported biofilm carefully on a glass slide.
- Stain the biofilm bacterial cells and EPS with a 15 µM of SYTO9 and 200 µg/ml of texas red-labelled ConA solution in PBS (pH 7.5). A stock solution 5 mg/mL of ConA can be prepared in 0.1 M sodium bicarbonate at pH 8.3).
- Incubate the samples under dark conditions for 30 min at room temperature.
- Visualize the samples by CLSM using 488- and 552-nm lasers and 40 or 60x oil immersion objectives. Fluorescence emission can be observed between 500–565 nm (for SYTO9) and 565–645 nm (for ConA).
- Capture images and analyze through the CLSM software.

2.2.3 Results description

When observed with CLSM, bactrial biofilms appeare as agglomerate (more or less widespread) of cells (green fluorescence) attached to the surface and embedded in a extracellular polymeric matrix containing EPS (red fluorescence) **(Figure 3).** Biofilm architecture is strain dependent (Tlais et al. 2022). The fluorescence qualitatively demonstrates the presence of the biofilm matrix and EPS. Such analyses also provide some information about relative quantity. For instance, when comparing different biofilms, the intensity and extension of red fluorescence signal caused by ConA, the quantity of EPS can be estimated..

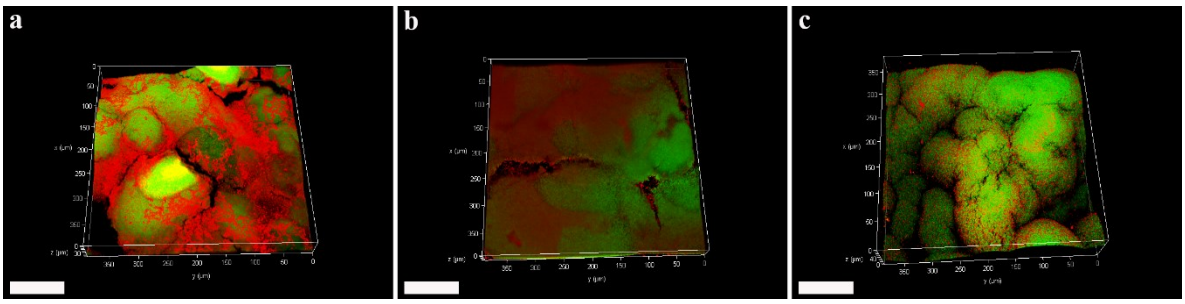

Figure 3. *CLSM analysis of biofilms produced by Apilactobacillus kunkeei BEE4 (panel a), BV61 (panel b) and PL13 (panel c). Bacterial cells show green fluorescence and EPS in biofilm extracellular polymeric matrix show red fluorescence. Scale bars represent 100 µm; units of x, y and z axes are µm (adapted from Tlais et al., 2022).*

2.3 Quantitative characterization of exopolysaccharides

2.3.1 Extraction

2.3.1.1 From solid agar medium (biofilms)

- Scrap the colony biofilms with a scraper.
- Suspend the colony biofilms with 1 ml of 1.5 M potassium phosphate buffer solution.
- Centrifuged the suspension at 5000 g for 10 min at 25°C without any incubation period.
- Collect the supernatants as extracellular matrix fractions.

2.3.1.2 From broth media

- Propagate the standardized microbe in broth medium from 24 h at 30°C.
- Boil the culture for 15 min.
- Treat the boiled culture with 17% (v/v) of trichloroacetic acid (85%) for 2 h.
- Save the supernatant by centrifugation at 18 000 g for 25 min.
- Incubate 5 mL of the supernatant overnight at 4°C with 5 volumes of 95% ethanol.
- Centrifuge at 18 000 g for 20 min to collect the precipitates.

- Re-dissolve the crude EPS precipitants in 5 mL of demineralized water.
- Purify the suspension by dialysis using a commercially available dialysis bag (12–14 kDa) at 4°C for 48 h.
- Remove the water using a freeze drier (Epsilon 2-6D LSC plus freeze-drier, Martin Christ, Osterode am Harz, Germany) for at least 24 h.
- The final powder corresponds to the EPS product.

2.3.1.3 From fermented cereals plant-based products

- Transform raw and fermented samples into powder using a freeze drier (Epsilon 2-6D LSC plus freeze-drier, Martin Christ, Osterode am Harz, Germany)
- Add 20 mL of chilled to 20 g of the dried samples then incubate for 12 h at 4 °C.
- Suspend the pellets containing EPS in 5 mL of distilled water with gentle heating at lower than 50 °C to achieve dissolvation after centrifugation at 6000 ×g for 30 min at 4 °C.
- In order to remove proteins, add trichloroacetic acid (TCA) at a final concentration of 20% and then incubate for 2 h at 4 °C under gentle agitation.
- Remove precipitated proteins by centrifugation at 13,000 ×g for 20 min at 4 °C and collect the supernatants.
- Add two volumes of cold ethanol and then centrifuge (13,000 ×g for 20 min) to achieve EPS precipitation.
- Purify the suspension by dialysis using a commercially available dialysis bag (12–14 kDa) at 4°C for 48 h.
- Dry the EPS using a freeze dryer.
- Resuspend the EPS in distilled water for further analysis.

2.3.2 Quantification

2.3.2.1 Phenol–sulfuric acid assay

- Mix 20 µL of EPS extract with 20 µL of 5% phenol in the 96 wells plate.
- Add 100 µL of sulphuric acid and incubate for 10 min at 25°C.
- Analyze the colorimetric reaction by measuring the absorbances at 492 nm with Infinite F200 Pro (Tecan).
- Generate a standard curve using solutions of known glucose concentrations (0.01-5 mg/mL).
- Calculate the EPS concentration based on the comparison to the standard curve, taking into account any dilution of the sample.

2.3.2.2 HPAEC-PAD

Principle

Dextran is homo- and exopolysaccharide (EPS) synthesized by extracellular dextransucrases produced by LAB of *Weissella*, *Leuconostoc*, *Streptococcus*, *Pediococcus* and *Lactobacillus* in presence of sucrose (De Vuyst & De Vin, 2007). Chemically, they are α-glucans with α-(1 → 6)-linked D-glucopyranosyl units in the main chain and variable amounts of α-(1 →2)-, α-(1 → 3)-, or α-(1 → 4)-branched linkages (Monsan et al., 2001). The enzyme-assisted method can be applied for the analysis of pure dextran; however, it can also be used as a semi-quantitative method of dextran in freeze-dried and homogenized cereal-based fermented samples. This method has been described in Katina et al (2009) for the quantification of dextran produced in-situ by *Weissella confusa*, and then performed in other research works, for instance in Wang et al (Wang et al., 2018), where dextran produced in-situ was evaluated in the quality improvement of wheat-faba bean composite bread. The quantification of the homopolysaccharide is based on initial washing

steps of the samples in aqueous ethanol (50% v/v) to remove free sugars and short oligosaccharides, and the enzymatic hydrolysis of dextran, with the release of glucose that is quantified via high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The method requires the action of two enzymes: dextranase and α-glucosidase. Dextranase catalyzes the endohydrolysishydrolyses of α-(1→6) linkages in dextran, releasing glucose, isomaltose, isomaltotriose and other oligosaccharides, while α-glucosidase hydrolyses maltose and linear isomaltooligosaccharides to final glucose; all hydrolysis reactions permit to quantify dextran depending on the amount of glucose released by breaking its original molecular structure (Katina et al., 2009).

Sample preparation
Fermented samples are freeze-dried and homogenised.

Material and instrument
Aqueous ethanol (50% v/v)
0.05 M Sodium citrate buffer pH 5.5 (0.05 M Citric acid solution is used to adjust the pH of Sodium citrate buffer to 5.5), stored at 4℃
2-Deoxy-D-galactose (ISD, 5.025 mg/mL)
D-glucose (Merck KGaA, Germany)
Commercial dextran (*Leuconostoc* spp. Sigma-Aldrich, Sweden)
Transglucosidase 16670 nkat/ml (α-glucosidase from *Aspergillus niger*, E-TRNGL, Megazyme Ltd., Wicklow, Ireland)
Dextranase 195 808 nkat/ml (dextranase from *Chaetomium erraticum, D0443,* Sigma-Aldrich, Germany)
HPLC: Corporation PA-1 Guard column (4×50mm, Dionex Corporation, Sunnyvale, CA, USA) Waters 2465 pulsed amperometric detector (Waters, Milford, MA, USA).

Standard preparation

Table 2. Glucose standard preparations and the final concentrations.

Standard solutions	**Concentrations (mg/ml)**
Standard 1	0.200
Standard 2	0.150
Standard 3	0.100
Standard 4	0.050
Standard 5	0.010
Standard 6	0.005

Enzymatic activity verification
Before performing the analysis on actual samples, activity of dextranase and α-glucosidase should be verified:

- First dilute both enzymes 1:100 in sodium citrate buffer (pH 5.5).
- Pure dextran (e.g 5 mg/ml) is then dissolved in sodium citrate buffer (pH 5.5).
- Transfer 500 µl of dextran solution into an Eppendorf.

- Add a mixture of dextranase (10 000 nkat/g) and α-glucosidase (1000 nkat/g). In 500 µl add 13 µl dextranase and 15 µl transglucosidase diluted.
- Hydrolyse the solution for 48 h with constant shaking at 30 ℃.
- After hydrolysis, place the samples in a boiling water bath for 10 min to inactivate enzymes.

Quantify the glucose released from the pure dextran by HPAEC-PAD. The amount of dextran is calculated as the sum of anhydro-glucose using correction factor 0.90. Recovery should be between 65-70%. Lower recovery may indicate that the enzyme did not function properly.

Enzyme dosage

The dosage of enzyme is calculated by assuming that the fermented sample contains approximately 5 mg/ml dextran (maximum amount of dextran produced from 10% sucrose addition in the sample).

Procedure to quantify dextran in fermented samples

- Weigh about 100 mg (99-102 mg) of freeze-dried and powdered sample into 15 ml centrifuge tube. Prepare 4 replicates per sample (A, B1, B2, B3). One replicate (A) will be used for correction of glucose background and the others (B1, B2, B3) will be parallel analytical replicates. For each series of samples, two control tubes are prepared weighing 10 mg of commercial pure dextran (by *Leuconostoc* spp.) + 90 mg of fermented sample (one EPS- sample is used) and analysed as other samples; they will be used to calculate the recovery rate of dextran in the analysis.
- In order to remove free sugars and short oligosaccharides, add 3 ml of aqueous ethanol (50% v/v) to all tubes. Vigorously vortex the samples, being careful that large aggregates are not formed, and the solution is dispersed.
- Place the samples in boiling water for 5 min.
- Vortex the sample and add other 3 ml of aqueous ethanol. Vigorously vortex until an even dispersion is formed. All aggregates should be broken down to permit that free sugars and oligosaccharides will be completely dissolved in the solution.
- Centrifuge the mixture at 10 000 rpm for 10 min.
- Discard the supernatant. Add 5 ml of aqueous ethanol, and vortex the sample.
- Centrifuge the sample again and discard the supernatant.
- For hydrolysis, re-suspend the pellet in 4.5 ml sodium citrate buffer (pH 5.5), ensure the samples are evenly dispersed and place them in a boiling water bath for 2 min.
- Vigorously vortex the tubes and place them in the boiling water bath for further 3 min.
- Allow the solutions to cool down before adding the enzymes.
- Dilute enzymes 1:100 to buffer right before using them. Add enzymes to fermented samples (B replicates): 25 µL dextranase (dextranase 10 000 nkat/g) and 30 µl transglucosidase (transglucosidase 1 000 nkat/g) and adjust the volume to 5 ml. For A replicates, only 30 µl of transglucosidase are added.
- Hydrolysis the samples for 48 h at 30 ℃ with constant shaking.
- After hydrolysis, place them in a boiling water bath for 10 min to inactivate the enzymes.
- Centrifuge the samples for 10 min at 10 000 rpm and collect the supernatants.
- Use the supernatants for glucose analysis with HPAEC-PAD. In detail, take 500 µl of supernatants and centrifuge with Amicon centrifuge filters. Dilute the samples before injecting into HPAEC-PAD. (For instance, pure dextran samples are diluted for 20 times, control 5 times, sourdough samples 10-20 times).

- The amount of dextran is calculated as the sum of anhydro-glucose using corrector factor of 0.90.
- Standard curve is built in the HPLC-software, followed by the integration of peaks to obtain the amounts of glucose detected in samples.

Calculation

Below the general equation applied to find dextran content (% dry weight):

Dextran content (% DW) =

(glucose in B – glucose in A) mg/ml x 5ml x 0.9 x 100 x correction factor/ 100 mg

0.9 is for correction of anhydro glucose, correction factor is based on the recovery rate

First calculate the recovery rate from pure dextran tubes amounts:
Amount is multiplied by the dilution done before injecting into HPAEC-PAD the sample, then it is converted from ng/µl to mg/ml dividing by 1,000 to find the mg of glucose/ml of sample. The value is then multiplied by 5 ml (volume when adjusted) to find the actual mg of glucose. In the meanwhile, same is done for B replicates of the sample used to weigh the control tubes (90 mg of sample + 10 mg of pure dextran). From the value of glucose mg is removed the average of values found for B replicates and then is multiplied by 0.9. To convert mg of glucose to mg of dextran, the value is multiplied by 0.9 and then divided by 10 (10 mg was the amount of pure dextran weighed in the control tube) to get the recovery rate that is converted to a percentage. The average of the two control tubes dextran recovery rates will be used as recovery rate for the actual samples.
When recovery rate is known, actual samples results can be calculated:
First steps are the same as control tubes, therefore conversion to mg/ml then multiplication by 5 ml. To calculate dextran mg in samples, from each B replicate value, glucose mg in A is removed, the result is then multiplied by 0.9. To obtain the actual dextran amount, the value is divided by the recovery rate, and finally the dry weight is considered for the result.

Limitation of the protocol

The enzyme-assisted method described above has been used with high accuracy to quantify dextran produced by *Weissella confusa* strains. The enzymes utilized are efficient to cause the breakdown of low-branched dextran, therefore when the molecular structure of this EPS is characterised by a high branching and linkages different from α-(1→6) this method does not permit to obtain an accurate estimation of dextran present in the sample. In fact, the activity of dextranase on other types of α-glycosidic linkages is still unknown (Katina et al., 2009).

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Chapter 9
Phytate and minerals

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Summary

- **Raw material**: Cereals (wheat & oat), pulses (faba bean & yellow pea)
- **Relevant applications:** dough, bread, meat and dairy alternatives, digesta
- **Fermentation-induced changes**: degradation of phytate, increased bio-accessibility of minerals
- **Analytical methods**:
    - Colorimetric determination of total phosphorus content before and after enzymatic hydrolysis (Megazyme)
        - Total phosphorus content
        - Phosphorus released by phytase and alkaline phosphatase
        - Approximation of the phytic acid content
    - Inductively coupled plasma mass spectrometry (ICP-MS)
        - Mineral content

1. Introduction

1.1 What is phytate and how does it complex minerals?

Phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate; IP6) is the major component to store phosphor in cereals and pulses and typically comprises 85% of the total phosphor content (Oatway, 2001; Lemmens et al., 2018). The phytate concentration of cereals and pulses depends on the growth conditions (climate, soil, year) and is on average between 0.2-3.4%dw, with measured concentrations varying between 0.39-1.35%dw for wheat, 0.42-1.16%dw for oat and 0.22-1.22%dw for peas (Schlemmer et al., 2009; Steiner et al., 2007). Phytate is stored in the form of phytate globoids and in cereals it can be found in the aleurone layer and the scutellum cells of the germ (De Brier et al., 2016; Skoglund et al., 2009). However, in legumes, phytate is found in protein bodies, present in the endosperm or the cotyledon (Schlemmer et al., 2009). In **Figure 1A**, the chemical structure of a fully protonated IP6 is shown. The number of protons present depends on the pH and therefore the fully protonated IP6 with 12 protons only exists at extremely low pH (**Figure 1B**) (Oatway et al., 2001; Wang and Guo, 2021). Under physiological conditions, phytate has a strong negative charge which leads to the chelation of cations (**Figure 1C**) and the formation of an insoluble salt, phytin, which forms globoids inside protein storage vacuoles. Phytate globoids confine potassium (K), Magnesium (Mg), Calcium (Ca), Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn), Sodium (Na), Sulfur (S) (Oatway, 2001; De Brier, 2016; Krog Madsen and Brinch-Pedersen, 2019; Lemmens et al., 2018). Despite the fact that cereals and pulses are important sources of minerals in the human diet, chelation of these micronutrients by phytate lowers the availability for absorption in the gastrointestinal tract (*bio-accessibility*) as they precipitate at neutral pH in the intestine, after being solubilized under the acidic

conditions of the stomach (Lemmens et al., 2018; McKie and McCleary, 2016; Schlemmer et al.; 2009). In plant-based diets, the anti-nutritional effect of phytate is considered the most important for inhibiting the absorption of Fe^{2+} and Zn^{2+}, hence, reducing bio-availability (Lemmens et al., 2018). Apart from the chelation of minerals, IP6 can interact with cationic amino acids or form cationic bridges with negatively charged proteins in the presence of divalent cations (Wang and Guo, 2021). Electrostatic interactions, hydrogen bonding or esterification reactions can occur between phytate and polysaccharides. Thanks to the interaction with digestion enzymes, phytate may influence the amino-acid and carbohydrate metabolism (**Figure 1C**) (Brouns, 2022; Wang and Guo, 2021; Oatway et al., 2021).

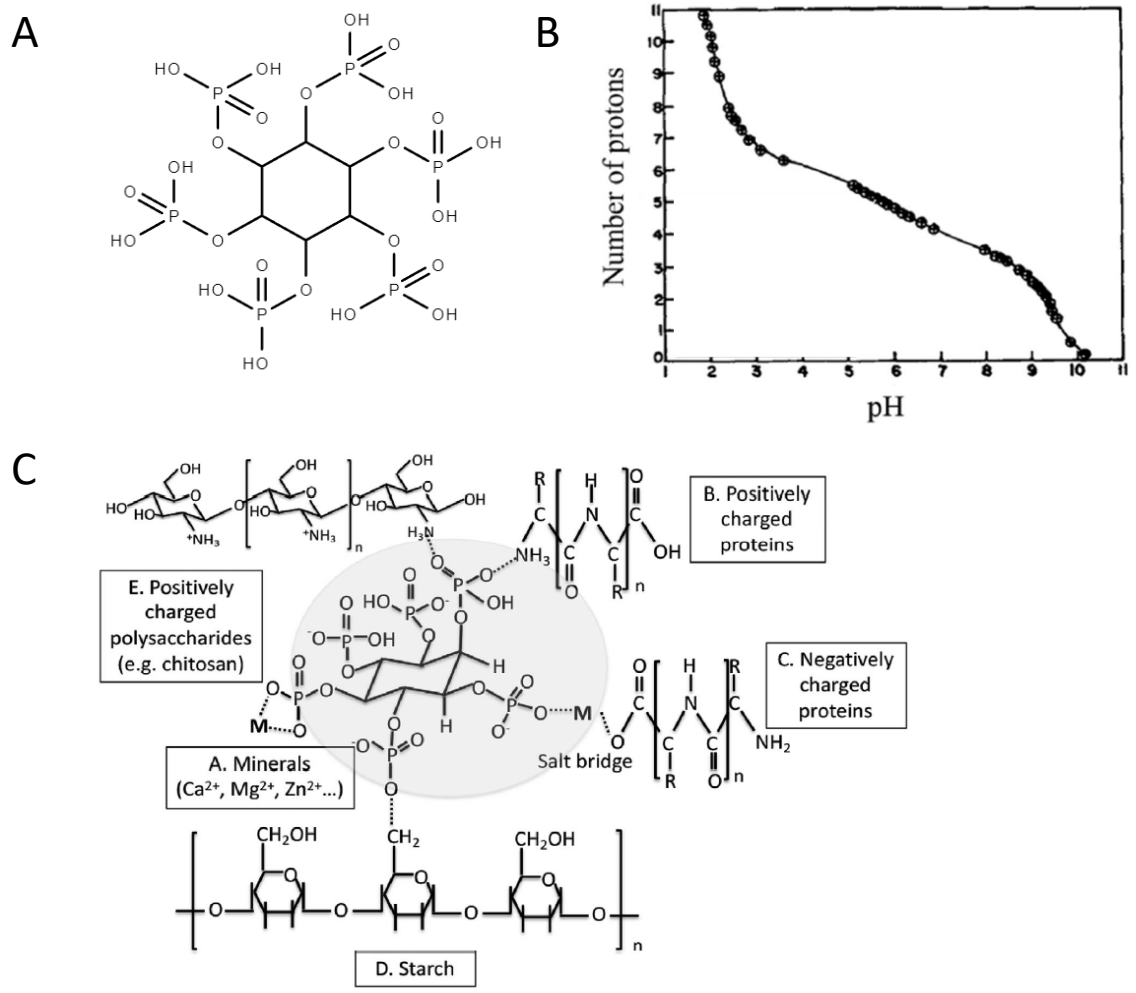

***Figure 1**. A. Molecular structure of fully protonated phytate, with 12 dissociable OH groups. B. Number of protons bound to a phytate molecule in function of the pH of the solution. C. possible interactions of negatively charged phytate with (a) minerals, (b and c) proteins, (d) starch and (e) charged polysaccharides. Adapted from Wang and Guo (2021).*

1.2 How can fermentation affect phytate and the interaction with minerals?

Cereal fermentation has proven to be a powerful tool to achieve dephytination during food processing (Krog Madsen and Brinch-Pedersen, 2019). The molecular structure of phytate and the interaction with minerals can be affected in different ways during fermentation.

First, **endogenous phytases**, which are present in cereals and pulses catalyse the stepwise breakdown of phytate to free phosphate and inositol phosphates during fermentation (Bohn et al., 2007; Greiner, 2002; Konietzny and Greiner, 2002). Phytase activity is mostly associated with the outer layers of cereal kernels and a higher phytase activity is described for bran, compared to the seed and the flour,

respectively (Steiner et al.; 2007). Phytases catalyse the stepwise breakdown of phytic acid (IP6) to free phosphate and inositol phosphates (Bohn et al., 2007). The resulting lower forms (IP5, IP4, IP3, IP2, and IP1) have a decreased chelating potential and the breakdown improves micronutrient bio-accessibility. However, humans and monogastric animals lack sufficient phytase activity in their digestive tract to absorb the micronutrients present as phytin in food and feed, respectively (Krog Madsen and Brinch-Pedersen, 2019; Schlemmer et al.; 2009). As such, the full potential of cereals as a nutrient-rich resource is not completely exploited if phytate is present in the food matrix, and opportunities can be found in the fermentation strategy.

Endogenous phytases in wheat and oats, identified as Histidine acid phosphatase (HAP) and the slightly less active purple acid phosphatase (PAP), have significant potential as stable and potent enzymes both in feed and food (Dionisio et al., 2011; Krog Madsen and Brinch-Pedersen, 2019; Faba-Rodriguez et al., 2022). However, the conventional heat treatment of oats to limit lipid rancidity inactivates the endogenous phytases to a large extent. This may explain the results of Steiner et al. (2007) on the native phytase activity in a variety of feed, harvested over different years, which indicate a higher phytase activity in wheat (2886 ± 645 U/kg dm) compared to oats (496 ± 35 U/kg dm) and peas (262 ± 73 U/kg dm). However, it should be noticed that considerable differences are found in the analysed phytase activity described in the literature, which can be attributed to the extraction procedure and pretreatment of the sample (Steiner et al.; 2007). As a lower endogenous phytase activity is described for legumes and oats, the contribution of microbial or exogenous phytases is relevant to facilitate phytate degradation in these food products (Augustin et al., 2000; Steiner et al., 2007; Verni et al., 2017; Frias et al., 2003).

In addition, the **acidification** that takes place during fermentation can affect the structure of phytate and the interaction with minerals. As shown in **Figure 1B**, more protons will be present at lower pH, which results in a smaller negative charge of phytate. This would decrease the chelating capacity of phytate in a product but will probably not affect the chelating capacity *in vivo* because the pH in the stomach will not be affected. Furthermore, acidification is believed to accelerate the phytase activity and several reviews describe that the pH optima range between 4.5 and 6 (Lemmens et al., 2018; Konietzny and Geiner, 2002). More specifically, the optimum pH of endogenous phytases in cereals and pulses ranges between 5 and 6 in wheat and barley and is described to be 6 in rye and 5 in oat and faba bean (Konietzny and Greiner, 2002).

Finally, phytate can be degraded during fermentation by the growth of **phytase-positive bacteria**, which express extracellular phytase activity (Reale, 2004). Microbial phytases have been identified as Histidine acid phosphatase (HAP) and are commercially used for feed supplementation. Previous research on sourdough described the accelerated breakdown of phytic acid after 12 h fermentation in wheat-based doughs inoculated with L. *plantarum,* L. *brevis*, and L. *curvatus,* compared to dough inoculated with S. cerevisiae (Reale et al., 2004). However, after 36 h fermentation, a yeast-inoculated sample showed the highest IP6 breakdown (Reale et al., 2004). Verni et al. (2017) studied the phytase activity expressed by different LAB strains, isolated from faba bean fermentation through traditional backslopping method and determined a large distribution ranging from 0 to 0.958 U (1 nmol of phosphate form Na-phytate/minute). They attributed the highest activity to strains of Leuc. m*esenteroides* and P. *pentosaceus*. However, strains of Lc. l*actis*, Enterococcus, Lb. s*akei*, W. *koreensis*, W. *cibaria*, and E; *casseliflavus* did not express significant phytase activity toward Na-phytate.

Noteworthy is that an improved bio-accessibility thanks to the breakdown of IP6 does not necessarily facilitate the bio-availability of divalent cations in the small intestine. First, in vitro digestion-absorption assays used to predict what may happen in vivo, report that a significant increase in bio-accessible minerals thanks to fermentation only show a negligible effect on absorption in Caco-2 cells. Second, from the outcome of in vivo trials that include other environmental and dietary factors, it is reasoned that IP6 is only one of many factors influencing overall mineral uptake by the human body (Brouns, 2022). Therefore, IP6 hydrolysis may contribute to an increased total bio-accessibility but may be ineffective in enhancing the bio-availability to reduce the prevalence of mineral deficiencies (Brouns, 2022).

2. Method(s) of analysis

Investigating phytate and its degradation products has received considerable attention during the last decades. Recently, Marolt and Kolar (2021) provided a systematic and chronological review of the analytical methods for the determination of phytic acid and other inositol phosphates. For an overview of the advantages and disadvantages of the techniques, we refer to this manuscript. In this chapter, we focus on the methods that are frequently used and suited to determine the low concentrations present in cereal and legume-based samples. As such, methods based on the quantification of total phosphor determination using colourimetry, the separation of inositol phosphates with HPIC, and the assessment of the mineral concentration with ICP-MS are described and discussed below (**Figure 2**).

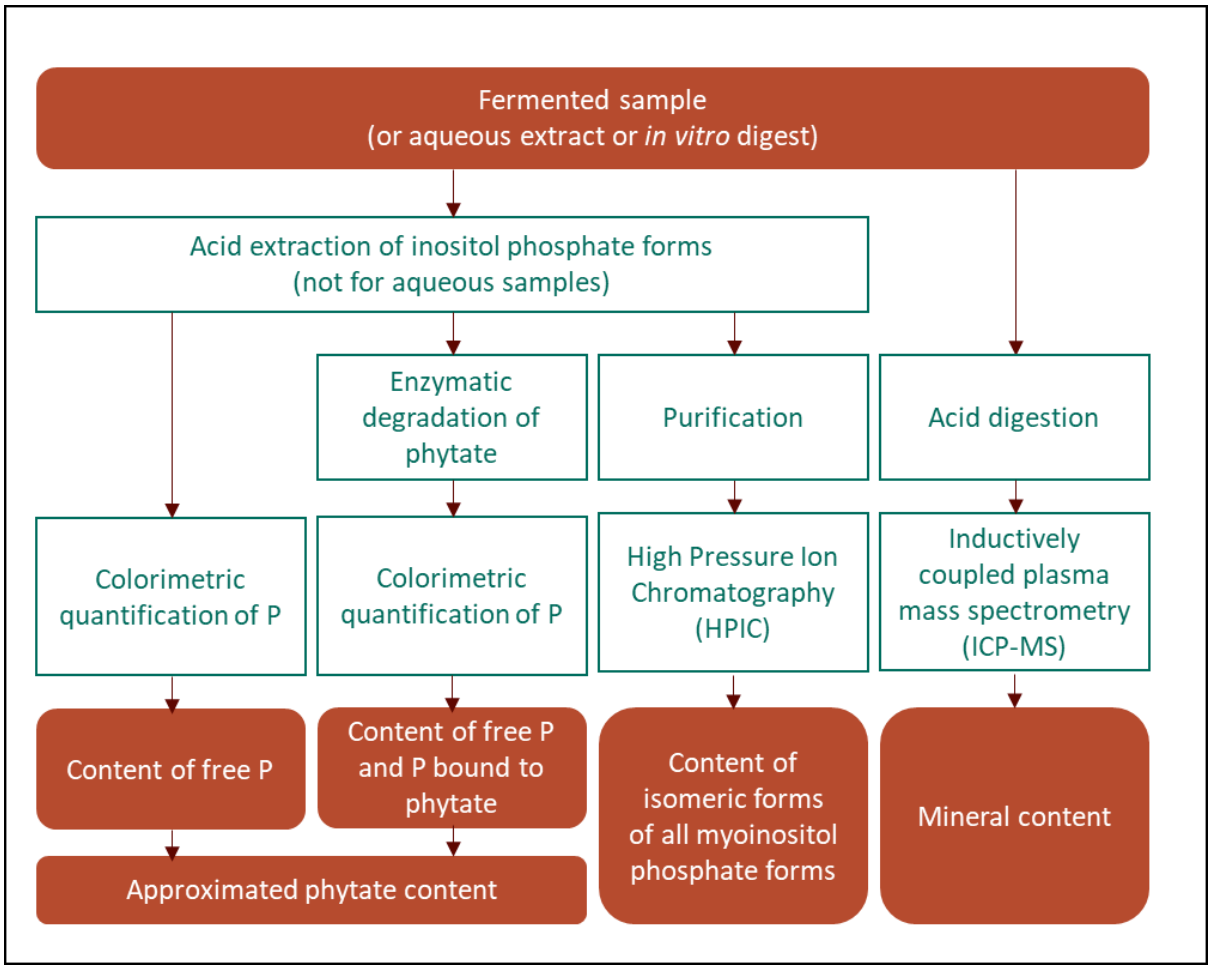

Figure 2. *Schematic overview of sample preparation steps and analytical method(s)*

2.1 Approximation of phytate content based on phosphorus content enzymatically released from inositol phosphates

The first method is a simple and high-throughput procedure to determine phytic acid based on the publication of McKie and McCleary (2016). In this method, the phosphorus content is determined after an acid extraction of inositol phosphates from food and feed samples, followed by an enzymatic treatment with phytase and phosphatase. As such, all phosphate groups are released from all myo-inositol phosphate forms present in the sample which are subsequently quantified using a colourimetric method. The reagents and enzymes are commercially available as the "Total Phosphorus and Phytic Acid Assay Kit" (Cat. No. K-PHYT) developed by Megazyme International (Bray, County Wicklow, Ireland).

2.1.1 General principle

The general principle of this method relies on the enzymatic dephosphorylation of all myo-inositol phosphate forms in the sample. First, the acid-soluble compounds are extracted from the sample during an overnight incubation. Phytic acid and lower (IP5-1) myo-inositol phosphate forms are obtained in the supernatant. Neutralizing the supernatant is required before starting the enzymatic treatment.

Next, phytase is added to the sample in combination with distilled water and a pH 5.5 buffer to optimise the degradation of all myo-inositol phosphate forms (IP6, 5, 4, 3, 2) to IP1 (**Figure 3A**). Second, a pH 10.4 buffer and a phosphatase solution are added to release the last phosphate group from each inositol in the sample (**Figure 3B**). To ensure the total enzymatic degradation, samples are incubated at 40°C for 1.5 h, compared to the proposed protocol by megazyme which applies 10 minutes (megazyme protocol). The enzymatic degradation is stopped by the addition of trichloroacetic acid (50% w/v) and remaining impurities are eliminated by centrifugation.
The colourimetric determination of free phosphate in the sample can be performed after the addition of the colour reagent. During incubation with this colour reagent, the free phophate groups interact with ammonium molybdate, forming 12-molybdophosphoric acid, which leads to a blue colour in the solution thanks to the formation of molybdenum blue in combination with H_2SO_4/ascorbic acid (**Figure 3C**). The amount of free phosphate is proportional to the amount of molybdenum blue and is quantified by analysis of the absorbance at 655 nm which is compared with a calibration curve of known phosphorus concentrations.

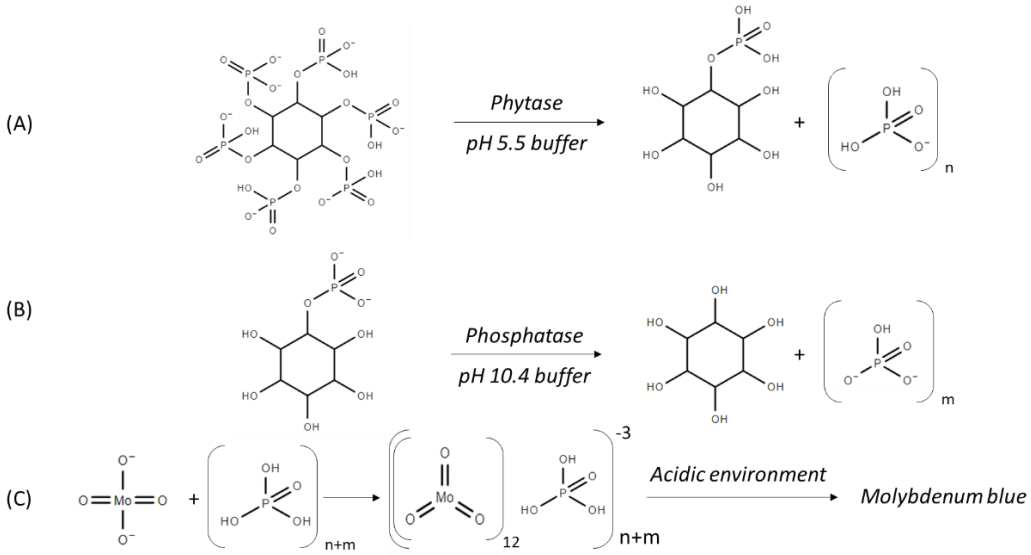

Figure 3. *Reaction mechanism of the procedure based on the enzymatic degradation of phytate and subsequent colourimetric phosphor determination. The charges on the molecules are indicative but vary due to solvent interactions. (A) Phytate and lower inositol phosphate forms are hydrolysed into myo-inositol and inorganic phosphate by phytase in an acidic environment. (B) Alkaline phosphatase hydrolyses myo-inositol phosphate into myo-inositol and inorganic phosphate in alkaline conditions. (C) Inorganic phosphate in combination with ammonium molybdate react to 12-molybdophosphoric acid, which is reduced to molybdenum blue in an acidic environment. Figure made with chemspider.*

Comparing the total phosphate released during the enzymatic treatment with the free phosphate groups in the original sample gives insight into the concentration of phosphorus bound to the myo-inositol in the sample. It is a suitable technique to assess and compare phytate degradation during fermentation. This method can be used to assess the phytic acid concentration in unfermented and unprocessed samples, assuming that all phosphate groups originate from IP6. However, fermented samples contain multiple lower forms of inositol phosphate which will lead to an overestimation of

phytate (IP6). A second drawback could be that alkaline phosphatase releases phosphate from monophosphate esters other than myo-inositol phosphate. However, for cereal flours and pulses, a high accuracy was obtained using the method, and the influence of monophosphate esters is hypothesised to be neglectable (McKie and McCleary, 2016).

2.1.2 Reagents

- Buffers:
  - PH 5.5 (200 mM sodium acetate)
  - pH 10.4 (400 mM glycine, 4 mM magnesium chloride, and 0,4 mM zinc sulphate)
- Enzyme suspensions:
  - phytase (12 000 U/ml)
  - alkaline phosphatase (80 U/ml)
- Standard: phosphorus standard solution (50 µg/mL)
- Colour reagent: 5 parts of Ascorbic acid (1°% w/v)/Sulphuric acid (1M) + 1 part of Ammonium molybdate (5% w/v)
- Extraction solvent: 0.66M Hydrochloric acid
- Neutralising solvent: Sodium hydroxide (0.75 M)
- Acidic solvent: Trichloroacetic acid (50% w/v)

2.1.3 Sample preparation

1. Accurately weigh 1 g* grounded sample (dry or known dm content**) in a 50 mL falcon tube and add 20 ml of hydrochloric acid (0.66 M).

   **The sample concentration during extraction should be adapted according to the amount of phosphate in the sample. The final concentration should be in the linear range of the analysis. ** When working with aqueous samples, one should make sure the final hydrochloric acid concentration approaches 0.66 M and the phosphate concentration in the sample is within the linear range of the analysis.*
2. Incubate overnight (16 h) on a shaking rack (150 rpm).
3. Transfer 1 mL of the extract to a 1.5 mL Eppendorf tube and centrifugate (13 000 rpm, 10 min).
4. Transfer 500 µL of the supernatant to a new 1.5 mL Eppendorf tube and neutralise by the addition of 500 µL sodium hydroxide (0.75 M).

2.1.4 Sample analysis

Label 2 new 1.5 mL eppendorf tubes for each sample with A and B to determine the free and total phosphate concentration, respectively.

1. Add the following solutions to the Eppendorf tubes:

   A. Free phosphate:
   - 620 µL water
   - 200 µL of the pH 5.5 buffer solution
   - 50 µL sample extract

   B. Total phosphate - Degradation of phytic acid and lower inositol phosphates to myo-inositol phosphate:
   - 620 µL water
   - 200 µL of the pH 5.5 buffer solution
   - 50 µL sample extract
   - 20 µL phytase solution (Vortex before use!)
2. Vortex each Eppendorf tube and incubate in a 40 °C water bath for 1.5 hours.
3. Add the following solutions to the Eppendorf tubes:

A. Free phosphate:
- 200 µL buffer solution pH 10.4
- 20 µL water

B. Total phosphate – Degradation of myo-inositol phosphate to inositol and free phosphate
- 200 µL buffer solution pH 10.4
- 20 µL alkaline phosphatase solution (Vortex before use!)

4. Vortex each Eppendorf tube and incubate in a 40 °C water bath for 1.5 hours.
5. Stop the reaction by the addition of 300 µL Trichloroacetic acid (50%, w/v)
6. Centrifuge the tubes (13000 rpm, 10 min) and transfer 1 mL of the supernatant to a new Eppendorf tube.

Colourimetric determination of the free phosphate in the samples

7. Prepare the calibration curve in 1.5 mL Eppendorf tubes.

	0 µg	**2.5 µg**	**5 µg**	**7.5 µg**	**10 µg**
Water (µL)	1000	950	900	850	800
Phosphorus standard* (µL)	0	50	100	150	200

* phosphorus standard with a concentration of 50 µg/mL.

8. Add 500 µL of the color reagent (5 parts Ascorbic acid (1°% w/v)/Sulphuric acid (1M) and 1 part Ammonium molybdate (5% w/v)) to all the Eppendorf tubes (samples and calibration curve), vortex, and incubate in a 40 °C water bath for 1 hour.
9. Vortex and transfer to a cuvette (1 cm light path, 1.5 mL) determine the absorption at 655 nm using a spectrophotometer. Read the absorption against air (without a cuvette) or against water. The absorption should be determined within 3 hours after the incubation.

2.1.5 Calculations

1. Determine the linear ($R^2 > 0.99$) absorbance curve of the phosphorus standards ($Absorption_{655\ nm}$ / µg phosphor), the slope of the curve is used in further calculations.
2. Calculate the concentration of phosphorus in both the "free phosphate" and the "total phosphate" sample using the following equation:

$$c_{phosphorus}\left(\frac{g}{100g}\right) = \frac{V_{initial} \cdot F}{S \cdot 10\,000 \cdot M \cdot V_{end}} \cdot E_{655}$$

With
$V_{initial}$ = Original sample extract volume (mL); 20 mL if using this procedure
F = Dilution factor; 55.6 using this procedure
S = Slope of the calibration curve
10 000 = Conversion from µg/g to g/100 g
M = Mass sample (g)
V_{end} = Sample volume used in the colourimetric determination of phosphorus; 1 mL using this procedure.

3. Expressing the phosphorus ratio of (free phosphate / total phosphate) of each sample can give insight into the degree of phytate breakdown in the sample during fermentation.
4. Under the assumption that the measured phosphate released during the enzymatic hydrolysis exclusively originates from phytic acid (IP6) and that the phosphorus comprises 28.2% of phytic acid [(6 x 32 g/mol)/660 g/mol)], the phytic acid concentration can be calculated with the following equation:

$$c_{\ phytate}\left(\frac{g}{100\ g}\right) = \frac{c_{total\ phosphorus} - c_{free\ phosphorus}}{0.282}$$

! One should take into account that these assumptions only hold for nonprocessed food and an overestimations of the phytic acid concentration will result when using this calculation for fermented samples.

2.2 Quantification of different inositol phosphates with High Pressure Ion Chromatography

Many researchers devoted their time to developing a technique to differentiate isomeric forms of inositol phosphates using HPIC. This sensitive technique based on the charge properties of ions and polar molecules, was first used to determine phytate (IP6) by Phillipy and Johnston in 1985. The separation described below, to determine the profile of phytate and lower inositol phosphate forms (IP2 – IP6) in wheat and a compound diet (barley, wheat, soybean meal, calcium carbonate, amino acids, sodium chloride and vitamin/mineral premix), is developed and validated by Blaabjerg, Hansen-Lokker, & Poulsen (2010). The analysis uses a gradient high-performance ion chromatographic method and is later adjusted with minor modifications to perform the analysis on legume samples by Wainaina et al. (2022).

This method allows the separation of different inositol phosphate forms and their different isomeric forms. However, not all isomers are commercially available and as such, some peaks should be identified by in-house reference standard solutions or based on separation sequences reported in the literature.

2.2.1 2.2.1. General principle

Measuring the hydrolysis products from phytate with HPIC requires well-controlled experimental conditions for the extended purification of the sample. The acid extraction, to release phytate from the complexes with minerals and protein, is followed by several centrifugation and purification steps. Afterwards, the extraction solvent is evaporated to protect the HPIC equipment, and the extract is dissolved in the elution solvent, methanesulfonic acid. This eluent leads to an almost horizontal baseline and thus, a more specific quantification compared to previously described methods using gradients of HCl. The separation involves a gradient of two mobile phases, allowing the separation of different myoinositol phosphate forms. The detection relies on a post-column derivatisation with a colour reagent based on the complexation of myoinositol phosphate forms with Fe^{3+} ions in a $HClO_4$ mixture, that can be measured by UV-detection at a wavelength of 290 nm.

Detection limit reported by blaabjerg: 0.9 - 4.4 mg / L

2.2.2 2.2.2. Procedure

2.2.2.1 Sample preparation

1. Cereals, pulses, and fermented material needs to be freeze dried and grounded to a fine powder with a particle size less than 125 µm using a ball mill.
2. Extract the phytate forms from the sample by incubation of 0.5 g sample into 20 mL of 0.5 M HCl during an overnight (16 h) stirred/shaken incubation. This step can be accelerated using ultrasonication, which should be optimised for the specific sample.
3. Collect the extract supernatant after centrifugation (13 000 rpm, 10 min) and store overnight at -40°C. After thawing, Transfer 1.2 mL of supernatant to an Eppendorf tube and centrifuge (13 000 rpm, 10 min) to eliminate remaining impurities.
4. An aliquot of the supernatant (1 mL) is evaporated to dryness at 50°C to eliminate the extraction solvent (HCl) that could harm the HPIC equipment.
5. Dissolve the dried extract 1 mL of the eluent used for the separation (1.5 M methanesulfonic acid solution, MilliQ water-methane sulfonic acid solution 95:5, v/v), vortex, and filter using 0.45 µm membrane. This eluent results in a more horizontal baseline, compared to using HCl, which improves the specificity of quantification.

2.2.2.2 Analysis

A Dionex BioLC system equipped with an AS50 autosampler, GS50 gradient pump, and an AD25 ultraviolet-visible (UV/Vis) detector can be used to separate the inositol phosphate forms in the sample. 50µL of the sample is injected on a CarboPac PA1 analytical column (50mmx4mm I.d., 10µm) with a Dionex CarboPac PA1 guard column (50mmx4mm I.d., 10µm) and a gradient of two mobile phases (A) 1.5 methanosulfonic acid and (B) MilliQ water at a flow rate of 0.6 mL/min is used. The elution program applied starts with increasing the solvent A concentration from 5 to 28% over 15 min, followed by an increase to 85% in 10 min, which is maintained for 25 min. The column can be re-equilibrated after separation by decreasing the concentration of 1 to 5% over 2 min and maintaining this for 8 min. The separation can be conducted on a column temperature of 30°C.
Detection and quantification of IP forms can be performed with a post-column derivatization reaction with a colouring reagent (0.1% Fe(NO3)3.9H2O and 2% HClO4). Using a UV detector at a wavelength of 290 nm, chromatograms can be generated. Here, InsP-Fe complexes are formed in a Dionex mixing tee and a knitted Dionex reaction coil (750µL, 0.019 mm I.D. x 0.050 mm o.d.). Identification and quantification can be done by analysing individual, external inositol phosphate standards (IP2-IP6)

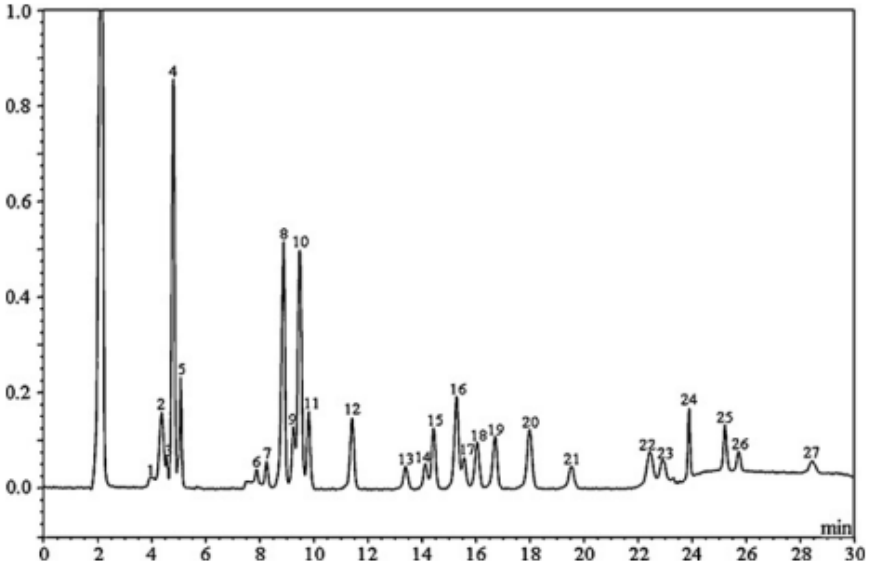

Fig. 3. Chromatogram of reference sample separated on a CarboPac™ PA1 using a gradient with methanesulfonic acid and water. (1–5) $InsP_2$, (6) $Ins(1,3,5)P_3$, (7) $Ins(2,4,6)P_3$, (8–11) $InsP_3$, (12) DL-$Ins(1,5,6)P_3$, (13) DL-$Ins(4,5,6)P_3$, (14) $Ins(1,2,3,5)P_4$, (15) DL-$Ins(1,2,4,6)P_4$, (16) DL-$Ins(1,2,3,4)P_4$, (17) $Ins(1,3,4,6)P_4$, (18) DL-$Ins(1,2,4,5)P_4$, (19) DL-$Ins(1,3,4,5)P_4$, (20) DL-$Ins(1,2,5,6)P_4$, (21) $Ins(2,4,5,6)P_4$, (22) DL-$Ins(1,4,5,6)P_4$, (23) $Ins(1,2,3,4,6)P_5$, (24) DL-$Ins(1,2,3,4,5)P_5$, (25) DL-$Ins(1,2,4,5,6)P_5$, (26) $Ins(1,3,4,5,6)P_5$, (27) $InsP_6$.

Figure 4. from Blaabjerg et al. (2010) (see marolt et al. For the reference – indicates "reprinted with permission from Elsevier) Chromatogram of reference sample analysed by Blaabjerg et al. (2010) shows the separation of the peak of IP6 and the multiple isomeric forms of the lower inositol phosphates.

2.2.3 Reagents

Hydrochloric acid, methanesulfonic acid and iron (III) nitrate nonahydrate minimum 98% are commercially available and needed for sample preparation and analysis.
D-myo-Inositol-1,4-diphosphate (sodium salt) (IP2), D-myo-Inositol-1,4,5-triphosphate (sodium salt) (IP3), D-myo-Inositol-1,3,4,5-tetraphosphate (sodium salt) (IP4), D-myo-Inositol-1,3,4,5,6-pentaphosphate (sodium salt) (IP5) and phytic acid sodium salt hydrate (IP6) standards are commercially available and can be used as external standards. However, it must be taken in to account

that various isomers of the different lower inositol phosphates result from enzymatic and acid degradation and will be present in the samples.

2.3 Determination of mineral content with ICP-MS

Inductively coupled plasma (ICP) - MS is used in the literature to assess the concentrations of minerals and phosphorus in (fermented) food samples or digested samples. The mineral content in a digested sample is a measure for the bio-accessibility of minerals in a food sample.

2.3.1 General principle

ICP-MS is a very precise instrument for multi-element analysis with limit of quantifications (LOQs) generally ranging between 0.01 and 1.6 mg/kg of dm depending on the mineral of interest. As a note, cations with a low mass (*e.g.* Na, Li) are difficult to quantify using the present technique. After acid digestion (see section 2.3.3), the purified sample is evaporated using a laser bundle and brought into an argon plasma (composed of ions and electrons with a high ionization energy) to ensure that all present elements are ionized. Subsequently, the (quadruple) MS separates the different ions based on their mass to load ratio. A quadrupole MS contains four parallel electrical conductive cylinders to which an oscillating electric field is applied. When the ions of the sample enter the (quadrupole) MS, they follow a certain course in the said field which can be detected. A specific profile (showing all isotopes) per element is obtained which finally can be used to calculate elemental concentrations.

2.3.2 Sample preparation

(Fermented) food samples first need to be subjected to acid digestion, for a complete destruction of the sample matrix, with ultrapure nitric acid (HNO_3) or aqua regia [1:3 *v/v* HCl (34% *w/w*): HNO_3 (67–69% *w/w*)] using for example an open digestion block (120 - 150 °C) or microwave digestion system (*e.g.* MARS 6). During microwave digestion, the temperature is gradually increased within 25 min from room temperature to 180 °C, then held constant at this temperature for 20 min and gradually decreased to 65 °C over a 25 min period. Next, the digested samples are diluted to appropriate elemental concentrations using Milli-Q water (18.2 MΩ) prior to analysis. Blank samples and reference samples are included during each acid digestion. The elemental recoveries for the chosen reference sample (*e.g.* NIST samples) should preferably range between 80 and 105%.

2.3.3 Sample analysis

Multi-element analysis in the acid digests (total elemental contents) or in the *in vitro* digests (bio-accessible elemental contents) can be performed using an ICP-MS (*e.g.* Agilent 7700) equipped with a collision cell, generally in the No Gas mode for ^{27}Al, ^{11}B, ^{137}Ba, ^{9}Be, ^{133}Cs, ^{115}In, ^{7}Li, ^{208}Pb, ^{121}Sb, ^{118}Sn, ^{205}Tl, ^{238}U and ^{90}Zr; in the Helium mode (5 mL He min^{-1}) for ^{75}As, ^{44}Ca, ^{111}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{56}Fe, ^{69}Ga, ^{39}K, ^{24}Mg, ^{55}Mn, ^{95}Mo, ^{23}Na, ^{60}Ni, ^{78}Se, ^{88}Sr, ^{47}Ti, ^{51}V and ^{66}Zn; and in the High Energy Helium mode (10 mL He min^{-1}) for ^{31}P and ^{34}S. He gas in the collision cell helps reducing interference by allowing collisions or reactions between elements and gas (Wilschefski & Baxter, 2019). An internal standard containing ^{72}Ge, ^{103}Rh and/or ^{193}Ir can be added online to correct for measurement drifts. The calibration for element quantification used to convert counts per second (CPS) to concentrations is performed using multi-element certified standard solutions (*e.g.* Certipur®). Instrument calibration can be validated using certified reference solutions (*e.g.* SRM 1643f Trace Elements in Water, TM-28.4 Trace Elements in Water). All solutions, including sample solutions, for ICP-MS analyses should be acidified to 1% HNO_3 (*v/v*).

2.4 Other techniques used in the literature

Phytate content and phytase activity have been studied with various analytical methods such as ion-exchange chromatography, complexometric titration, P NMR techniques (Reale et al., 2004).
The NMR-analysis requires the stepwise extraction of phytate with HCL and EDTA, from the dough and a dispersion of the dispersion of the freeze-dried supernatant, respectively. After increasing the pH

with NaOH and filtration, the freeze-dried sample is dissolved in D_2O and the inositol species are stabilised adjusting the pH to 12.6. ^{1}H and ^{31}P NMR spectra can give insight into the IP6 content using 2-aminoethylphosphonic acid (9.4 mM) as an internal reference (Reale et al., 2004). It should be considered that NMR-analyses are expensive and require expertise to correctly interpret the results of complex systems such as foods.

3. Tips & tricks

Phytic acid concentration and the ratio of free phosphate over total phosphate in the sample are generally expressed on dry matter weight of the sample. This facilitates comparison between different studies and new research in the field should respect this to enhance conformity in the field. Therefore, the dry matter weight of the sample should be analysed in a preliminary experiment. Starting the phytic acid analysis after (freeze)-drying the sample standardises the moisture content and could facilitate more accurate measurement.

It is important that all phytic acid is extracted from the sample during the acid hydrolysis step. If large variations are observed within the sample set, it could be due to the coarseness of the samples. It should be noted that extraction is facilitated by smaller particle sizes and that milling the sample could improve the accuracy of the measurement.

Avoid using metal containers, as phytic acid can retain on the surface thanks to its high chelating properties (Healthgrain).

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Chapter 10
Saponins and tannins

Fabio Tuccillo[1], Minnamari Edelmann[1], Silvia Cera[1], Ndegwa H. Maina[1], Anna-Maija Lampi[1], Kati Katina (University of Helsinki, Finland)

Summary

- **Raw material**: Faba bean flour, protein concentrate, protein isolate
- **Relevant applications:** Dairy and meat alternatives.
- **Fermentation-induced changes**: Expected degradation.
- **Analytical methods**:
  - Determination of saponins
    - UHPLC-PDA
  - Total assay of condensed tannins
    - Spectrophotometer

1. Introduction

Saponins and tannins are a diverse group of compounds that are found in varying sources in the plant kingdom. Beans, peas, grains, and quinoa are just a few examples of plant protein sources that include saponins (Price et al., 1987). Tannins are mainly non-volatile secondary metabolites that protect plants from predators and environmental aggressions (Soares et al. 2020). They can be found in dark chocolate, wine, tea, and coffee (Wei et al., 2022), all of which are frequently characterized as having astringent properties. Indeed, both these classes of compounds can create sensory challenges in protein-rich and plant-based ingredients, contribuiting also to bitter taste and acrid flavor (Drewnowski Gomez-Gomez, 2000; Tuccillo et al., 2022; Wang et al., 2022). However, saponins have emerged as comercially demanded compounds with expanding applications in the food, cosmetics, and pharmaceutical sectors because of thier physicochemical (surfactant) properties and their biological activity (Güçlü-Üstündağ & Mazza, 2007). While saponins were once deemed antinutrients, they are now being extensively researched for a their antioxidant, hypocholesterolemic, and anticancer properties (Shi et al., 2004). Tannins are also applied worldwide, from medicial to food use (Khanbabaee & Van Ree, 2001) .

1.1 What are saponins?

Saponins are nonvolatile compounds consisting of oligosaccharide chains connected to an aglycone group, also known as a sapogenin (Shi et al., 2004). Featuring a hydrophilic sugar chain and a hydrophobic, lipid-soluble aglycone group, saponins constitute amphiphilic molecules. The term "saponin" originates from the Latin word 'sapon,' which translates to soap, and 'in,' implying one of (Tay & Koh, 2012). This name is attributed to their capacity to generate soap-like foam when present in aqueous solutions (Shi et al., 2004). Sapogenins, or aglycone groups, exist in two variants: triterpenic and steroidic forms (Figure 1). The classification of saponins is determined by the structure of the aglycone they contain. In triterpenic aglycones, three terpene units form the structure, while in steroidic aglycones, a sterol group characterizes the composition. As a result, saponins fall into the categories of triterpenic or steroidal glycosides, featuring a sugar (monosaccharide or oligosaccharide) attached to a functional group through a glycosidic bond (Shi et al., 2004). Apart from their core structure, saponins are categorized based on the quantity of sugar chains linked to them. Monodesmosidic saponins possess a single simple sugar chain (Güçlü-Üstündağ & Mazza, 2007),

exemplified by the DDMP saponin, which have a 2,3-dihydro-2,5-dihydroxy-6-methl-4H-pyran-4-one (DDMP) moiety at the C-22 position. On the other hand, bidesmosidic saponins, such as the group A saponins, are characterized by having two simple sugar chains. These chains are typically connected to carbon 3 in core structure through an ether linkage and to either carbon 26 or carbon 28 through an ester linkage. The extensive range of saponin configurations results from the differences in aglycone structures, the characteristics of side chains, and their diverse placements on aglycones (Tay & Koh, 2012). As a result, the categorization of saponins can be also subdivided based on the specific food source being examined. For instance, legumes contain a rich variety of soyasaponins, which are grouped into categories A, B, and E. Group A encompasses naturally existing saponins, while groups B and E are derived from the hydrolysis of DDMP saponin forms (Murphy et al., 2008).

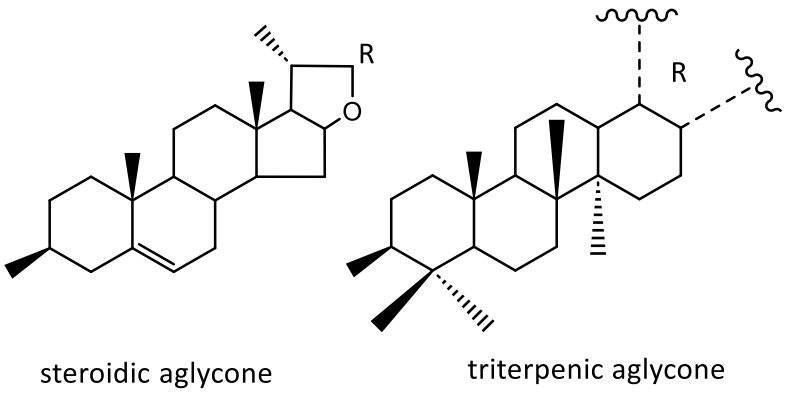

***Figure 1**. Aglycone structures*

1.2 What are tannins?

According to Bate-Smith (1954), tannins are polyphenolic compounds that have a unique capacity to precipitate and agglomerate proteins. Non-flavanoid and flavonoid groups make up the broad classification of tannins. Condensed, hydrolysable, phlorotannins, and complex tannins are located beneath these two major classes (Soares et al., 2020). In this chapter we will focus on condensed tannins, which are classified as flavonoid tannins. They present a flavanic core (Figure 2) and different subunits that make isoflavones, flavonols, flavan-3-ols, and anthocyanins (Soares et al. 2020). Within the flavan-3-ol structural units are (-)-epicatechin and (+)-catechin subunits that further compose proanthocyanidins by interflavonic bonding (Porter, 2017). The type of interflavanic linkages and mean degree of polymerization vary as a result of the wide variety of plants and foods (Soares et al., 2020). A studied chemical property of tannins is the ability to bind with salivary proteins resulting in protein aggregation and precipitation. Whether precipitation or aggregation occurs is due to the protein concentration. In low protein concentrations, a thin layer that is less hydrophilic than the protein itself forms when the tannin binds to several sites on the protein, and aggregation ensues (Spencer, 1988). In higher protein concentrations, the tannins cross-link to various proteins creating a hydrophobic surface layer and causing precipitation of the protein (Spencer, 1988).

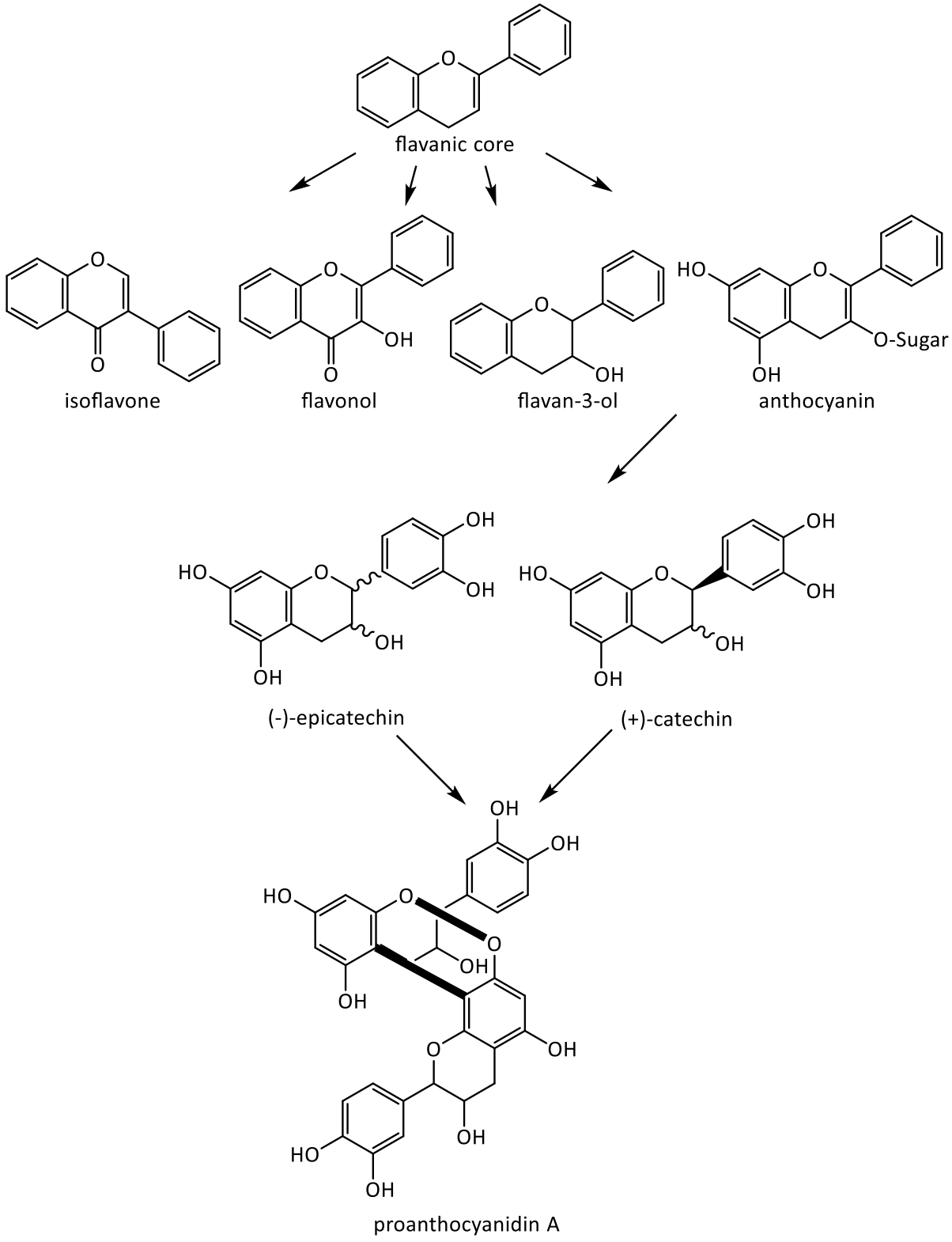

***Figure 2**. Chemical structures of condensed tannins: from the flavonic core to proanthocyanidin A. Modified from Soares et al., 2020.*

1.3 How can fermentation affect saponins and tannins?

Saponins showed a significant function in immune system regulation, reducing cholesterol, antitumor and antiviral activity (Singh et al., 2017). According to del Hierro et al. (2018) and several other studies, saponins are characterized by low bioavailability. However, microbiota was found to hydrolyze saponins to secondary glycosides or sapogenins that showed higher bioavailability and bioactivity than their precursors (Yang et al., 2015). Certain microorganisms have the ability to break down saponins by their glycosidase enzymes, specifically glucosidase, rhamnosidase, or xylosidase. Among microbial genera, *Lactobacillus spp*., *Bifidobacterium spp*., *Bacteroides spp*., *Eubacterium spp*., *Fusobacterium spp*. and *Prevotella spp*. may possess the capacity to hydrolyze saponins (Kim et al., 2018; Navarro del Hierro et al., 2019). Fermentation can reduce, therefore saponin content (Reddy& Pearson, 1994). Moreover, according to De Pasquale et al. (2020), a high decrease in total saponins concentration can be reached when fermentation is combined with gelatinization.

Fermentation can have various effects on tannins depending on the specific context. In the case of lactic fermentation of high-tannin cereals, such as sorghum grains, it has been reported that fermentation can eliminate most assayable tannins (Svanberg et al., 1993). However, this reduction in tannin content is attributed to the formation of insoluble tannin-protein complexes rather than the degradation or loss of tannins themselves (Svanberg et al., 1993). Several lactobacilli having tannase activity were isolated from fermented foods and they included *Lactobacillus plantarum, L. paraplantarum,* and *L. pentosus* (Osawa et al., 2000). This enzymatic functionality is a shared phenotypic trait among these three species.

2. Method(s) of analysis

2.1 Sample preparation

Sample preparation for tannins and saponin analysis requires careful consideration, especially when dealing with fermented raw materials or foods. Fermentation can alter the composition and structure of compounds, potentially affecting their extraction and analysis. Non-fermented materials like faba bean flour can be directly used for analysis without extensive pre-processing. For fermented materials, such as sourdough, begin by freeze-drying the sample. Freeze-drying preserves the sample's composition by removing water without significantly altering the compounds of interest. For solid-model samples like extrudates, start by milling the sample to achieve a homogeneous particle size. Then proceed with freeze-drying. Before the extraction, ensure that the samples are stored properly at -20 °C in airtight containers to prevent moisture uptake.

2.2 Determination of soysaponins B and βG using UHPLC-PDA: General Principle

This analysis focuses on determining the concentrations of soyasaponins B and βG (mg/g) in samples. The procedure involves extracting the saponins using aqueous MeOH, followed by chromatographic separation and quantification using an Ultra-High Performance Liquid Chromatography system with a Photodiode Array detector (UHPLC-PDA). Calibration curves are constructed to relate detected signals to saponin concentrations. The method described is based on Tuccillo et al. (2022).

2.2.1 Reagents

Prepare the following reagents:

- 20% aqueous MeOH solution
- Water (solvent A) and acetonitrile (solvent B), each with 0.025% trifluoroacetic acid

2.2.2 Extraction

- Weigh precisely 0.1 g of the flour into a small Eppendorf tube. Prepare three replicates for each sample to ensure accuracy.
- Add 1.5 ml of a methanol-water (80:20) solution to each tube. This extraction solvent facilitates efficient saponin extraction.
- Thoroughly mix the contents of each tube using either a vortex mixer or a tube shaker. This ensures uniform distribution of the extraction solvent.
- Place the tubes into a table shaker and allow them to shake for 20 minutes. This step enhances the extraction of soyasaponins from the flour.
- After shaking, centrifuge the tubes at 4000 rpm for 5 minutes. Centrifugation separates the solid components from the liquid phase.
- Carefully collect the supernatant from each tube and transfer it into a 5-ml volumetric flask. Repeat steps 2 to 6 for a total of three extractions to ensure comprehensive saponin recovery.
- To ensure thorough extraction, wash the residual precipitate in each tube with 1.5 ml of the methanol-water solution. Gently mix the contents and return the tubes to the shaker for an additional 20 minutes.
- Centrifuge the tubes again and collect the supernatant as before.
- Fill each volumetric flask to the mark using the methanol-water solution. This step standardizes the volume of the extracted solution.
- To remove any particulate matter, filter the samples using a 0.2 µm filter. Collect 1 ml of the filtered solution and transfer it to macro UHPLC vials. This ensures that the samples are free from particulate interference during analysis.
- For storage, transfer the remaining portion (or 1-2 ml) of the extracted solution to Eppendorf tubes. Store these tubes in a freezer at -20°C to preserve the stability of the saponin samples for future analyses.

By following these steps, you can effectively extract and preserve soyasaponins B and βG from legume flours for subsequent UHPLC-PDA analysis, enabling accurate quantification of these compounds.

2.2.3 Standard Curve Preparation

A six-point calibration curve covering the concentration range of 5–10 µg/mL is created for soyasaponin B. This curve acts as a reference for quantifying both soyasaponins B and βG.

2.2.4 UHPLC-PDA

Chromatographic separation and quantification are achieved using an Acquity Waters UPLC system equipped with an HSS T3 C18 column (1.8 µm, 2.1 × 150 mm) and a Photodiode Array detector (PDA) set to monitor in the 190–600 nm range. The mobile phase consists of modified water (solvent A) and acetonitrile (solvent B) with 0.025% trifluoroacetic acid. A gradient elution system at a flow rate of 0.3 mL/min is used with the following mobile phase gradient: 0–1 min (95:5); 1–8 min (50:50); 8–10 min (50:50); 10–12 (20:80); 12–13 min (20:80); 13–14 min (95:5). The autosampler and column are temperature-controlled, and 30 µL injections are made.

2.2.5 Determination

Soyasaponin B is identified based on retention time and UV spectrum (maximum at 200 nm). Soyasaponin βG is identified by its maximum absorption at 294 nm. The UHPLC-PDA setup allows for precise quantification based on these identifying features.

2.2.6 Calculations

Utilize the six-point calibration curve created for soyasaponin B at 200 nm to calculate concentrations of both soyasaponins B and βG in the samples.

2.3 Total condensed tannins assay: General principle

The analysis aims to quantify condensed tannins in a sample using a spectrophotometric method, also known as the vanillin method, developed by Price et al. (1978) and modified by Sun et al. (1998). The tannins are first extracted from the sample using a combination of sulfuric acid in methanol. This extraction breaks down the complex tannins into simpler compounds. The breakdown products react with a vanillin reagent, forming a colored complex. The intensity of this color is directly proportional to the concentration of tannins in the sample. By preparing a standard curve with known concentrations of a tannin-related compound, the absorbance of the sample's-colored complex can be measured and used to determine its condensed tannin concentration. This method allows for accurate assessment of tannin content in various samples.

2.3.1 Reagents

In the preparatory stage, various reagents are assembled for the subsequent analysis:

- A catechin solution is formed by dissolving 6 mg of (+)-catechin in 50 mL of MeOH. This compound acts as a reference for condensed tannins.
- 7.2 N H_2SO_4 is created by mixing 21 mL of concentrated sulfuric acid with 79 mL of MeOH. This strong acidic solution aids in the extraction process.
- A 1% H_2SO_4in MeOH solution is formulated, serving as a milder extraction medium.
- The Vanillin reagent is prepared by dissolving 0.5 g of vanillin in 50 mL of MeOH. Vanillin will react with the tannin breakdown products to produce a colored complex.

2.3.2 Extraction

Sample extraction involves the following steps:

- Accurately weigh a sample of 0.1 g and place it into a 5 ml Eppendorf tube.
- Add 3 ml of the 1% strong H2SO4 in MeOH solution to the sample for extraction.
- Vortex the mixture to ensure thorough mixing.
- Shake the sample for 90 minutes at room temperature using a table shaker to facilitate tannin extraction.
- Following shaking, centrifuge the sample for 10 minutes at 8000 rpm, and collect the supernatant in 2-ml Eppendorf tubes.

2.3.3 Standard Curve preparation

To establish a quantifiable relationship between absorbance and condensed tannin concentration, a 6-point standard curve is generated. This calibration curve provides a reference for converting absorbance measurements into meaningful tannin concentration values. To create this curve, a series of standard solutions are prepared by pipetting varying volumes of the catechin solution along with MeOH, as shown in Table 1.

Table 1. *Concentrations of Standard Solutions for Calibration Curve.*

Catechin solution (µL)	MeOH (µL)	mg/mL
500	0	0.12
400	100	0.096
300	200	0.072
200	300	0.048

100	400	0.024
50	450	0.012

Each standard solution corresponds to a known concentration of the tannin-related compound. After preparation, the absorbance of each standard solution is measured using the spectrophotometer at 500 nm. These absorbance values are then plotted against their respective concentrations to generate the standard curve. This curve serves as the reference for converting the absorbance of unknown samples into condensed tannin concentrations.

2.3.4 Determination

The quantification of condensed tannins in the sample is achieved through a meticulous process. The spectrophotometer is first autozeroed using a sample containing the following components:

- 0.5 ml of 1% H2SO4 in MeOH
- 1.25 ml of the vanillin reagent
- 2.5 ml of 7.2 N H2SO4

This zeroing step compensates for any background absorbance originating from the reagents themselves. Subsequently, for both the sample and the standard solutions, a reaction mixture is assembled as follows:

- 0.5 ml of the prepared sample or standard solution
- 1.25 ml of the vanillin reagent
- 2.5 ml of 7.2 N H2SO4

This reaction mixture is incubated for 15 minutes at a controlled temperature of 35 degrees Celsius. During this incubation, the tannin breakdown products react with the vanillin reagent, forming a colored complex. This complex's color intensity is directly proportional to the tannin concentration. Following incubation, the absorbance of the reaction mixture is measured at a specific wavelength, 500 nm, using the spectrophotometer. The resulting absorbance value is then compared to the standard curve, allowing the determination of the condensed tannin concentration in the sample. This comprehensive process ensures accurate and reproducible quantification of condensed tannins.

2.3.5 Calculations

The concentration of condensed tannins in the sample is calculated using the standard curve generated earlier. This curve correlates known concentrations of the catechin solution with their respective absorbance values, allowing the absorbance of the sample to be converted into a concentration value. To report the results accurately, it's crucial to take into consideration the weight of the original sample, the dilution factor applied during the extraction, and the dry matter content of the sample. The best way to present the results is as milligrams of condensed tannins (catechin equivalents CE) per 100 grams of dry matter (mg/100g dm). This unit accounts for both the concentration of tannins and the weight of the sample's dry matter, providing a standardized and meaningful representation of the tannin content.

3. Tips & tricks

Maintain consistency in sample extraction procedures and instrument parameters to ensure reproducibility. Attention to accurate dilutions and meticulous calibration is crucial for obtaining reliable results.

Regarding total tannin assay, in case of recorded absorbance values under the lowest point of the calibration curve, repeat the analysis and increase the amount of sample weighted (e.g., 0.2-0.5 g). If,

after that, the absorbance is still lower than the lowest point of the standard curve, the concentration of tannins is under the limit of quantification (<5 ng/100g dm).

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Chapter 11
(Con)vicine

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Summary

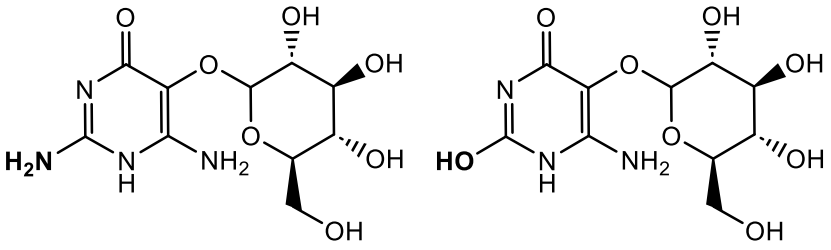
- **Raw material**: Fava bean (*Vicia faba L.*)
- **Relevant applications:** Solid, semi-solid, liquid fermented products containing Faba bean flours, isolates, concentrates
- **Fermentation-induced changes**: It may reduce convicine and vicine contents if selected lactic acid bacteria having specific enzymatic activities are used
- **Analytical methods**:
  - RP-HPLC-UV

1. Introduction

Vicine and convicine are compounds found mainly in the faba bean, a legume plant from the Fabaceae family that grows in various climates. These two compounds are mainly present in the cotyledons of faba beans at 1% of dry matter (Purves et al., 2018). Vicine and convicine are precursors of the aglycones divicine and isouramil (Crépon et al., 2010), which induce severe hemolysis after ingestion in people who suffer from favism, a genetic deficiency of the glucose-6-phosphate dehydrogenase (G6PD)-enzyme (Arese et al., 1981).To enhance the use of faba bean with high nutritional quality for food and feed, several studies investigated different processing methods and fermentation to reduce the content of vicine and convicine in the faba bean. Cardador-Martínez et al. (2012) found that roasting and boiling can affect the content of these two undesired compounds, which result partially thermostable with the convicine that is the most sensitive to thermal treatments. Hegazy & Marquardt (1983) investigated soaking in glacial acetic acid solution at 40°C for 72 h, finding that reduction can be complete. Fermentation and enzymatic hydrolysis were also studied, for instance, by Coda et al. (2015), Rizzello et al. (2016), and Pulkkinen et al. (2019), who studied the application of selected lactic acid bacteria having β-glucosidase activity in different conditions.

1.1 Chemical structure of vicine and convicine

Vicine and convicine are two pyrimidine glycosides, chemically defined as 2,6-diamino-4,5-dihydroxypyrimidine 5-β-D-glucopyranoside and 2,4,5-trihydroxy-6-aminopyrimidine 5-β-D-glucopyranoside, respectively (Fig. 1) (Pulkkinen et al., 2019). Björnsdotter et al. (2021) recently identified the key gene in the biosynthesis of vicine and convicine, moreover they demonstrated that these pyrimidine glycosides derived from purine metabolism, from the riboflavin pathway.



vicine convicine

Figure 1. Molecular structure of vicine and convicine.

1.2 How can fermentation affect vicine and convicine?

Fermentation is a pivotal food technology that can enhance pulses' nutritional profile, for instance, by reducing antinutritional factors naturally present in the raw material. Regarding the vicine and convicine, Coda et al. (2015) observed a decrease of more than 91% of vicine and convicine in faba bean flour samples fermented by *L. plantarum* strain able to synthesize ß-glucosidase. Rizzello et al. (2016) confirmed the potential of *L. plantarum* fermentation in this respect and investigated the proper fermentation conditions to gain more knowledge in the application of this species.
Pulkkinen et al. (2019) found that sourdough fermentation can induce losses of these two compounds, which depends on hydrolysis efficiency. Therefore, it changes based on the strains and the fermentation conditions. Controlled acidification plays a significant role in obtaining acceptable sensory quality of the fermented food matrix.

2. Method(s) of analysis

2.1 General principle

Vicine and convicine are water soluble compounds that can be extracted with water, separated by reversed phase (RP) HPLC with acidic eluents and detected by UV set at e.g. 273 nm. To combine extraction of the analytes and precipitation of proteins, extraction is often conducted with acids, e.g. 7 % percholoric acid. The method is described in detail by Pulkkinen et al. (2015).

2.2 Standard preparation

The standards purchased are vicine (≥98%, Cfm Oskar Tropitzsch GmbH Germany) and uridine (≥99%, Sigma Aldrich, USA). Uridine is used as an internal standard. For calibration, vicine is dissolved in 7% perchloric acid (10 µg/ml) and uridine in Milli-Q water (5 µg/ml) prior to making standard solutions containing vicine (0.0125—0.50 µg/µl) and uridine (0.25 µg/µl). Since convicine was shown to have similar UV absorptivity than vicine at 273 nm, convicine is quantified using vicine calibration curves (Pulkkinen et al. 2015). Uridine solution of 8 mg/ml in water is prepared to be added to samples as an internal standard. Stock solutions of standards are stored at -18°C.

2.3 Sample preparation

Faba bean samples are milled to particle size of 0.5 mm, and sourdough and bread samples are freeze-dried and ground prior to vicine and convicine analysis (Pulkkinen et al 2015; Pulkkinen et al. 2019).

2.4 Reagents and instruments

- Milli-Q water (MilliQPlus system; 0.22 µm, Millipore Corporatioin, Bedford, MA, USA)
- Formic acid
- Acetonitrile
- GHP filter (0.45-µm GH Polypro, GHP, filter; Acrodisc Pall, Cornwall, UK)
- Reversed phase HPLC column: Atlantis T3 C18 (3 µm particle size, 4.6 x 100 mm) with a guard column (4.6 x 20 mm) (Waters, Milford, USA)
- HPLC instrument with diode array detector (or UV detector)
- Data analysis software

2.5 Extraction of vicine and convicine

Homogenized faba bean samples of 0.5 g are weighed into 50-ml centrifuge tubes and 1 ml of uridine solution (8 mg/ml) is added. Samples are extracted with 15 ml of 7% perchloric acid three times. In each step, extraction is done by vortexing for 1 min followed by centrifugation (13 000 g, at 4—8°C for 10 min). Supernatants are collected and filtered using GHP filters prior to HPLC analysis.
Each sample is extracted in triplicate.

2.6 HPLC analysis of vicine and convicine

HPLC separation is performed using reversed phase HPLC and isocratic elution using 0.1% formic acid followed by gradient elution with increasing proportion of acetonitrile to wash the column. Elution is done at the rate of 0.8 ml/min and at 30°C. Injection volumes of 10 µl are used. Vicine and convicine are detected with an UV detector set at 273 nm.

Steps of elution:

0—8 min 100 % 0.1% formic acid
8—15 min increasing proportion of 0.1% formic acid in acetonitrile to 70%
15—22 min 30% 0.1% formic acid; 70% 0.1% formic acid in acetonitrile
22—23 min increasing proportion of 0.1% formic acid to 100%
23— 35 min 100 % 0.1% formic acid

Results are calculated using linear calibration curves and an internal standard method. The results are given as mg/g of the sample.

3. Interpretation and analysis of results

The analytes and the internal standard elute in the order of vicine, convicine, and uridine. According to Pulkkinen et al. (2015), average retention times of these compounds were 5.40 ± 0.02 min, 6.05 ± 0.02 min, and 10.30 ± 0.04 min (n=185), corresponding to retention factors of 2.10, 2.38 and 4.28, respectively.

4. Tips & tricks

Extraction can also be carried out using Milli-Q water, but then proteins need to be removed prior to chromatographic analysis. Purification can be done by using, e.g., Amicon Ultracel® 0.5-ml filters (10 kDa cutoff; Merck, Millipore, Darmstadt, Germany). With fermented materials extracted with water, enzymes i should be denaturated, e.g., by placing test tubes in a boiling water bath for a few minutes prior to filtration.
With fermented samples, it may be good to run the samples first without the internal standard to be sure that fermentation would not produce compounds that would coelute with uridine.
To shorten the run time and to improve separation of the analytes in chromatography, UHPLC may be used instead of HPLC.

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Chapter 12
Vitamin B12

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Summary

- **Raw material**: Cereals (e.g., oat) and pulses (e.g., faba bean, pea), as flours or protein-rich fractions (e.g., protein concentrate, protein isolate)
- **Relevant applications:** Solid (meat, breads and other baked products), semi-solid (dairy products, plant-based yoghurt), and liquid (dairy products, plant-based milk) fermented food
- **Fermentation-induced changes**: *In situ* production of vitamin B12 in plant-based food is achievable tailoring fermentation induced by certain bacteria that are able to synthetize this compound.
- **Analytical methods**:
    - Extraction
    - Immunoaffinity purification
    - UHPLC/UV analysis
    - UHPLC-MS/MS analysis
    - Microbiological Assay

1. Introduction

Vitamin B12, also known as cobalamin (O'Leary & Samman, 2010), is an essential water-soluble micronutrient that is naturally present in food of animal origin, such as meat, seafood, eggs, milk and dairy products (Watanabe, 2007). Both humans and animals require vitamin B12 as a cofactor for properly functioning two enzymes: methyl malonyl-CoA mutase and methionine synthase (Banerjee & Ragsdale, 2003). Since humans cannot acquire this compound from their enteric bacteria, as some animals can, it is essential for humans to obtain it through their diet (Stabler & Allen, 2004). Adults' recommended daily dietary allowance (RDA) is 2.4 µg/day (Institute of Medicine, 1998). Only certain bacteria ad archaeons can synthesize vitamin B12 (Martens et al., 2002). Plant-origin foods do not contain this vitamin (except for algae); however, via the design of fermentation processes or fortification (Watanabe et al., 2013) also, plant-based products can acquire a significant content of vitamin B12. Nowadays, subclinical vitamin B12 deficiency is worldwide spread, particularly among individuals with limited consumption of animal products, such as vegetarian and vegan subjects (Hermann., 2003; Rizzo et al., 2016; Smith et al., 2018). The chemical synthesis of vitamin B12 is complex and expensive. Therefore, commercial vitamin B12 is produced via a biotechnological process. Fermentation with specific starter cultures is, in fact, a cost-effective method for fortifying food materials with micronutrients directly during the production process. A tailored fermentation process represents a powerful tool to prevent vitamin B12 subclinical deficiency in the future worldwide population.

1.1 Chemical structure of vitamin B12

Vitamin B12, also known as cyanocobalamin, was initially identified in the 1920s as a nutrient or extrinsic factor by Minot, Murphy, and Whipple, who conducted research on the alleviation of symptoms associated with pernicious anaemia by incorporating the liver into the diet (Minot &

Murphy, 1926; Whipple & Robscheit-Robbins, 1925). However, the understanding of the structure of vitamin B12 came later, thanks to the pioneering work of Dorothy Hodgkin's research team, who utilized X-ray crystallography (Hodgkin et al., 1956). The scientists revealed that the vitamin has a cyanolated, cobalt-containing, amidated tetrapyrrole structure, leading to its cyanocobalamin designation. Vitamin B12 refers to a group of corrinoids characterized by a cobalt ion at the centre of the corrin ring and axial ligands coordinated with the cobalt ion (Combs & McClung, 2017). In nature, the final products of vitamin B12 biosynthesis are 5'-deoxyadenosylcobalamin (coenzyme B12) and MeCbl. In contrast, the term "vitamin B12" specifically denotes cyanocobalamin (CNCbl), which is predominantly produced by the industry (Fig. 1). The CN group is a result of the extraction process used to isolate the compound from bacterial cultures. The vitamin B12 molecule can be, therefore, divided into three parts: a central corrin ring that contains four ligands for the cobalt ion, a lower (alpha) ligand contributed by 5,6-dimethylbenzimidazole (DMB), and an upper (beta) ligand derived from either an adenosyl group or a methyl group (Martens et al., 2002).

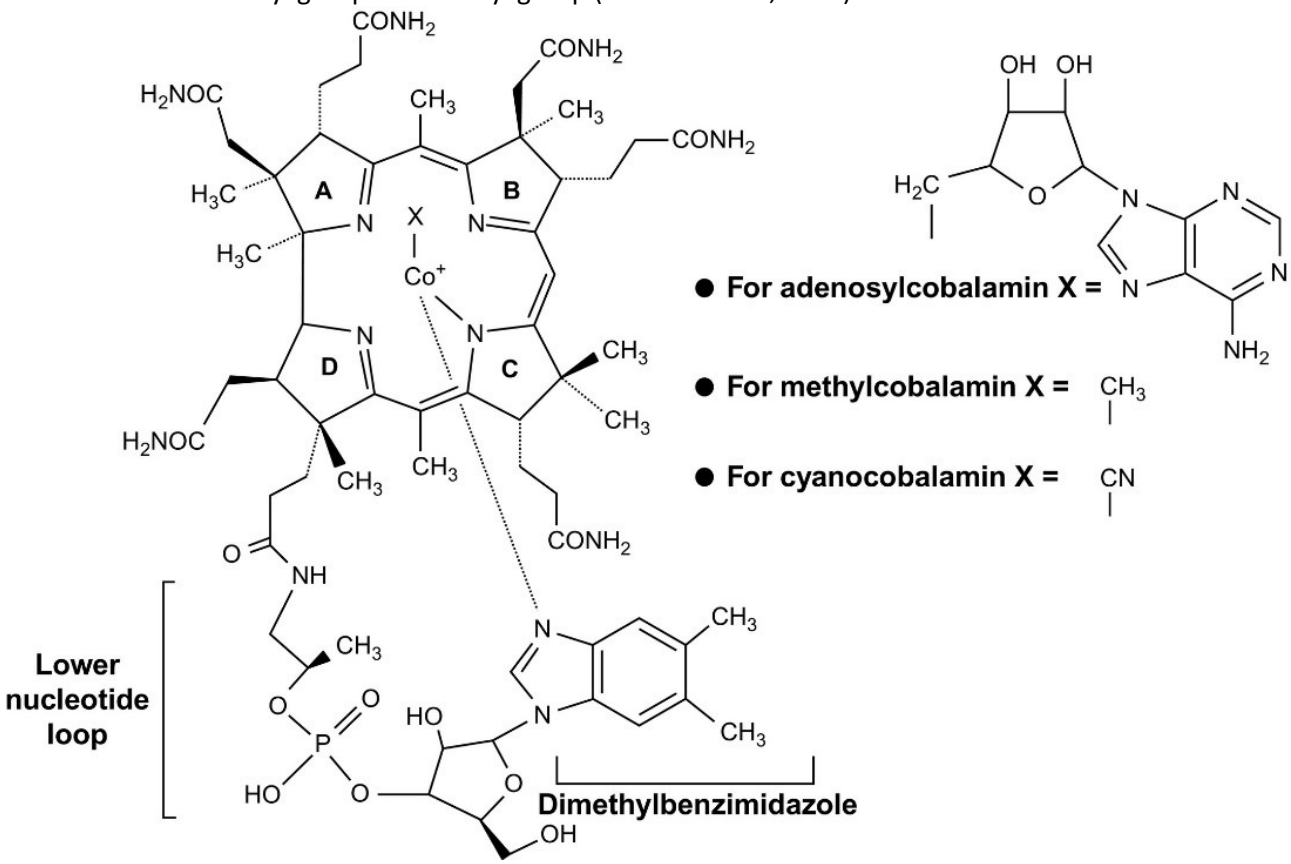

Figure 1. Structure of vitamin B12 as cyanocobalamin when X is CN. The primary biological forms of cobalamin have the cyano group replaced with an adenosyl, methyl, or hydroxyl group, which are found in adenosylcobalamin, methylcobalamin, and hydroxocobalamin, respectively (Smith et al., 2018).

In this vitamin, the cyano group represents the upper ligand, while the nitrogen from DMB is the lower ligand. In biological systems, the upper cyano ligand can be substituted by an adenosyl group, resulting in adenosylcobalamin (AdoCbl), a methyl group, leading to methylcobalamin (MeCbl), or water, giving rise to hydroxocobalamin (Smith et al., 2018). Some biological systems employ a different lower base to which the cobalt is bound; for instance, the DMB can be substituted with bases such as adenine, substituted benzimidazoles that encompass hydroxy or methoxy benzimidazole, or phenolic compounds including phenol or cresol (Hazra et al., 2013). However, the coenzyme forms of vitamin B12 are limited to MeCbl and AdoCbl. MeCbl is essential for the remethylation reaction of homocysteine to methionine, facilitated by methionine synthase. This reaction is significant for DNA synthesis, working in conjunction with folate. On the other hand, AdoCbl is utilized as a cofactor for methyl malonyl-CoA mutase, enabling the conversion of methyl malonyl-CoA to succinyl-CoA. This

reaction is involved in the metabolism of cholesterol, odd-chain fatty acids, and various amino acids (Pawlak et al., 2013).

1.2 *In situ* vitamin B12-fortification of food via fermentation

Chemical synthesis of vitamin B12 is technically challenging and expensive; therefore, the current commercial form is produced via a biotechnological process, which involves using selected microorganisms. Microorganisms belonging to 20 genera were identified as B12-producing strains; however, due to the higher yields achieved, the industrial production via microbial fermentation is mainly induced by strains belonging to *Propionibacterium freudenreichii*, *Bacillus megaterium* and *Pseudomonas denitrificans* species (Hugenholtz & Smid, 2002). After microbial fermentation, extraction, purification and conversion to cyanocobalamin are performed (Martens et al., 2002). Due to the cost-effectiveness and efficiency of the procedure, the in situ production of vitamin B12 via fermentation represents an essential tool for food fortification during processing. Several studies about the *in situ* production of vitamin B12 in cereal and food matrices were conducted in the last decade, and among these, the use of *P.freudenreichii* strains was extensively investigated (Chamlagain et al., 2015, 2018; Xie et al., 2018, 2019, 2021; Edelmann et al., 2016; Wang et al., 2022). Propionic acid bacteria (PAB) are mesophilic and aerotolerant Actinobacteria, characterised by the ability to produce propionic acid as the main metabolic end-product. Among *P. freudenreichii* strains, two subspecies can be distinguished due to their metabolic features: *P. freudenreichii subsp. freudenreichii*, which cannot use lactose but can reduce nitrates, and P. freudenreichii subsp. shermanii can utilise lactose but cannot reduce nitrates (Thierry et al., 2011). Although the high yield of these strains for vitamin B12 production, the in situ fortification of cereal food induced by them involves some challenges to face. The first example is the optimal pH for cell growth of *P. freudenreichii*, which is around 7. Cereal fermentation is commonly characterised by a low pH, reached due to the production of acidic compounds by lactic acid bacteria, and this may inhibit the synthesis of vitamin B12 in the matrix. Therefore, it becomes essential to ferment sterilised raw materials with neutral or slightly acidic pH (Chamlagain et al., 2018). In this chapter, the analysis procedure for vitamin B12 from fortified fermented foods described is originally reported by Chamlagain et al. (2015).

2. Methods of analysis

2.1 Sample preparation

Analysis can be conducted on flour ingredients or freeze-dried samples. If sourdough or other fermented samples are analysed, firstly remove the water with freeze-drying.

2.2 General principle of vitamin B12 analysis

Several studies have previously shown the reliability of high-performance liquid chromatography (HPLC) for vitamin B12 analysis in different food types. Furthermore, ultra-high-performance liquid chromatography (UHPLC) enhances the sensitivity and efficiency of vitamin detection (Chamlagain et al., 2015; Wang et al., 2021). For this reason, the Association of Official Analytical Chemists (AOAC) adopted a method that combined HPLC with immunoaffinity extraction as the recommended internationally recognised technique (Campos-Gimenes et al., 2012). In addition, mass spectrophotometry (MS) combined with the HPLC permits the identification of the molecule, possibly distinguishing it from inactive corrinoid compounds (Watanabe & Bito. 2018; Edelmann et al., 2019). This chapter aims to describe a suggested method of analysis, reported by Chamlagain et al. (2015), that is sensitive and can be adopted when vitamin B12 is produced in situ in plant-based food products.

2.3 Chemicals, materials and reagents (for all preparation and analyses to conduct)

- Sodium hydroxide, analytical grade (Merck, Darmstadt, Germany)
- Acetic acid, analytical grade (Merck, Darmstadt, Germany)
- Vitamin B12 assay medium, analytical grade (Merck, Darmstadt, Germany)
- Ethanol (Altia, Rajamäki, Finland)
- Acetonitrile, HPLC grade (Sigma-Aldrich, Steinheim, Germany)
- Trifluoroacetic acid (TFA) (Sigma-Aldrich, Steinheim, Germany)
- Sodium cyanide (Sigma-Aldrich, Steinheim, Germany)
- Cyanocobalamin (Supelco, Bellefonte, USA)
- Tween 80 (Sigma, Aldrich, USA)
- α-amylase (EC 232-588-1, A9857-5MU, *Aspergillus oryzae* (Sigma-Aldrich, Steinheim, Germany)
- Certified reference material BRC 487 (lyophilised pig liver; Institute for Reference Materials and Measurements, Geel, Belgium; Sigma-Aldrich
- MilliQ water (MilliQ Plus system, Millipore corporation, Bedford, MA, USA)
- Cyanocobalamin stock solution (200 µg/mL) prepared in 25% ethanol/MilliQ water and the concentration is measured by a spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Inc., USA) at 361 nm, according to Indyk et al. (2002)
- Immunoaffinity column Easy extract® (RBiopharma, Glasgow, Scotland)
- Paper filters (Ø 90 mm, VWR, Leuven, Belgium)
- Filters 0.45 µm (Pall, Cornwall, UK)
- Methanol
- Syringe filters 0.2 µm (Pall, Cornwall, UK)
- Waters UPLC vials
- Waters Acquity UPLC system (Milford, MA, USA) equipped with a photodiode array detector (PDA; 210-600 nm)
- Waters Empower 2 software
- Two reversed-phase C18 columns, high strength silica T3 (HSS) and ethylene bridged hybrid (BEH) (Waters, Milford, MA, USA) (2.1 x 100 mm; 1.8 µm and 1.7 µm particles)
- Esquire-LC quadrupole ion trap mass spectrometer with an electro-spray ionization (ESI) interface (Bruker Daltonics, Bremen, Germany)
- LC-MSD Trap version 5.2 (Bruker, Daltonics)
- 96 well microtiter plate (Corning, NY, USA)
- *L. delbrueckii* ATCC 7830
- Microplate reader (Multiskan EX; Labsystems, Finland) with Ascent software version 2.6 (Labsystems)

2.4 Vitamin B12 extraction

- All analytical processes need to be carried out under subdued light or protected from direct light;
- The protocol for vitamin B12 extraction (as cyanocobalamin) involves using 1-5 g of cereal matrix or 0.5 g of freeze-dried sample (Wang et al., 2022). The sample is weighed in duplicate in extraction tubes;

- Add 10 (Chamlagain et al., 2015) or 15 mL (Wang et al., 2022) of buffer (8.3 mM sodium hydroxide and 20.7 mM acetic acid; pH 4.5 containing 100 µL 1 % sodium cyanide (1% w/v in water) and mix the samples on a stirrer;
- Place the samples in a boiling water bath for 30 minutes;
- Place the samples in an ice bath to cool them down until approximately 37°C;
- If sample is cereal-based, to ease the purification with the immunoaffinity system, add 300 µL of α-amylase (50 mg/mL) and incubate at 37°C for 30 min. to digest the starch;
- Centrifuge at 6,900 × g for 10 min and carefully collect the supernatant into new tubes;
- Suspend the pellet remained in 5 mL of extraction buffer, vortex, and centrifuge the mixture again (6,900 × g, 10 min);
- Filter the supernatants with paper filters (Ø 90 mm), combine the supernatants, and adjust the final volume to 25 mL with extraction buffer. 10 mL of these supernatants will be used for the next purification step by immunoaffinity method.

2.5 Immunoaffinity purification for UHPLC quantification

- Drain the buffer in the immunoaffinity column;
- Filter 10-15 mL of the extract with 0.45 µm filters;
- The column is washed with 10 mL of MilliQ water, and 50 mL of air is applied;
- Vitamin B12 is eluted with 3 mL of methanol, and the elution is completed with an additional 0.5 mL of methanol;
- The eluate is evaporated at 50°C under a stream of nitrogen, and the residue is reconstituted with 300 µL of water, which is then syringe filtered (0.2 µm) to a Waters total recovery UPLC vial.

2.6 Ultra high performance liquid chromatography and UV method (UHPLC/UV analysis)

2.6.1 Protocol of UHPLC/UV

1. A Waters UHPLC system (Milford, MA, USA) equipped with a photodiode array detector (PDA; 200-600 nm), which will record the absorption spectra, and an Acquity HSS T3 C18 column (2.1 × 100 mm, 1.8 µm) are used for analysis; the detection is performed at 361 nm;
2. The autosampler is set up at 4°C, while the column is operated at 30°C;
3. The mobile phase consisted of 0.025% trifluoroacetic acid (TFA) in water (solvent A) and 0.025% TFA in acetonitrile (solvent B), with a flow rate of 0.32 mL/min. An external calibration curve for quantitation was prepared by injecting six cyanocobalamin standards (0.015–0.75 ng/µL) in duplicate;
4. Load the UPLC vials containing the sample into the sample holder;
5. The autosampler will inject the sample solution (2-15 µL) onto a column via a 20 µL injection loop, operated in a partial-loop mode.

2.6.2 Ultra high performance liquid chromatography and tandem mass spectrophotometry (UHPLC-MS/MS analysis)

1. The mobile phase is modified with 0.5% formic acid, replacing 0.025% TFA;
2. Ion positive mode is used for MS analysis on the Esquire-LC quadrupole ion trap mass spectrometer with an electronic spray ionisation (ESI) interface. At the same time, the data are analysed by the LC-MSD Trap version 5.2.;
3. The scanning is carried out for a range of m/z 900-1400, and the tandem mass spectrometry (MS/MS) is performed for a specific m/z according to the target

molecule using helium as the collision gas. Other corrinoids, such as pseudovitamin B12, were recognised in the chromatograms based on their retention times and absorption spectra. For the analysis of cyanocobalamin ([M+H]+ m/z 1356) and pseudo cyanocobalamin ([M+H]+ m/z 1345) the settings are described below:

Nebulizer (nitrogen)	Dry gas (nitrogen)	Dry temperature	Capillary	End plate offset	Trap drive
50 psi	8.0 L/min.	300 °C	4500 V	-250 V	84

2.7 Microbiological assay (MBA)

The MBA is performed using a 96-well microtiter plate (Corning, NY, USA), according to Kelleher and Broin (1991). *L. delbrueckii* ATCC 7830 is used as an assay organism, and cyanocobalamin as a calibrant.

1. Dilute the sample extracts at two levels with the extraction buffer (pH 6.2);
2. A certified reference material, BCR 487 (pig liver) extract, served as a reference sample in each set of samples.
3. A blank extract is used to obtain the actual readings of the samples.
4. Inoculate 100 µL of cyanocobalamin solutions of increasing concentration (0–8 pg/well) and sample extracts into the wells of the microtiter plate (4 wells for each concentration);
5. Add 200 µL of freshly prepared sterile filtered vitamin B12 assay medium (pH 6.2) inoculated with the cryopreserved assay organism was introduced into each well.
6. Incubate the plate at an optimised condition (35 °C; 19 h);
7. Measure turbidity with a micro-plate reader (Multiskan EX; Labsystems, Finland) at 595 nm. A calibration curve of 8 concentration levels and the amount of vitamin B12 in each well were obtained automatically by Ascent software version 2.6 (Labsystems).

3. Tips & tricks

The repeatability of the experiment can be checked by carrying out two independent experiments on 2 days.

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Chapter 13
Free fatty acids

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Summary

- **Raw material**: Cereals (e.g., oat) and pulses (e.g., faba bean, pea), as flours or protein-rich fractions (e.g., protein concentrate, protein isolate)
- **Relevant applications:** Solid (meat, breads and other baked products), semi-solid (dairy products, plant-based yoghurt), and liquid (dairy products, plant-based milk) fermented food
- **Fermentation-induced changes**: Certain microbial strains have lipase and lipoxygenase enzymes that can release free fatty acids into the food matrix, in addition some may be able to enhance the antioxidant capacity of food environment
- **Analytical methods**:
    - NP-HPLC-ELSD

1. Introduction

Fatty acids (FA) are the major components of lipids containing an aliphatic chain with a carboxylic acid group (Damodaran et al., 2007). In cereals grains, fatty acids are present in all parts, such as in the intercellular membranes, spherosomes, starch and protein bodies (Chow, 2007). In pulse grains, lipids are located in the embryo axis, while the cotyledons and seed coat have these components in low amount. In cereal and pulse grains, among natural occurrences, lipid degradation plays a significant role in the release of free fatty acids (FFAs) that are more susceptible to chemical or enzymatic oxidation (Tiwari & Singh, 2012). Lipid oxidation occurs by three ways: autoxidation, enzymatic-catalysed oxidation, and photo-oxidation. According to several studies, reported by the review of Shadidi & Oh (2020), during food processing Strecker degradation and Maillard reaction may involve lipids resulting in the formation of multiple volatile compounds. Lipids significantly contribute to food flavor formation, especially in the presence of high amounts of unsaturated fatty acids. In certain foods, such as pulses (Tiwari & Singh, 2012) and meat (Shadidi, 2002), lipid autoxidation via a free radical chain releases hydroperoxides, which are unstable compounds that decompose to aldehydes, hydrocarbons, alcohols, ketones, acids, esters, furans, lactones, and epoxy compounds, which are flavor-active. In particular, aldehydes are highly reactive with other compounds, forming off-flavor molecules. Regarding the enzymatic-catalyzed oxidation of lipids, it is mainly due to the activity of lipid-modifying enzymes, such as lipase, lipoxygenase and peroxygenase. Endogenous lipase leads to the release of FFAs (from their esters) that undergo oxidative reactions and the formation of hydroperoxides, due to lipoxygenase (LOX) activity. Peroxygenase (POX) leads to the formation of non-volatile flavor molecules, known as epoxy and hydroxyl fatty acids, by degrading unsaturated fatty acids in presence of hydroperoxide. The activity of these three enzymes in oat and faba bean samples was studied and described by Yang et al. (2017) to determine their role in the lipid-derived off-flavor release. Among the three lipid-modifying enzymes mentioned above, in oat, the activity of lipase and POX occurred significantly, while in faba bean lipase and LOX were the most active enzymes. Oat-based raw materials commonly undergo hydrothermal treatments, for instance, kilning, to inactivate lipases and other enzymes, due to high presence of polyunsaturated fatty acids (PUFAs), e.g., linoleic

and linolenic acids that are easily oxidized (Lampi et al., 2015; Yang et al., 2023). Similarly, heat treatment was considered effective for controlling LOX activity in legumes (Roland et al., 2017).

1.1 Chemical structure of free fatty acids

Fatty acids are organic molecules consisting of a hydrocarbon chain with a carboxyl group (-COOH). The hydrocarbon chain can vary in length and degree of saturation, leading to different types of fatty acids (Figure 1) (Damodaran et al., 2007). The carboxylic acid group is polar and hydrophilic (water-attracting), while the hydrocarbon chain is nonpolar and hydrophobic (water-repelling). This amphiphilic nature (having both hydrophilic and hydrophobic parts) allows FFAs to play crucial roles in lipid-based structures and processes.

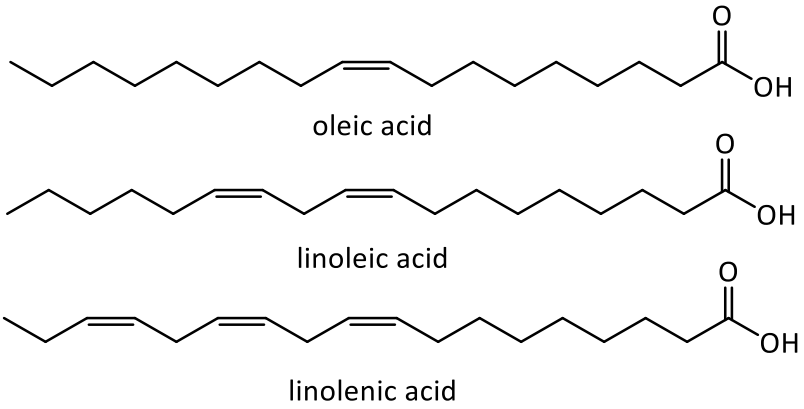

Figure 1. *Three free fatty acids with different unsaturation levels*

1.2 How can fermentation affect FFAs?

Fermentation plays a significant role in the development of the typical flavor in fermented food products. Microbial starters have the ability to synthetize lipases and phospholipases that can release free fatty acids, which contribute to desirable flavor compounds formation. However, in case the oxidation of lipids becomes excessive the food product undergo an evident decrease of nutritional and sensorial quality, resulting in spoilage. On the other hand, some microorganisms may be able to increase the antioxidant activity in food matrixes. Zhao et al., (2021) reported several studies, which focused on this specific potential of microbial starters. For instance, *L. rhamnosus* showed a significant influence on lipid peroxidation inhibition in cereals (Đorđević et al., 2010), and lead to the formation of antiradical compounds in soymilk (Marazza et al., 2012). Moreover, other studies found other microorganisms with this ability, e.g. *L. plantarum* in cowpea, *Cordyceps militaris* in chickpea, and *Streptomyces* sp., *Acetobacter* sp., *Lactobaillus* sp. and *Saccharomyces* sp. in soybean.

2. Method(s) of analysis

2.1 Sample preparation

When preparing samples for free fatty acid analysis, meticulous attention is required to ensure accurate results. The nature of plant-based materials, particularly those susceptible to oxidation, demands careful handling to prevent alterations in FFA composition and content. Plant-based materials are prone to oxidation especially during storage and exposure to heat, oxygen, and light. These factors can promote fatty acid oxidation, leading to undesirable changes in the composition and concentration of FFAs. Therefore, plant-based materials should be stored in airtight containers, in the dark, and at low temperatures, ideally at -20°C prior to analysis. Flours, protein concentrates, and protein isolates can be extracted as such. Sourdoughs should be freeze-dried before analysis, whereas food models (e.g., meat alternatives) should be first homogenized and then freeze-dried.

2.2 General principle

The method for analyzing (Figure 2) FFAs involves extracting and quantifying the fatty acid present in a sample, as presented in previous research (Lampi et al., 2020; Tuccillo et al., 2022; Yang et al., 2019) The principle of this method relies on the separation of FFAs from the sample matrix using an extraction process and subsequently quantifying these FFAs using NP-HPLC (Normal Phase High-Performance Liquid Chromatography) analysis coupled with an ELSD (Evaporative Light Scattering Detector) system. The advised extraction process is Accelerated Solvent Extraction (ASE), which involves an automated system that reduces extraction time and manual handling (Lampi & Piironen, 2009). In ASE, a sample is placed in a sample cell, which is then filled with a solvent or mixture of solvents. The entire cell is then placed in an extraction chamber after the sample cell has been sealed. An inert gas, such as nitrogen, is used to pressurize the extraction chamber. The efficient extraction of analytes from the sample matrix is made possible by the interaction of heat, pressure, and solvent penetration. The UHPLC separates the FFAs based on their physical and chemical properties such as molecular weight and polarity. The ELSD detects the analytes by measuring the scattering of light due to the presence of the analytes in the mobile phase. During the UHPLC-ELSD analysis, the FFAs are eluted from the chromatographic column and are carried into the ELSD detector. In the ELSD, the eluted FFAs are nebulized into a spray of fine droplets. The solvent is then evaporated from these droplets, leaving behind the analytes in the form of solid particles. These particles scatter light when exposed to a laser beam. The scattered light is then collected and measured by the detector. The intensity of the scattered light is proportional to the concentration of the analytes, in this case, the FFAs.

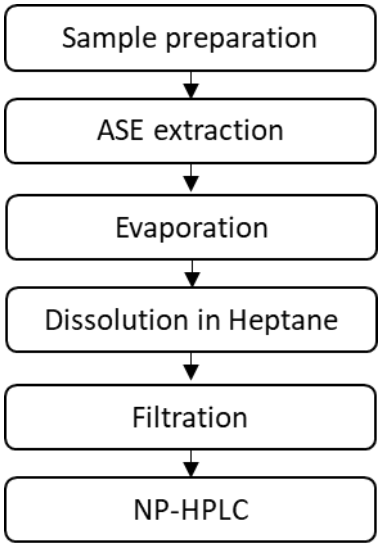

Figure 2. *Schematic overview from sample preparation to normal phase high performance liquid chromatography*

2.3 Reagents

- Eigh Ethanol
- Acetone
- Heptane
- 0.1% Acetic acid in heptane
- 3% 2-Propanol in heptane
- Oleic acid

2.4 ASE Extraction

Carry out the analysis in triplicate:

- Weigh the sample, ranging from 0.5 to 2 grams of flour/freeze dried sample, into an ACE cell with a cellulose filters at the bottom.
- Mix the sample with an equal volume of drying agent (e.g., Sand) to aid in the extraction.
- Add 1 ml of ethanol to the sample and fill the cell with more drying agent.
- Assemble the cell with the top part and tighten it.
- Repeat the process for each flour sample, ensuring triplicate extractions.
- Place the prepared cells in an ASE extractor in consecutive positions.
- Prepare marked vials with proper septa for sample extraction and rinsing.
- Set the following extraction parameters:
    - Solvent: Acetone
    - Temperature: 100°C
    - Pressure: 1000 psi
    - Heating time: 5 min
    - Static cycle time: 10 min
    - Number of static cycles: 2
    - Flush volume: 60%
    - Purge time: 60 s
    - Rinsing time: 1 min

2.5 Preparation for UHPLC-ELSD analysis

- Carefully transfer each extract from the ACE vials to a round-bottom flask using ethanol. Ensure thorough rinsing to maximize analyte transfer and minimize losses.
- Evaporate the lipid solution using a rotary evaporator at a temperature below 37°C.
- Re-dissolve the resulting lipid residue in about 2 ml of heptane.
- Transfer the re-dissolved lipid solution to a 10 ml volumetric flask with a Pasteur pipette. Rinse the round-bottom flask with heptane multiple times to ensure no residue remains and then fill the flask to the mark.
- Filter the extract into an HPLC vial.

2.6 NP-UHPLC-ELSD

Separation is achieved as described by Yang et al. (2019) and Tuccillo et al. (2022), using a NP-HPLC method, ELSD detection, and a diol column suitable for polar and slightly hydrophobic interactions. Use the following gradient elution with solvents A (0.1 % of acetic acid in heptane) and B (0.1 % acetic acid, 3 % of 2-propanol in heptane):

- 0–5 min (97:3)
- 5–15 min (97:3 to 0:100)
- 15–35 min (0:100)
- 35–40 min (0:100 to 97:3)
- 40–50 min (97:3)

2.7 Calculations

For quantification, this method uses a second order standard curve using oleic acid. Lipid contents are given as mg/g.

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Chapter 14
Organic acids

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Summary

- **Raw material**: Sourdough, fermented food matrix, liquid medium
- **Relevant applications:** Measurement of concentrations of organic acids in samples
- **Analytical method**:
  - Ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)

1. Introduction

Organic acids are a broad class of low-molecular-mass compounds with a general R-COOH structure that are found in all living organisms (Hajati, 2018; Jones, 1998). Some organic acids only posses a single carboxyl group (*e.g.*, formic acid, acetic acid, propionic acid, and butyric acid), whereas others contain multiple carboxyl groups and/or hydroxyl groups (*e.g.*, citric acid, lactic acid, and malic acid), or even double bonds (*e.g.*, fumaric acid).

Within a fermented food context, for example sourdough, the microorganisms present can produce and consume a variety of organic acids (De Vuyst *et al*., 2021). Yeasts are also able to produce organic acids. Concerning lactic acid bacteria (LAB), a distinction has to be made between homofermentative and heterofermentative LAB species. Homofermentative LAB species produce lactic acid from glucose using the Embden-Meyerhof-Parnas pathway and a further reduction of pyruvate to lactate for redox balancing. Heterofermentative LAB species produce both lactic acid and acetic acid from pyruvate and acetyl-phosphate, respectively, through the phosphogluconate pathway. Furthermore, heterofermentative LAB can convert citric acid and oxaloacetic acid into malic acid, fumaric acid, and succinic acid through the reductive tricarboxylic acid cycle. Lastly, acetic acid bacteria can produce acetic acid and gluconic acid.

2. Method of analysis

Within the following paragraphs, a method, based on De Bruyn *et al.* (2017), for the analysis of organic acid concentrations using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) is described. The method uses an Acquity system equipped with an HSS T3 column for compound separation. Detection is performed using a triple-quadrupole (TQ) tandem mass spectrometer with a ZSpray electrospray ionization source operating in the negative ionization mode (Waters, Milford, MA, USA).
The current method has been validated for the quantification of citric acid, fumaric acid, gluconic acid, glucuronic acid, lactic acid, malic acid, succinic acid, and oxalic acid (Zhang *et al*., 2019).
The organic acids can come from both liquid and solid matrices. An example of a liquid matrix can be a liquid fermentation broth. When using such a sample, centrifugation at 5000 x *g* for 20 min is needed to remove the cells present in the broth. The resulting supernatant is used in the next steps. In the case of more solid samples, such as sourdough, an appropriate extraction from the sourdough matrix is needed. For a sourdough sample, a 1:5 dilution of the sample in ultrapure water (MilliQ; Merck,

Darmstadt, Germany) should be prepared and mixed, for example using a rotating mixer, at 40 rpm for 15 min to make an aqueous extract of the metabolites from the sourdough matrix. A similar centrifugation as for liquid samples needs to be performed (5000 x *g*, 20 min) to remove the cells and the sourdough matrix material. The resulting supernatant is used in the next steps.

Sample preparation is done, in triplicate, by diluting the supernatant with ultrapure water to reach an organic acid concentration that falls in the linear range of the detector. Then, 40 µl of sample is added to 760 µl of an organic acid solution (500 ml of ultrapure water, 500 ml of methanol, and 20 mg/l of salicylic acid added as internal standard). Samples are vortexed for 2 min after which they are centrifuged (19,400 x *g*, 15 min, 10°C). Samples are filtered using a 0.2-µm PTFE filter (Millex, Merck, Darmstadt, Germany) into capped chromatography vials (PP screw neck vial N9 with an inner cone and screw closure N9 without slit; Macherey-Nagel, Düren, Germany) before injection (10 µL) into the column. The eluents used are ultrapure water (MilliQ, Merck) with 0.2 % (v/v) formic acid (Merck) as eluent A and a mixture of methanol (Merck) and water (95:5) with 0.2 % (v/v) formic acid (Merck) as eluent B. The elution gradient is as follows: 0.0 to 1.5 min, isocratic 10 % B; 1.5 to 3.0 min, linear from 10 to 90 % B; 3.0 to 4.0 min, isocratic 90 % B; 4.0 to 4.1 min, linear from 90 to 10 % B; and 4.1 to 6.0 min, isocratic 10 % B. The flow rate is kept constant at 0.3 ml/min.

Quantification of the organic acids is performed through external calibration with pure compounds (Zhang *et al*., 2019).

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Chapter 15
Biogenic amines

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Summary

- **Raw material**: Sourdough, fermented food matrix, liquid medium
- **Relevant applications:** Measurement of concentrations of biogenic amines in samples
- **Analytical methods**:
  - Ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)

1. Introduction

Biogenic amines (BA) are derivatives of amino acids produced through a decarboxylation reaction (Biji *et al*., 2016; Barbieri *et al*., 2019). These compounds are regularly found in food products that have a high protein content, such as fish, meat, dairy and other fermented food products (Biji *et al*., 2016; Barbieri *et al*., 2019; Decadt and De Vuyst, 2023). BA can be produced by a range of different microorganisms including enterobacteria, lactic acid bacteria, and coagulase-negative staphylococci. The latter two microbial groups are often present in fermented food products, such as cheese and fermented sausages (Van der Veken *et al*., 2020; Decadt *et al*., 2023).

The presence of BAs in food products is generally unwanted, as some biogenic amines have a toxic nature (Benkerroum, 2016). Bas such as histamine, tyramine, and β-phenylethylamine exert a vasoactive effect on the human body, presenting intoxication issues when those BAs are consumed in too high amounts. Other BAs, such as cadaverine, putrescine, and spermine, do not pose a similar intoxication threat but they can sometimes lead to the formation of carcinogenic nitrosamines (Biji *et al*., 2016; Van der Veken *et al*., 2020). Also, their foul-smelling odour is detrimental for the quality of the food product (Van der Veken and Leroy, 2022).

2. Method of analysis

BAs can be determined by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) (Van der Veken *et al*., 2020). This can be done using an Acquity UPLC system equipped with an HSS T3 column for compound separation (Waters, Milford, MA, USA). Detection can be performed using a TQ tandem mass spectrometer with a ZSpray electrospray ionization source operating in the positive ion mode (Waters). The current method has been validated for the identification and quantification of agmatine, cadaverine, histamine, β-phenylethylamine, putrescine, tryptamine, tyramine, spermidine, and spermine (Van der Veken *et al*., 2020).

The following protocol is based on the method of Van der Veken *et al*. (2020). Samples need to be first centrifuged to obtain a cell-free supernatant (5000 x *g*, 20 min, 4°C). When dealing with a (semi-)solid sample, such as sourdough, the centrifugation needs to be preceded by an extraction of the metabolites from the solid matrix. This can be done by making a 1:5 dilution of the sample in ultrapure water, which is mixed, for example using a rotating mixer, at 40 rpm for 15 min. To subsequently remove the cells and matrix particles, centrifugation is performed (5000 x g, 20 min, 4°C). Cell-free supernatant (250 µl) is added to 250 µl of acetonitrile containing 0.15 % (v/v) heptafluorobutyric acid (HFBA, ≥ 99 %; Merck, Darmstadt, Germany) to precipitate the proteins present in the sample. This

mixture is then centrifuged again (13,000 × *g* for 15 min at 4°C) and filtered (0.2-µm PTFE filters, Millex, Merck) and collected in chromatography vials (PP screw neck vial N9 with an inner cone and screw closure N9 without slit; Macherey-Nagel, Düren, Germany). Finally, the samples are injected (2 µl) into the column. Compound quantification is done through external calibration. All standards and samples are prepared in triplicate. Standard solutions are prepared using pure compounds and processed identically as the samples (Van der Veken *et al*., 2020).

The mobile phase used for compound separation is an ultrapure water-acetonitrile mixture (95:5, v/v) with 0.1 % (v/v) HFBA as an ion-pairing reagent (eluent A) and an ultrapure water-acetonitrile mixture (5:95, v/v) with 0.1 % (v/v) HFBA (eluent B). The following gradient (0.23 mL/min) is applied: 0.0–5.0 min, isocratic 95 % A and 5 % B; 5.0–7.0 min, linear from 95 % to 80 % A and from 5 % to 20 % B; 7.0–8.0 min, linear from 80 % to 30 % A and from 20 % to 70 % B; 8.0–11.0 min, isocratic 30 % A and 70 % B; 11.0–12.0 min, linear from 30 % to 95 % A and from 70 % to 5 % B; 12.0–15.0 min, isocratic 95 % A and 5 % B. The selected reaction monitoring (SRM) method was optimized via IntelliStart (Waters), and the dwell time was adjusted to ensure a minimum of ten scans for each compound peak. The following mass spectrometric settings were used: capillary voltage, 3.50 kV; source temperature, 150°C; desolvation temperature, 450°C; cone gas flow, 0 l/h; desolvation gas flow, 800 l/h.

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Chapter 16
Ethanol

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Summary

- **Raw material**: Sourdough, fermented food matrix, liquid medium
- **Relevant applications:** Measurement of concentrations of ethanol in samples
- **Analytical methods**:
    - Gas chromatography with flame ionization detection (GC-FID)

1. Introduction

Within a fermented food context, ethanol is mostly produced by yeasts through the glycolysis and subsequent pyruvate metabolism as a way to produce ATP and balance the redox equilibrium (De Vuyst *et al*., 2017, 2021). As end-products of these metabolic pathways, ethanol and carbon dioxide are formed. Another group of microorganisms that produce ethanol in fermented food matrices are heterofermentative lactic acid bacteria, although they produce not only ethanol but rather a mixture of ethanol, lactic acid, acetic acid and/or formic acid as the end-products of their carbohydrate metabolism.

2. Method of analysis

Ethanol can be determined by gas chromatography with flame ionization detection (Moens *et al*., 2014). This can be done using a Focus gas chromatograph (Interscience, Breda, The Netherlands) equipped with an AS 3000 autosampler (Interscience), a Stabilwax-DA column (Restek, Bellefonte, PA, USA), and an FID-80 detector (Interscience).

The following protocol is based on the method of Moens *et al*. (2014). Sample preparation is done, in triplicate, by using cell-free supernatant obtained after centrifugation at 4168 x *g* for 20 min at 4°C. This supernatant is mixed (1:4) with acetonitrile (Merck, Darmstadt, Germany) supplemented with 1 % (v/v) formic acid (Merck) and 0.2 % (v/v) 1-butanol (Merck) as an internal standard. After mixing, the samples are centrifuged (18,894 x *g*, 15 min, 4°C), filtered (0.2-µm PTFE filters, Millex, Merck), and collected in chromatography vials (glass screw neck vial N9 with a flat bottom and screw caps N9 without slit; Macherey-Nagel, Düren, Germany). To perform a quantification, external calibration is performed. All samples are analyzed using an injection volume of 1 µl in split flow with a 40:1 split ratio.

During the analysis, hydrogen gas (Nippon Gases, Madrid, Spain) is used as carrier gas with a constant flow rate of 1 ml/min and nitrogen gas (Nippon Gases) is used as make-up gas. The injector and detector temperatures were set at 240°C and 250°C, respectively. The column temperature program was 0 min, 40°C; 10 min, 140°C; 12 min, 230°C; and 22 min, 230°C.

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Chapter 17
Sugar alcohols

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Summary

- **Raw material**: Sourdough, fermented food matrix, liquid medium
- **Relevant applications:** Measurement of concentrations of sugar alcohols in samples
- **Analytical methods**:
  - High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD):

1. Introduction

Sugar alcohols, also called polyols, are carbohydrate derivatives in which the aldehyde or keto group has been substituted with a hydroxyl group (Grembecka, 2015; Bieleski, 1982). These polyols can be found naturally in small quantities in vegetables, fruits, and mushrooms (Grembecka, 2015). Furthermore, sugar alcohols are produced by microorganisms fulfilling different functions, such as regeneration of cofactors (glycerol in yeasts and mannitol and erythritol in lactic acid bacteria) and acting as osmoregulatory compounds (glycerol in yeast) (De Vuyst *et al.*, 2017, De Vuyst *et al.*, 2021). They have a lower caloric value than carbohydrates, can be used as sugar replacer, have a cooling down effect in the mouth, are laxative, and display diabetic- and teeth-friendly properties (Vrancken *et al*., 2010).

2. Method of analysis

Sugar alcohols can be determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (De Bruyn *et al*., 2017; Comasio *et al*., 2019; Díaz-Muñoz *et al*., 2021). This can be done using an ICS 3000 chromatograph equipped with an AS-autosampler for automatic sample loading (Thermo Fisher Scientific, Waltham, MA, USA) and a CarboPac MA1 column for compound separation (Thermo Fisher Scientific). The current method has been validated for the identification and quantification of arabitol, erythritol, galactitol, glycerol, mannitol, sorbitol, and xylitol.

The following protocol is based on the method of Comasio *et al*. (2019). When working with a liquid sample, for example a fermentation broth, centrifugation at 5000 x *g* for 20 min is first performed. The supernatant obtained is used in the subsequent steps. When working with a sourdough sample, however, typically, a 1:5 dilution of sample in ultrapure water is prepared. This dilution is mixed, for example, using a rotating mixer, at 40 rpm for 15 min to extract the metabolites from the sourdough matrix before centrifugation (5000 x *g*, 20 min) to remove the cells and sourdough matrix particles.

Further sample preparation is done, in triplicate, by diluting the supernatant with ultrapure water to reach a sugar alcohol concentration that is within the linear range of the detector. Then, 100 µl of sample is added to 900 µl of a mixture (1:1, v/v) of acetonitrile (Thermo Fisher Scientific) and ultrapure water, supplemented with 0.05 g/l of rhamnose as an internal standard (Merck, Darmstadt, Germany). The addition of acetonitrile precipitates the proteins present in the sample. Samples are shaken for 5 min by vortexing and microcentrifugation (14000 rpm, 15 min, 10 °C) is carried out before filtering (0.2-µm PTFE filters, Millex, Merck) and collection in chromatography vials (PP screw neck vial N9 with

an inner cone and screw closure N9 with slit; Macherey-Nagel, Düren, Germany). The sample injection volume is 10 µl. For quantification of the sugar alcohols, external calibration is applied. Therefore, samples with known concentrations of the pure sugar alcohols of interest are used, following the same sample preparation procedure as described above.

The parameters for the CarboPac MA1 column are as follows: column temperature is set at 30.0 °C, the flow rate of the mobile phase, consisting of ultrapure water (eluent A) and 500 mM NaOH (Thermo Fisher Scientific; eluent B), is set at 0.4 ml/min. The following gradient is applied: 0.0 min, 50 % A and 50 % B; 15.0 min, 50 % A and 50 % B; 25 min, 0 % A and 100.0 % B; 45.0 min, 0 % A and 100.0 % B; 45.5 min, 50.0 % A and 50.0 % B; and 55.0 min, 50.0 % A and 50.0 % B.

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Chapter 18
Volatile compounds

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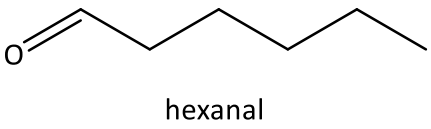
Summary

- **Raw material**: Cereals (e.g., oat) and pulses (e.g., faba bean, pea), as flours or protein-rich fractions (e.g., protein concentrate, protein isolate)
- **Relevant applications:** Dough, bread, meat- and dairy-alternatives
- **Fermentation-induced changes**: Synthesis and loss of volatile compounds
- **Analytical methods**:
    - HS-SPME-GC-MS

1. Introduction

1.1 What are volatile compounds?

Flavor is an important characteristic of food, and volatile compounds play a significant role in it (Wang et al., 2022). Plant raw materials contain a wide array of flavor-active volatile compounds, which are mainly produced as secondary metabolites. The volatile profile is specific to each raw material, and it can be influenced by genetic factors, environmental conditions, agricultural practices, storage, and food processing. Certain volatile compounds contribute positively to the flavor of foods. Others, instead, are considered off-flavors. Aldehydes, alcohols, ketones, organic acids, pyrazines, and sulfur compounds are the main groups responsible for the off flavors of pulses (Rackis et al., 1979). Many of these compounds are generated during oxidation of lipids, and contribute to the development of grassy, beany, and earthy flavors. Hexanal (Figure 1) is one instance; it is an odor-active monocarbonyl compound associated to the presence of aroma defects in food. (Belitz et al., 2008).



hexanal

***Figure 1**. Chemical structure of hexanal*

The choice of raw materials can significantly impact the volatile profile of the food products. When comparing cereals and pulses as raw materials, for instance, variations in volatile compounds can arise due to inherent differences in their biochemical composition (Karolkowski et al., 2021). Also, the processing methods to transform the raw materials into flour, protein concentrate, and protein isolates can further modify the volatile compound composition, thereby influencing the overall flavor of the final food products (e.g., bread, meat-, and dairy-alternatives) (Tuccillo et al., 2022). For example, the thermal processing involved in the production of several food applications may lead to the generation of Maillard reaction products, which contribute to the characteristic aroma and flavor of baked goods (Liu et al., 2022). For bread, especially, Maillard reaction products play a crucial role in consumer acceptance and preference (Starowicz & Zieliński, 2019). Similarly, in the development of meat and dairy alternatives, volatile compounds can help recreate the characteristic flavors and aromas associated with animal-based products (Harper et al., 2022).

1.2 How can fermentation affect volatile compounds?

Fermentation greatly impacts the composition of volatile compounds. This biological transformation occurs through the activity of microorganisms, such as bacteria, yeasts, and molds. They utilize carbohydrates, proteins, and lipids as source of energy. They convert organic compounds into different degradation products, including volatile compounds (Petel et al., 2017). The specific volatile compounds generated during fermentation are influenced by factors such as the type of microorganisms involved, fermentation conditions (temperature, pH, oxygen availability), duration of fermentation, and the composition of the raw materials themselves (McFeeters, 2004). On the one hand, spoilage microorganisms can produce volatile compounds that contribute to the unpleasant odors and off-flavors associated with food deterioration (Singh et al., 2004). For instance, certain bacteria can produce volatile sulfur compounds, such as hydrogen sulfide and dimethyl disulfide, which are responsible for the characteristic rotten egg-like smell in spoiled eggs or seafood (Xia et al., 2017). Similarly, the presence of volatile amines, such as putrescine and cadaverine, produced by spoilage bacteria in protein-rich foods, can result in the development of foul odors (Bulushi et al., 2009). On the other hand, other microorganisms can produce volatile compounds that enhance sensory properties and consumer liking (Ranadheera et al., 2019). In fact, fermentation has been also employed as a sustainable bioprocessing tool to mask off-flavors (Verma et al., 2022). Remarkably, the presence of lactic acid bacteria has been found to induce significant modifications in the volatile profile of plant protein ingredients, resulting in perceptible sensory enhancements (Artega et al., 2021; El Youssef et al., 2020; Leroy & De Vuyst, 2004; Wang et al., 2022).

2. Method(s) of analysis

2.1 Sample preparation

This method entails minimal sample preparation. Samples obtained before, during, or after fermentation should be stored at -20 °C in glass containers to prevent contamination from volatile compounds originating from plastics. On the day of analysis, the samples are quickly brought to room temperature and directly weighed into 20-mL amber SPME vials. These vials are selected to ensure sample stability and minimize potential interactions with light. The sample amount should be weighed precisely up to two decimal places. The vials are immediately placed on the temperature-controlled sample tray set at 4 °C to prevent fermentation-induced changes. The analysis is performed in three or four analytical replicates. To optimize efficiency, it is recommended to organize the sample order so that samples of the same replicate are analyzed consecutively.

2.2 General principle

The volatile analysis method described here utilizes headspace solid-phase microextraction gas-chromatography mass-spectrometry (HS-SPME GC-MS) as previously reported (Tuccillo et al., 2022). This technique involves extracting volatile compounds from the sample's headspace using a solid-phase microextraction fiber, followed by their separation and identification using gas chromatography-mass spectrometry. This method leverages the unique property of volatile compounds to evaporate into the headspace, allowing their extraction using a solid-phase microextraction fiber. This fiber acts as a sorbent, capturing the volatile compounds in its coating. Subsequently, the volatile compounds are introduced into a gas chromatograph (GC), where they are separated based on their differing affinities for the chromatographic stationary phase. This separation is essential to resolve the complex mixture of volatile compounds present in the sample. The separated compounds are then identified using mass spectrometry (MS), a technique that quantifies the mass-to-charge ratio of ions to provide insights into the composition and structure of the compounds. HS-SPME GC-MS offers distinct advantages, such as its non-destructive nature, minimal sample handling, and high sensitivity. It enables the detection and quantification of a wide range of volatile compounds, from simple aliphatic hydrocarbons to complex aromatic compounds.

2.3 Equipment and Reagents

Gather the necessary equipment and reagents:

- HS-SPME injector with a 1 cm divinylbenzene/carboxen/polydimethylsiloxane fiber (DVB/CAR/PDMS) of 1 cm with 50/30 µm film thickness
- Gas chromatograph (GC) Mass spectrometer (MS equipped with a mid-polar/polar column
- 20-mL amber SPME vials
- Temperature-controlled sample tray
- Helium gas for carrier gas supply
- Hexane
- MQ-water (Milli-Q water)
- Alkane series in heptane (C7-C30) (Sigma 49784-U)
- Mass-spectra database for compound identification

2.4 HS-SPME-GC-MS analysis of the samples

After sample preparation as described in section 2.1, position the samples in the sample tray for analysis. Then, Perform the analysis using the following HS-SPME-GC-MS conditions:

- Incubation time: 10 minutes
- Extraction time: 30 minutes with agitation (250 rpm)
- Incubation and extraction temperature: 50 °C
- Splitless injection
- Initial flow rate of helium: 0.7 mL/min
- Temperature profile: 40 °C (5 min hold), 200 °C (5 °C/min), and 200 °C (10 min hold)
- MS scan range: m/z 40-300 amu
- Electron ionization mode at 70 eV with ion source temperature at 230 °C and quadrupole temperature at 250 °C.

2.5 HS-SPME-GC-MS analysis of the alkane's series

Conduct analysis of the alkane series as listed below:

- Dilution: Dilute the alkane series 1:10 in hexane.
- Sample Preparation: Prepare duplicate samples by pipetting 1 mL of MQ-water and 1 µL of the diluted alkane series directly into the SPME vial.
- Vortex: Vortex the vials to ensure proper mixing.
- Analysis Conditions: Perform the analysis using the same conditions as described in section 2.4.Identification and calculations

2.6 Identification and calculations

In the identification process, compounds are typically minimally integrated to account for the potential impact of even small peaks on flavor perception. Compound identification is primarily based on their mass correspondence with internal standards or publicly available databases. The mass spectra obtained from the analysis are compared to spectral libraries, such as Wiley's library software (Wiley 7N, Wiley Registry of Mass Spectral Data, 7th Edition), for compound identification. Additionally, retention times can be utilized as supporting information for identification, especially in studies that adopt the same column and chromatographic conditions. By comparing the retention times of target compounds with those of known standards or established references, confident identifications can be made. Moreover, retention indexes (RI) can be employed as standard indicators for identification. These indexes are calculated using the retention times of specific reference compounds, often from an alkane series (7-30), which are injected separately under the same chromatographic conditions. The RI values of target compounds can then be compared to the RI values

of the reference compounds to aid in compound identification. Results are usually expressed as relative peak area (%) of all integrated peaks for each measurement.

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