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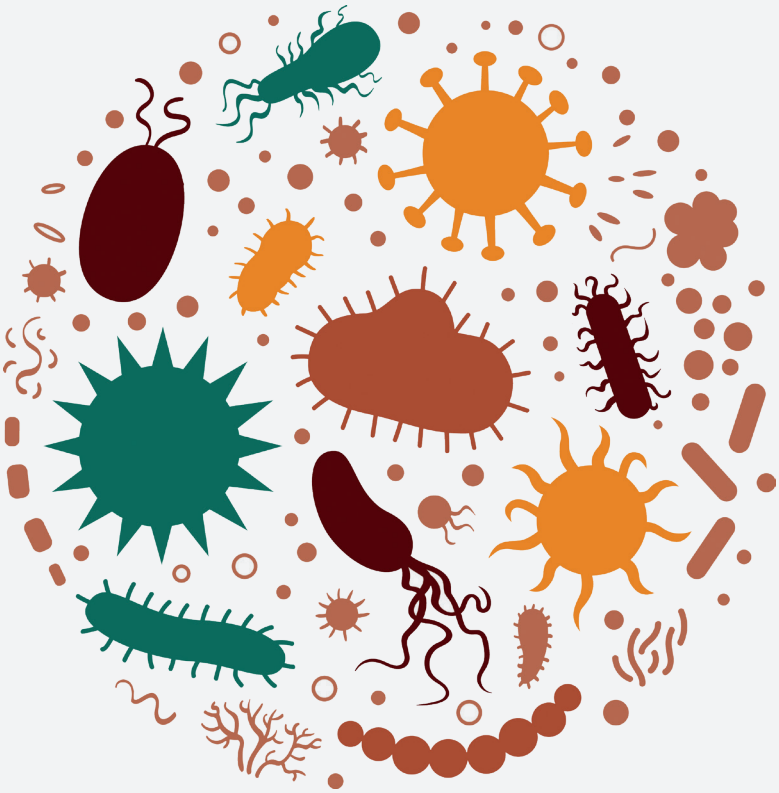

CHARACTERISATION OF FERMENTED GRAIN-BASED RAW MATERIALS AND FOODS

ANALYTICAL METHODS FROM THE HEALTHFERM PROJECT

Leuven University Press

Characterisation of Fermented Grain-based Raw Materials and Foods
Analytical Methods from the HealthFerm Project

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Edited by
Arno G. B. Wouters, Yamina De Bondt, Kati Katina
& Christophe M. Courtin

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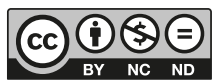

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Preface

Fermented foods are widely consumed in Europe and across the globe because of their sensory characteristics, cultural value and – especially in the past few decades – perceived nutritional and health benefits. In this light, but also in the context of the ongoing (partial) transition from animal towards plant protein–based foods, interest in plant-based fermented foods has recently surged. This edited volume positions itself in the context of the Horizon Europe project HealthFerm. HealthFerm aims to improve knowledge on the interaction between food fermentation microbiomes, fermented grain-based foods and the human gut microbiome and how it benefits human health, while developing novel, healthy and nutritious fermented foods based on pulses and cereals.

To understand the impact of food fermentation processes on the properties of plant-based raw materials and foods, an in-depth understanding of the (bio)chemical conversions that take place in a food fermentation process is needed. Understanding these conversions requires the availability of an in-depth analytical toolbox tailored towards (i) plant-based raw materials and foods and (ii) the specific context of raw materials and food fermentations.

This edited book comprises a comprehensive overview of (bio)chemical analytical methods targeted towards characterising changes in constituents in plant-based raw materials or food fermentations. While analytical methods for the characterisation of food constituents are available in the literature, these often require adaptations to be useful (i) in the context of food fermentation and (ii) for specific raw materials (in this case, grains). To the best of the editors' knowledge, a comprehensive overview of such state-of-the-art methods is not available at present. This edited volume aims to fill this gap, comprising contributions by researchers from seven European research institutes and universities.

The book consists of 20 chapters, grouped into 5 sections. Section 1 comprises an introduction to the field and the scope of the manuscript. The focus is twofold, with one chapter focusing on the targeted raw materials and one chapter focusing on food fermentations, two key elements of the text. Subsequent sections comprise different chapters with different

analytical methods targeted towards the characterisation of different constituent categories: Section 2 deals with proteins; Section 3, with carbohydrates; Section 4, with minor grain constituents; and Section 5, with fermentation metabolites and intermediates.

We hope that this edited volume will end up being a reference work for the chemical analysis of fermented grain-based raw materials and foods to be used by food technologists and fermentation specialists worldwide. In time, this will, we hope, contribute to profound knowledge on the impact of fermentation processes on the sensory and health-related aspects of plant-based foods, in turn leading to the development of novel, appealing and healthy plant-based fermented foods.

Arno G. B. Wouters
Yamina De Bondt
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SECTION 1

Introduction

CHAPTER 1

HealthFerm raw materials

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Summary

- The HealthFerm project focuses on raw materials originating from four different crops, namely wheat, oats, faba bean and yellow pea.
- These grains are important raw materials for the production of novel plant-based fermented foods.
- The methods described in the book were optimised for and are applicable to one or multiple of these raw materials. It may well be possible to apply these methods to the analysis of other raw materials, but such application is likely to require some further optimisation.

1. Introduction

As we note above, this book is part of the broader context of the Horizon Europe HealthFerm project (2022–2026), which aims for improved knowledge on the interaction between food fermentation microbiomes, fermented grain-based foods and the human gut microbiome and how fermentation benefits human health, while developing novel, healthy and nutritious fermented foods based on pulses and cereals. Within this multidisciplinary research project, grain-based fermented foods take up a central position in the research. Although various micro-organisms represent an important factor in the production of such foods, the raw materials used are crucial. Food fermentation processes induce numerous changes in the chemical structure of the many different constituents that occur in grain-based raw materials and foods. These changes, in turn, affect the sensory properties of the fermented foods obtained from them, as well as their potential impact on human health. Thus, there is a need

for analytical methods to study changes in chemical structures of grain-based raw-material constituents. In HealthFerm, the strategic choice was made to focus on raw materials originating from four different crops, i.e. wheat, oats, faba bean and yellow pea. In what follows, each of these crops is introduced briefly, both in general terms and in the context of their use as raw materials for food fermentation processes.

2. Wheat

Wheat is one of the oldest domesticated food crops and is the third most produced crop worldwide (FAOSTAT, 2022). The major wheat species grown worldwide is *Triticum aestivum* L., which is also known as "common" or "bread" wheat. The second most grown wheat species is *Triticum durum*, which is adapted to the Mediterranean climate and is often referred to as "pasta wheat", given its use in that application, or "durum wheat" (Delcour & Hoseney, 2010). Wheat provides 20% of the daily food calories and protein and thus takes a central place in human nutrition (Poutanen et al., 2022). Wheat is grown primarily for human consumption and is, in most cases, milled into flour. After the harvest, wheat kernels are typically milled into white flour or wholemeal. White flour consists of the inner part of the wheat kernel (the endosperm), while wholemeal consists of the entire kernel (Delcour & Hoseney, 2010). Wholemeal contains more dietary fibre, vitamins, minerals and bioactive compounds than white flour (Poutanen et al., 2022). The most important application of wheat in biotechnological applications in Europe is its use as raw material for the production of bread and baked goods (Goesaert et al., 2005). In this context, the main proteins of wheat (i.e. the gluten proteins) are important, as they possess the unique ability to develop a viscoelastic network upon hydration and mixing (Delcour et al., 2012). Significant amounts are also used for the production of pasta products and breakfast cereals.

In a food fermentation context, wheat dough is fermented during (sourdough) bread production, where yeast (and lactic acid bacteria) are responsible for gas production but also affect the flavour and the structure of bread (Poutanen et al., 2009; Struyf et al., 2017). Wheat can also be fermented to make beer or fermented wheat porridges. It has been shown that fermentation can positively affect the taste and texture (Katina et

al., 2006) and health-related properties (Poutanen et al., 2009) of wheat-based products. Furthermore, fermentation could represent a new way to expand the use of wheat proteins in emerging plant-based foods, such as dairy alternatives and meat analogues.

3. Oats

Oats (*Avena sativa* L.) are among the oldest crops known to human civilisation. Their current production ranks sixth in the worldwide and fourth in the European production statistics (FAOSTAT, 2022). The best growth performances of common oat are achieved in acid soil and cool and moist climates, as it is sensitive to hot and dry weather. For all these reasons, oat production is concentrated between latitudes 35 and 65°N (including Finland and Sweden) and 20 and 46°S (including Argentina and Brazil) (Mäkinen et al., 2017; Stewart & McDougall, 2014). After the harvest, oats are typically dehulled and heat-treated (kilned) into oat groats, which can be cut and converted into flakes or milled into wholemeal or flour. Traditionally used for animal feed in many countries, oats have gained popularity in the last decades as food ingredients due to their versatility in several applications and their unique nutritional properties. Indeed, oats have a well-balanced amino acid composition, low allergenicity and gluten-free nature of its proteins (Spaen & Silva, 2021). Moreover, consuming oat dietary fibre (DF) is associated with a reduced risk of non-communicable diseases, with arabinoxylan and β-glucans being the main species, the latter of which is associated with several European Food Safety Authority (EFSA) –approved health claims (Paudel et al., 2021).

Nowadays, oats are consumed as porridge, snacks, breakfast cereals and flakes, bakery products, baby foods, gluten-free foods, and meat and dairy analogues. The characteristics of oats have made it possible in recent years to use these cereals more and more in the fermentation processes for novel food products, such as functional beverages and plant-based yoghurt. At the same time, the rise of the functional food market has induced increased interest in oat fermentation and the consequent development of several oat-based fermented foods, including beverages and yoghurt alternatives (Djorgbenoo et al., 2023). However, current oat-based (fermented) beverages and yoghurt alternatives generally have very low protein and fibre levels, and their taste and texture need to be

improved. Fermentation technology has the potential to help overcome some of these challenges.

4. Faba bean

One of the first domesticated plants from the Fertile Crescent is the faba bean (*Vicia faba* L.). The crop expanded throughout North Africa and Eurasia and was brought to the Americas and Australia by European colonists. Faba bean today is utilised as both food and animal feed. It is traditionally consumed in Mediterranean and Middle Eastern countries, but it can grow in cold climates. Faba bean ranks seventh among grain legumes in terms of harvested area on a worldwide scale (FAOSTAT, 2022). Different varieties are cultivated, varying primarily by seed size. The largest seeds are historically found in var. major (broad beans), whereas the smallest are found in var. minor (tick beans). Var. equina (horse beans) has seeds of intermediate size. Faba bean has gained popularity as a promising crop for many agroecological reasons. The potential nutrient intake, nodulation, soil nitrogen balance, better soil fertility and structure, and increased biological diversity are but a few of these. Moreover, faba bean's ability to fixate nitrogen in symbiosis with alpha-proteobacteria relieves farmers from the need to apply a large quantity of nitrogen fertiliser (Köpke & Nemecek, 2010).

As a raw material for the production of plant-based foods, faba beans stand out, with a high protein content, of up to 35%; a well-balanced amino acid composition (Warsame et al., 2018); relatively high levels of complex carbohydrates, DF, and mineral nutrients; and a low saturated fat content. At the same time, faba bean has several disadvantages, including its off-flavours (bitterness, astringency, beany flavour) and antinutritional compounds (vicine, convicine, oligosaccharides) (Multari et al., 2015; Tuccillo et al., 2022). Interestingly, fermentation has the potential to mitigate some of the disadvantages associated with the use of faba beans. For example, fermentation with lactic acid bacteria has been found to have the potential to improve the sensory and nutritional quality of faba bean–based foods (Rizzello et al., 2016; Wang et al., 2024). In general, faba bean seeds can be processed into flour, protein concentrate, protein isolate, or fibre- and starch-rich fractions. Notably, depending on the type of prior processing, antinutritional factors can be substantially reduced in

faba bean ingredients (Vioque et al., 2012). Faba bean–based ingredients can be used in various processes aimed at producing i.a. meat and dairy alternatives or at fortifying cereal-based and gluten-free products.

5. Yellow pea

Yellow peas (*Pisum sativum* L.) are one of the oldest-known legumes, originating in Southwest Asia. Canada is the leading producer of yellow peas worldwide, while France is the main producer in Europe. It is a sustainable crop that can fix nitrogen during cultivation and hence contributes to soil fertility, with increased crop yields (Tidåker et al., 2021). Yellow pea is a nutritious crop rich in proteins; starch; dietary fibres; minerals, particularly potassium; and vitamins (A, B and C). After harvesting and dehulling, yellow peas can be processed through various wet or dry fractionation methods into protein- or starch-enriched isolates or concentrates (Asen et al., 2023).

Yellow pea–based ingredients are an often used raw materials for producing foods that serve as alternatives to animal-based products, such as meat and dairy alternatives. In addition, they can be used for protein-fortification purposes in, amongst others, bread and other baked goods. Fermentation technology has the potential to overcome some of the challenges associated with the use of yellow peas, such as their flavour profile or the presence of some antinutrients. Indeed, fermentation of yellow pea flour can reduce some of its antinutritional factors (e.g. raffinose, phytic acid and tannins), increasing the overall bioavailability of nutrients and improving digestibility. The use of fermented yellow pea flour as a replacement for wheat flour in breadmaking has been shown to impact the bread quality and flavour characteristics (Bourré et al., 2019).

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CHAPTER 2

Plant-based food fermentations

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Summary

- In the context of food science, the term fermentation refers to three different distinct concepts: biomass fermentation, precision fermentation and food fermentation. The focus of this book and the HealthFerm project is food fermentation.
- Fermented foods are defined as "foods made through desired microbial growth and enzymatic conversions of food components" (Marco et al., 2021) and can be produced by spontaneous fermentation, by back-slopping or by using starter cultures.
- Fermentation of foods can have beneficial effects on food safety and shelf life, enhance organoleptic properties, and offer health benefits. However, for plant-based foods, knowledge regarding the impact on human health and mechanisms behind such impact is limited.
- Possible mechanisms behind the benefits of food fermentation include (i) the transformation of food constituents; (ii) the synthesis of new bioactive compounds; and (iii) the delivery of micro-organisms to the gastrointestinal tract.
- A comprehensive toolbox for characterising the changes in constituents that occur as the result of plant-based raw material and food fermentations is essential for understanding the impact of fermentation on sensory and health-related aspects of foods.

1. Introduction

Micro-organisms play a central role in our food system, ranging from food production and processing to digestion. In the context of food science, the term fermentation refers to three distinct concepts, each of which involves microbial activity. The first is biomass fermentation, in which microbial biomass is produced from a defined sugar source, such as glucose, or from different waste streams or atmospheric gases. The microbial biomass is then harvested and processed into foods. Quorn, made from fungal biomass, is an example of a food produced through this method. The second is precision fermentation, in which micro-organisms are selected and (genetically) optimised to produce specific compounds. These compounds are typically purified and used for various applications, for example as

enzymes for baking. The third and final category, and the focus of the HealthFerm project and this book, is food fermentation, in which different food-relevant substrates are transformed by micro-organisms to create fermented raw materials and foods (Jahn et al., 2023).

Fermented foods have been a part of the human diet for millennia. Today, there are roughly 5,000 varieties of fermented foods and beverages prepared and consumed worldwide, contributing to 5–40% of the human diet (Rul et al., 2022). Fermented foods and beverages were defined in, 2021 by the International Scientific Association for Probiotics and Prebiotics as "foods made through desired microbial growth and enzymatic conversions of food components" (Marco et al., 2021). A broad range of fermented foods exists, produced from diverse food raw-material substrates, such as vegetables, grains, soybean, milk, fish and meat. Fermented foods often contain live micro-organisms, as is, for example, the case in yoghurt, cheese and kombucha. However, in many fermented foods, the micro-organisms are not alive or present anymore, for example, in bread, pasteurised fermented vegetables, soy sauce and wine. During fermentation, micro-organisms transform the raw material or food matrix and will synthesise new components. A wide diversity of micro-organisms can be used for the production of fermented foods. The most common fermented foods and beverages require lactic acid bacteria, acetic acid bacteria, bacilli or other bacteria, yeasts, or filamentous fungi (Marco et al., 2021).

The process of fermentation has developed significantly over the years. Traditionally, fermented foods and beverages have been produced by relying on the microbiota naturally occurring on the food substrate. Many types of fermented foods, such as sauerkraut and kimchi, are still produced using spontaneous fermentation, especially in small-scale settings and in developing countries. The start of the fermentation process may involve transferring a small amount of a previously successful fermented batch into fresh ingredients as an inoculum, which is called backslopping. With progress in microbiological techniques, specific starter cultures have been isolated and characterised from fermented foods. Selected efficient and well-characterised starter cultures are currently used, especially in industrial practice, to ensure that the fermentation process and product quality are controlled. The fermentation process has further evolved as the use of starter cultures has undergone significant improvement with the advancement of molecular biology techniques. Recently, genetic

improvement of starter cultures has resulted in the selection of better-performing and well-adapted starter cultures for improved fermentation and desired food properties (Mannaa et al., 2021).

2. Benefits of fermentation

Fermented foods have historically been valued because of their improved shelf life, safety and sensory properties. However, it is increasingly understood that fermented foods may also have enhanced nutritional and functional properties (Marco et al., 2017). This section provides several examples of the various benefits of fermentation for plant-based foods; it is not an exhaustive list.

2.1 Food safety and shelf life

Licandro et al. (2020) summarise in a review how fermentation by lactic acid bacteria can address safety issues in legume-based food products. These bacteria can play a role in the reduction of allergenic compounds and they can prevent the accumulation of mycotoxins by inhibiting pathogen growth and activity. The preservative role of lactic acid bacteria fermentation technology has also been reported for several cereal products. This has been attributed to the production of acids, hydrogen peroxide and antibiotics (Blandino et al., 2003).

2.2 Sensory properties

Blandino et al. (2003) describe in their review on cereal-based fermented foods and beverages how fermentation often leads to a general improvement in the texture, taste and aroma of the final product. During cereal fermentations, several volatile compounds are formed, which contribute to a complex blend of flavours in the products.

For legumes, it has been described that fermentation can be used to substantially improve the flavour profile (Senanayake et al., 2023). For example, fermentation of soy milk resulted in a reduction of the sensory strength of “green odour”, and 60% of the sensory panellists were unable to detect off-notes (Nedele et al., 2020).

2.3 Health benefits

There are several reviews describing the (potential) health benefits of fermented foods (Marco et al., 2017; Rul et al., 2022; Şanlier et al., 2019). These include antioxidant, anti-microbial, anti-fungal, anti-inflammatory, anti-diabetic and anti-atherosclerotic effects. However, some studies have shown no relationship between fermented foods and health benefits. One of the key reasons why analysing potential health benefits is a complex task is the extreme diversity in type and composition of fermented foods, on the one hand, and possible effects, on the other hand. While for yoghurt and cultured dairy products, there is clear evidence on the impact of food fermentation on human health, for most plant-based fermented foods, knowledge regarding the impact on human health and the mechanisms behind such impact is limited due to a lack of dedicated and integrated studies. Certainly, results are lacking to confirm the widespread idea that fermented foods have general health benefits (Marco et al., 2017, 2021; Rul et al., 2022).

3. Mechanisms behind possible beneficial effects of food fermentation

To understand and explain the various potential benefits of fermented plant-based foods, it is essential to identify the underlying mechanisms. Possible mechanisms include (i) the transformation of food constituents; (ii) the synthesis of new bioactive compounds; and (iii) the delivery of micro-organisms to the gastrointestinal tract.

3.1 Transformation of food constituents

During fermentation, various food components undergo transformations due to the endogenous enzymatic activity of the raw materials and/or the metabolic activity of micro-organisms (Adebo et al., 2022; Marco et al., 2017). Adebo et al. (2022) provide an overview of how major constituents, such as proteins, carbohydrates and fats, are altered during the fermentation of cereals and legumes. Through proteolysis, complex proteins can be broken down into amino acids and peptides, and bonds between proteins and antinutritional factors (e.g. phytate) may be disrupted. However,

different trends of modification of proteins and amino acid compositions have been observed because of the large variability in raw materials and fermentation conditions. During fermentation, carbohydrates can be degraded, used as an energy source and converted into more available carbohydrates. Again, the type and extent of conversions greatly depend on the raw material and the fermentation conditions. Most studies on fermented cereals and legumes reported a reduction in the fat content attributed to increased activity of lipolytic enzymes, whereas some studies on fibre content report that fermentation results in increased values while other studies reported decreased values. A decreased fibre content or solubilisation of fibres can be attributed to an increase in the activity of hydrolysing enzymes and utilisation of fibre as a carbon source. Finally, it has been shown that fermentation can decrease the levels of antinutritional factors, such as phytate or (con)vicine (Adebo et al., 2022; Marco et al., 2017).

3.2 Synthesis of new bioactive compounds

Fermentation can lead to the synthesis of new bioactive compounds. Studies have shown that fermentation often increases levels of several vitamins, including the B, E and K types (Adebo et al., 2022; Marco et al., 2017; Şanlier et al., 2019). Additionally, bioactive peptides, antioxidants and exopolysaccharides can be produced, each associated with specific health advantages. However, further research is needed to determine the consumption levels required to realise these benefits. Selecting optimal microbial strains and ensuring optimisation of fermentation processes are also crucial to steer the capacity to produce certain components (Adebo et al., 2022; Marco et al., 2017; Şanlier et al., 2019).

3.3 Delivery of micro-organisms to the gastrointestinal tract

In addition to transforming and synthesising various food components, the micro-organisms used in fermentation can also be involved in delivering specific health benefits directly (Marco et al., 2017; Rul et al., 2022). Fermented foods do not necessarily have live micro-organisms at the time of consumption, as their manufacture can include (heating) steps that remove micro-organisms from finished products or kill them (Marco et al., 2021). However, several fermented foods, such as sauerkraut, kimchi

and miso, can contain viable micro-organisms in quantities ranging between 10^6 and 10^9 cells/g (Marco et al., 2017). Fermented foods are often erroneously equated with probiotics. Although they may act as delivery vehicles for probiotics or other "biotic" substances, including prebiotics, synbiotics and postbiotics, stringent criteria must be met for a fermented food to be considered a "biotic" (Vinderola et al., 2023). Fermentation-associated microbes could exert influence on epithelial cells and the intestinal composition or function of the gut microbiome in a similar way to existing probiotic strains; however, more research is needed to understand underlying mechanisms (Marco et al., 2017). A study estimating dietary intake of live microbes indicated that more than 90% of the microbes in our diet originate from fermented foods (Marco et al., 2022). Consumption of foods with higher microbial concentrations seems to be associated with different health benefits, including lower systolic blood pressure, C-reactive protein, plasma glucose and body mass index levels. However, additional research to disentangle the benefits of the food from the microbes they provide is needed to find a possible causal relationship between live dietary microbes and health (Hill et al., 2023). This book does not focus further on the possible effect of live micro-organisms in fermented raw materials and foods. Instead, it focuses on (bio)chemical transformation of constituents.

4. Conclusion

Fermenting plant-based foods offers numerous potential benefits, including in terms of food safety, shelf life, organoleptic properties and health. However, the wide variability in raw materials and fermentation conditions presents a challenge in consistently predicting and attributing specific advantages to fermented plant-based foods. To advance our understanding, it is crucial to uncover the underlying mechanisms responsible for these positive effects. To understand the impact of food fermentation processes on the properties of plant-based raw materials and foods, an in-depth understanding of the (bio)chemical conversions that take place in a food fermentation process is needed. Understanding these conversions requires the availability of an in-depth analytical toolbox tailored towards (i) plant-based raw materials and foods and (ii) the specific context of raw materials and food fermentations. In the remaining chapters of this

edited book, the contributors provide a comprehensive overview of (bio) chemical analytical methods targeted towards characterising changes in constituents in plant-based raw materials or food fermentations.

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SECTION 2

Proteins

CHAPTER 3

Determination of free amino acids and total amino acid composition in plant-based raw materials

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Summary

- Raw materials: Plant raw materials, fractions, products
- Relevant applications: Determination of protein quality and nutritional value
- Fermentation-induced changes: Amino acid profile of proteins and free amino acid content due to (i) hydrolysis of peptides, proteins, or both by plant endogenous or microbial peptidases, proteases, or both, and (ii) 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
    - Derivatisation of amino acids
    - Determination of amino acid composition: UHPLC analysis

1. Introduction

The analysis of total amino acid (TAA) composition, including protein-bound and free amino acids (FAA), is an important tool for the assessment of protein nutritional quality, especially the content of the essential amino acids (EAA). The Food and Agriculture Organisation 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).

The analysis of FAA from plant-based materials can be useful because they directly or indirectly affect the nutritional, sensory and health-promoting properties of plant-based foods and beverages. The hydrolysis of

plant protein to FAA can improve protein digestibility (Christensen et al., 2022). FAA can also enhance the immune system, prevent liver damage, balance blood sugar levels, act as neurotransmitters and have many other effects (FAO, 2013). They also affect the taste and aroma of fermented plant ingredients and products (Emkani et al., 2022; Zhao et al., 2016). FAA can impart sweet, bitter, acidic and umami properties in fermented foods, depending on the amino acid (Kato et al., 1989; Zhao et al., 2016), and can serve as precursors for the synthesis of various volatile flavour compounds.

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 synthesised by the body to supply the demand and that must, therefore, come from the diet are called EAA. The nine EAA for humans are valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, threonine, histidine and lysine.

Plant proteins are considered an incomplete source of proteins since they lack some of the EAA (Table 3.1). Cereals are typically deficient in lysine but rich in sulphur-containing amino acids. In contrast, legumes provide a good source of lysine (64 mg/g of protein) and threonine (38 mg/g of protein) but are low in sulphur-containing amino acids, such as methionine, cysteine and tryptophan (Havemeier et al., 2017).

Table 3.1. Digestible indispensable amino acid scores of various protein sources according to the 0.5- to 3-year-old reference pattern score. Adapted from Havemeier et al., 2017.

Protein source	Histidine	Isoleucine	Leucine	Lysine	Met + Cys	Phe + Tyr	Threonine	Tryptophan	Valine	DIAAS	Limiting AA
Corn	110 ± 29.7	90 ± 14.6	162 ± 58.2	36 ± 14.9	126 ± 22.2	140 ± 42.8	86 ± 10.2	52 ± 35.4	90 ± 14.4	36	Lys
Rice	93 ± 7.0	89 ± 17.4	80 ± 12.4	47 ± 2.3	104 ± 11.0	119 ± 29.6	75 ± 4.1	114 ± 28.6	95 ± 18.0	47	Lys
Wheat	118 ± 21.7	91 ± 10.5	87 ± 11.1	48 ± 10.6	127 ±, 19.4	109 ± 16.9	78 ± 7.1	127 ± 17.8	92 ± 9.8	48	Lys
Hemp	124 ± NA	106 ± NA	85 ± NA	54 ± NA	121 ± NA	131 ± NA	87 ± NA	-	99 ± NA	54	Lys
Faba bean	108 ± 4.1	106 ± 2.2	95 ± 5.4	95 ± 4.3	55 ± 5.1	119 ± 3.4	91 ± 6.2	68 ± 7.8	83 ± 2.2	55	Met + Cys
Oat	91 ± 11.4	100 ± 4.2	94 ± 4.9	57 ± 5.8	151 ± 52.9	135 ± 9.2	85 ± 5.9	110 ± 17.2	102 ± 3.4	57	Lys
Rapeseed	107 ± 8.0	90 ± 4.9	78 ± 5.0	67 ± 10.3	125 ± 14.3	92 ± 12.3	97 ± 6.5	106 ± 9.4	92 ± 4.6	67	Lys
Lupin	121 ± 16.1	104 ± 27.2	89 ±, 19.3	75 ± 12.3	68 ± 12.7	121 ± 35.6	97 ± 22.7	72 ± 22.5	78 ± 14.6	68	Met + Cys
Pea	99 ± 9.7	101 ± 13.1	87 ± 11.5	110 ± 10.8	70 ± 12.3	116 ± 16.3	94 ± 7.9	77 ± 7.1	83 ± 9.8	70	Met + Cys
Canola	105 ± 6.9	93 ± 9.9	79 ± 7.8	72 ± 9.2	121 ± 10.4	97 ± 6.1	97 ± 12.2	112 ±, 19.5	87 ± 9.1	72	Lys
Soy	119 ± 9.4	124 ± 8.3	102 ± 6.1	96 ± 9.0	91 ± 11.5	147 ± 8.3	105 ± 6.0	132 ± 21.1	95 ± 7.3	91	Met + Cys
Potato	100 ± 7.3	156 ± 9.2	143 ± 11.2	122 ± 4.6	115 ± 6.0	210 ± 18.2	165 ± 12.0	128 ± 13.7	138 ± 5.1	100	NA
Gelatin	34 ± 9.5	34 ± 10.6	35 ± 8.7	60 ± 11.5	27 ± 10.3	36 ± 13.0	46 ± 4.9	2 ± 3.0	46 ± 8.6	2	Trp
Whey	85 ± 10.8	166 ± 23.2	138 ± 22.9	131 ± 25.2	132 ± 21.6	101 ± 14.0	174 ± 22.8	180 ± 47.0	116 ± 14.3	85	His
Egg	101 ± 11.7	129 ± 25.5	103 ± 16.2	133 ± 58.4	123 ± 53.2	144 ± 18.9	106 ± 14.1	129 ± 49.7	105 ± 32.3	101	NA
Casein	147 ± 9.4	153 ± 4.3	141 ± 6.6	134 ± 4.3	117 ± 5.0	201 ± 8.0	130 ± 4.3	159 ± 13.4	148 ± 2.7	117	NA
Pork	197 ± 13.6	153 ± 11.1	122 ± 9.2	157 ± 10.7	128 ± 10.7	148 ± 10.4	145 ± 10.1	144 ± 17.1	117 ± 9.0	117	NA

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

Fermentation can modify TAA, FAA and EAA (both total and free) content and composition in plant materials and thereby affect the nutritional and sensory properties as well as the health benefits of plant-based foods and beverages (Feng et al., 2023). The fermentation microbiota, substrates and conditions strongly affect the outcome (Feng et al., 2023; Gänzle et al., 2008).

Fermentation typically results in partial hydrolysis of plant proteins into (bioactive) peptides and FAA. Acidification during lactic acid fermentation can activate endogenous proteases of plant materials, which mainly hydrolyse plant protein to peptides (Gänzle et al., 2008; Rizzello et al., 2019). Microbial metabolites generated during fermentation, such as organic acids, can increase the susceptibility of plant proteins to enzymatic degradation (Emkani et al., 2022; Gänzle et al., 2014). Various micro-organisms involved in plant fermentations, such as lactic acid bacteria (LAB), *Bacillus* species, yeasts and filamentous fungi, also produce proteinases or peptidases, or both, and these enzymes hydrolyse plant proteins into peptides and FAA (Zheng et al., 2023). The proteolytic systems of LAB consist of various extracellular proteinases, amino acid transporters and intracellular peptidases, and their composition varies from strain to strain (Gänzle et al., 2008; Christensen et al., 2022). Fungi can secrete a range of acid, alkaline and neutral proteinases (Christensen et al., 2022; Feng et al., 2023). Multiple extracellular proteinases with broad pH and temperature activity and stability range have also been characterised from food-fermenting *Bacillus* species (Li et al., 2023).

Several studies have shown an increase in total FAA content in a wide range of legumes, cereals and seeds after fungal or lactic fermentation, which improves the bioavailability of amino acids (Emkani et al., 2022; Feng et al., 2023; Garrido-Galand et al., 2021; Harper et al., 2022). Many studies have also shown an improvement in free EAA content in various legumes after fermentation (Coda et al., 2015; Emkani et al., 2022; Sözer et al., 2019; Verni et al., 2019). For instance, fermentation of faba bean flour by *Lactiplantibacillus plantarum* increased the amount of free FAA and EAA, including the limiting sulphur-containing amino acids (Coda et al., 2015). Fermentation of cereals, such as maize, can significantly enhance the quality of protein and especially the concentrations of certain EAA, such as lysine (Singh et al., 2015). However, selection of an appropriate

starter culture is critical to achieving the target EAA content. Micro-organisms, especially filamentous fungi, can also convert carbohydrates in the plant matrix into microbial protein and extracellular enzymes during fermentation, which can increase the content of TAA and EAA in plant-based materials (Feng et al., 2023).

Fermentation can also lead to a decrease in the content of FAA or TAA or specific AA levels when micro-organisms use AAs for growth and metabolism, which can negatively affect nutritional value. The decrease in selected AAs can result from the preferential use of specific AAs by fermentation micro-organisms. For example, LAB cannot synthesise all AAs, and the need of LAB for AAs varies from species to species. The fermentation conditions can also favour the use of AAs for growth. For instance, low pH is known to shift lactic metabolism from hexose fermentation to the utilisation of AAs, especially glutamate, glutamine and arginine (Gänzle, 2015). FAA are also precursors of different flavour compounds and can be converted to alcohols, aldehydes, acids, esters and sulphur compounds via oxidative deamination and/or transamination reactions, reducing their concentration (Emkani et al., 2022; Thiele et al., 2002). Thus, a careful selection of a combination of micro-organisms, substrate and fermentation conditions is necessary to achieve the desired goal of improving the AA content and composition in the finished product.

2. Methods of analysis

2.1 Analysis of free amino acids in plant-based materials

2.1.1 General principle

Analysis of FAA in dry samples requires, first, an efficient extraction with a solvent in which different amino acids are well soluble. As in addition to FAA a plant-based material contains also proteins or peptides, protein precipitation is needed prior to analysis of FAA. Amino acids are in general well soluble in acid, and therefore precipitation with acid is preferred.

Due to amino acids' high water solubility, their ionic properties, and the absence of a selective chromophore, chromatographic separation of amino acids entails challenges. Therefore, in liquid chromatography (LC) methods, either pre- or postcolumn derivatisation is used to enable UV/Vis

or fluorescence detection. A derivatisation-free approach commonly used for amino acid analysis is a high-performance anion-exchange chromatography-coupled pulsed amperometric detection (HPAEC-PAD). However, both the LC and the HPAEC method typically require close to 1 h analysis time per sample, limiting their practicality for large-scale projects.

Hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry (HILIC-MS/MS) offers a fast, specific and sensitive alternative for amino acid analysis without requiring derivatisation (Prinsen et al., 2016). The HILIC separation mode allows retention of underivatised amino acids and good sensitivity, also due to the high content of organic solvent in the mobile phase. Twenty proteinogenic and other naturally occurring amino acids, such as γ-aminobutyric acid (GABA), are separated with the chromatographic method with a total assay time of 13 min. Detection and quantification are conducted through electrospray ionisation/tandem mass spectrometry in multiple reaction monitoring (MRM) mode, monitoring transitions from protonated molecules to specific product ions. To ensure the accuracy of the method, a heavy isotope–labelled standard mixture is spiked into the standards and samples.

Note that the method described below involves the use of specific column and high-performance liquid chromatography (HPLC) types. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems and/or columns, although this would require some optimisation, which is not included within the scope of this chapter.

2.1.2 Reagents

- Acetonitrile LC-MS Ultra
- Formic acid
- Ammonium formate
- Amino Acid Standard mixture solution (Sigma-Aldrich, Darmstadt, Germany)
- Labelled Internal Standards: DL-Alanine-2,3,3,3,-d4, DL-Glutamic acid-2,3,3,4,4-d5, and DL-Valine-2,3,4,4,4,5,5,5-d8, obtained from Sigma-Aldrich, and 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, and DL-Methionine-3,3,4,4-d4, obtained from C/D/N Isotopes (Quebec, Canada)

2.1.3 Extraction of free amino acids from plant-based materials

The protocol is developed for dry, powdered plant-based samples for the determination of FAA and other usual amines.

- Weigh accurately 10–15 mg dry biomass sample. Prepare triplicates.
- Add 750 µL of 30% (v/v) ethanol to the sample and mix thoroughly.
- Sonicate for 2 h (35 kHz).
- Precipitate dissolved proteins by adding sulfosalicylic acid (final concentration of 5%, w/v) and centrifuge.
- Transfer the clear supernatant to a new tube and dilute as needed with 10 mM ammonium formate.

2.1.4 Quantification of free amino acids via UHPLC-MS/MS

Analysis of the samples is performed on a Waters (Milford, MA, USA) Acquity ultra high-performance liquid chromatography (UHPLC) system and a Waters (Milford, MA, USA) Xevo TQ-XS using a Waters (Milford, MA, USA) ACQUITY UPLC BEH Amide Column, 130Å, 1.7 µm, 2.1 × 100 mm, kept at 35 °C. Injection volume is 1 µL. Separation is performed using gradient elution with 10 mM ammonium formate and 0.5% formic acid in water (A solvent) and 10 mM ammonium formate and 0.5% formic acid in 90% acetonitrile (B solvent) at a flow rate of 0.5 mL/min. Total time of run is 13 min. The gradient elution starts at 4% A and maintains at 4% A for 2 min, after this, the proportion of A increases to 70% A within 8 min, then directly returns to the initial percentage (4% A) and maintains there for 3 min.

Mass spectrometry is performed in positive polarity using the capillary voltage of 1.0 kV, desolvation temperature of 550 °C, and source temperature of 150 °C. Desolvation gas flow is 1,000 L/h, and cone gas flow is 150 L/h. Analytes are detected using MRM using auto dwell time function. For data acquisition and data processing, MassLynx software (V4.2) is used. Additional details of the method are presented in Table 3.2.

Analytes are quantified by internal standard method. Internal standards mix is added at the same concentration to all samples throughout a quantitative analysis. Compounds concentration in the internal standard mix are 0.05 ug/ml for L-Phenyl-d5-alanine, L-Leucine-d10, DL-Valine-2,3,4,4,4,5,5,5-d8, DL-Histidine-α,β,β-d3, 4-Aminobutyric-2,2.3,3,4,4,-d6 and DL-Methionine-3,3,4,4-d4; 0.5 ug/ml for DL-Alanine-2,3,3,3,-d4,

DL-Glutamic acid-2,3,3,4,4-d5, DL-2-Aminobutyric-d6 Acid and DL-Aspartic-2,3,3-d3 Acid; 1.5 ug/ml for DL-Cystine-2,2,3,3,3,3-d6; and 4 ug/ml for Glycine-d5. Internal standards for each compound are chosen based on the availability and retention times of analysed compounds. Calibration curves are prepared in the range from 0.01 to 100 µmol/L. Standards are prepared by diluting a stock standard mixture with 10 mM ammonium formate to the required concentrations. The limits of detection and quantification of the method are presented in Table 3.3.

Table 3.2. Mass spectrometry conditions of free amino acids and related compounds included in the analysis. The precursor and product ions used for MRM and the internal standard are listed for each compound. CE = collision energy; ISTD = internal standard; Rt = retention time.

	Component	Abbreviation	Precursor ion m/z	Product ion, m/z	Rt, min	Cone (V)	CE (eV)	ISTD
1	Alanine	Ala	90.1	44.1	3.71	15.0	8.0	L-Ala-d4
2	β-Alanine	β-Ala	90.0	30.1	2.93	15.0	15.0	L-Ala-d4
3	α-Aminobutyric acid	AABA	104.0	58.1	3.2	15.0	15.0	AABA-d6
4	γ-Aminobutyric acid	GABA	104.0	69.0	1.98	15.0	15.0	GABA-d6
5	Arginine	Arg	175.1	115.9	5.17	25.0	20.0	His-d3
6	Asparagine	Asn	133.0	74.0	4.47	16.0	14.0	L-Ala-d4
7	Aspartic acid	Asp	134.0	74.0	4.59	15.0	15.0	AspA-d3
8	Citrulline	Cit	176.1	159.1	4.59	15.0	15.0	L-Glu-d5
9	Cystathionine	Cyst	223.1	134.0	5.71	20.0	20.0	Cys-d6
10	Cysteine	Cys	122.0	76.0	3.17	12.0	10.0	Cys-d6
11	Ethanolamine	EA	62.0	44.1	2.20	15.0	15.0	L-Ala-d4
12	Glutamic acid	Glu	148.0	56.0	4.24	18.0	16.0	L-Glu-d5
13	Glutamine	Gln	147.1	84.1	4.35	15.0	15.0	L-Glu-d5
14	Glycine	Gly	76.0	30.1	4.08	15.0	6.0	Gly-d3
15	Histidine	His	156.1	110.0	5.2	15.0	15.0	His-d3
16	Isoleucine	Ile	132.1	86.1	2.00	15.0	15.0	L-Leu-d10
17	Leucine	Leu	132.1	86.1	1.85	15.0	10.0	L-Leu-d10
18	Lysine	Lys	147.1	84.1	5.32	20.0	15.0	His-d3
19	Methionine	Met	150.0	104.0	2.27	16.0	10.0	Met-d4
20	Ornithine	Orn	133.1	70.1	5.4	15.0	15.0	His-d3
21	Phenylalanine	Phe	166.1	120.1	1.86	20.0	15.0	L-Phe-d5
22	Proline	Pro	116.0	70.1	2.8	20.0	10.0	L-Ala-d4
23	Serine	Ser	106.0	60.0	4.4	15.0	10.0	L-Ala-d4
24	Threonine	Thr	120.0	74.1	3.99	15.0	10.0	L-Ala-d4
25	Tryptophan	Trp	205.1	146.0	1.86	18.0	18.0	L-Leu-d10
26	Tyrosine	Tyr	182.1	136.1	2.89	20.0	15.0	L-Ala-d4
27	Valine	Val	118.1	72.1	2.63	18.0	10.0	DL-Val-d8

Table 3.3. Limit of detection (LOD) and limit of quantitation (LOQ) determined for the method.

	Component	LOD µmol/L	LOQ µmol/L
1	Alanine	1.0	2.0
2	β-Alanine	0.5	2.0
3	α-Aminobutyric acid	1.0	5.0
4	γ-Aminobutyric acid	0.02	0.05
5	Arginine	0.1	0.5
6	Aspargine	0.02	0.1
7	Aspartic acid	0.05	0.2
8	Citrulline	0.02	0.1
9	Cystathionine	0.05	0.2
10	Cysteine	0.5	1.0
11	Ethanolamine	0.1	0.5
12	Glutamic acid	0.05	0.5
13	Glutamine	0.1	0.5
14	Glycine	5.0	10.0
15	Histidine	0.01	0.1
16	Isoleucine	0.05	0.1
17	Leucine	0.02	0.1
18	Lysine	0.05	0.5
19	Methionine	0.01	0.02
20	Ornithine	0.1	0.5
21	Phenylalanine	0.01	0.02
22	Proline	0.1	0.5
23	Serine	1.0	2.0
24	Threonine	0.2	0.5
25	Tryptophan	0.02	0.05
26	Tyrosine	0.1	0.2
27	Valine	1.0	2.0

2.2 Analysis of total amino acid content and composition in plant-based materials

2.2.1 General principle

To analyse the amino acid composition or total protein concentration in a complex sample, the proteins and peptides must be hydrolysed first, to release the individual amino acids. Proper hydrolysis of proteins and peptides is a critical step in the analysis of TAA, but so far, there is not one single hydrolysis method suitable for all amino acid residues. Conventional acid hydrolysis is performed using 6N hydrochloric acid (HCl), but the protocol needs modifications in order to achieve complete hydrolysis and avoid the degradation of labile amino acids. Cysteine and cystine are typically partially degraded in acid hydrolysis, and therefore they are often oxidised to form cysteic acid, a stable compound. Also, methionine will be oxidised to methionine sulfone in the oxidation step prior to acid hydrolysis. Tryptophan (Trp), being sensitive to acid, needs to be determined after a separate alkaline hydrolysis method, and recovery of Trp is further improved by the addition of an antioxidant (La Cour et al., 2019).

Derivatisation of amino acids prior to liquid chromatographic analysis improves the method sensitivity when using UV detection and allows the use of reversed-phase chromatography. Waters' AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) reagent converts both primary and secondary amino acids to stable derivatives.

The general principle of the TAA content and composition determination is presented in Figure 3.1. The derivatisation step and UPLC analysis are performed according to the validated Waters AccQ-Tag™ method. The Waters AccQ-Tag method is used also in Association of Official Analytical Chemists (AOAC) Official Method, 2028.06, which recently obtained Final Action status from AOAC International (Jaudzems & Fuerer, 2022). Since the analysis of TAA composition usually does not require high method sensitivity, a method based on mass spectrometry detection, similar to the analysis of FAAs, is not considered necessary.

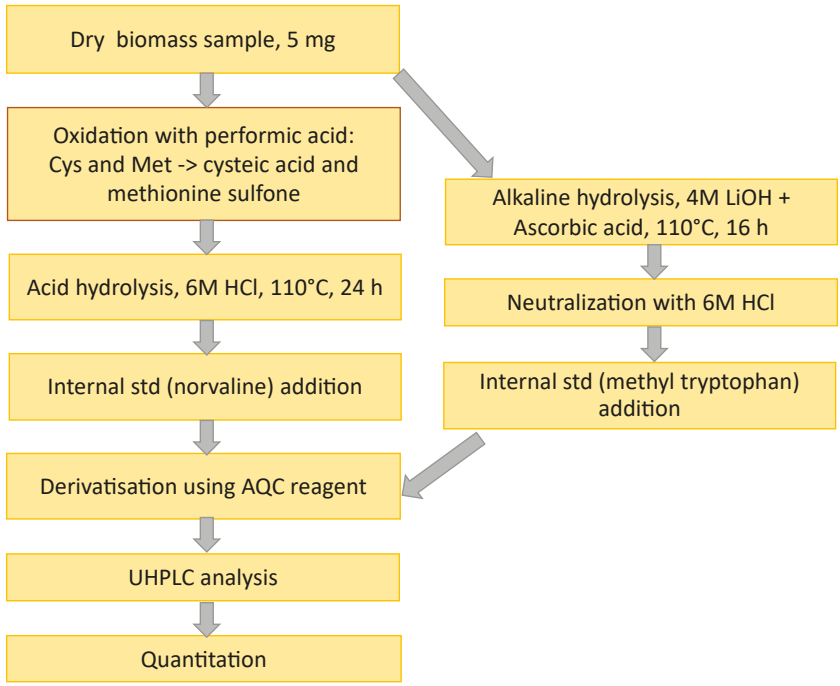

Figure 3.1. General principle of amino acid composition analysis, including the protein hydrolysis steps.

2.2.2 Reagents

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

2.2.3 Acidic hydrolysis

- Weigh accurately ~5 mg of dry (freeze-dried) sample.
- Prepare a performic acid solution by mixing 5 mL of 30% (w/w) hydrogen peroxide with 45 mL of formic acid–phenol solution (88%, w/v formic acid and 0.5%, w/v phenol).
- Add 0.5 mL fresh performic acid solution to the sample and mix.
- Incubate at 0 °C for 12 h.
- Add 1 mL of ice-cold H_2O.
- Evaporate to dryness under nitrogen flow.
- Add to the dry sample 1 mL of 6 N HCl solution that contains 0.1% (w/v) phenol.
- Hydrolyse at 110 °C for 20 h.
- Evaporate to dryness under nitrogen flow.
- Reconstitute in 1 mL of H_2O.
- Dilute 1/10 with H_20. Further dilution, e.g. 1/50, may also be needed depending on the sample.

2.2.4 Alkaline hydrolysis

- For the determination of tryptophan, prepare 4 M lithium hydroxide (LiOH) solution containing ascorbic acid (95 mM).
- Weigh accurately 2–5 mg of dry (freeze-dried) sample.
- Dissolve the sample in 0.9 mL of freshly prepared hydrolysis solution.
- Remove excess oxygen in the vial headspace by nitrogen gas and close the vials.
- Hydrolyse at 110ºC for 16 h.
- Neutralise by adding 600 µl of hydrochloric acid (6 N).

2.2.5 Derivatisation of amino acids

Derivatisation is performed according to the Waters (Milford, MA, USA) AccQ-Tag method.

- Reconstitute AccQ-Tag reagent (i.e. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) in acetonitrile (350 µl): Vortex the mixture for 10 sec, heat on a heating block at 55ºC and vortex occasionally until the reagent is dissolved.
- Pipet 60 µL of AccQ-Tag borate buffer and add 10 µl of sample solution as well as 10 µl of internal standard solution. Add internal standard norvaline (250 µM) to the samples that underwent acidic hydrolysis and methyl-tryptophan (50 µM) to the samples originating from alkaline hydrolysis.
- Vortex the mixture for 30 sec.
- Add 20 µL of AccQ-Tag reagent.
- Vortex the sample–reagent mixture instantly for 10 sec.
- Allow to stand at room temperature for 1 min.
- Heat in a heating block for 10 min at 55 °C.
- Derivatised amino acid standard mixtures are derivatised as samples by adding 10 µl of standard solution and 10 µl of internal standard solution.

2.2.6 Determination of amino acid composition via UHPLC

Analysis is performed on Waters (Milford, MA, USA) Acquity UHPLC system with a diode array detector. For chromatography, a Waters Acquity BEH C18 Column, 130Å, 1.7 µm, 2.1 × 100 mm is used and the column temperature is kept at 55 °C. Separation is performed at a flow rate of 0.7 mL/min using certified Accq-Tag Ultra eluents for amino acids analysis by Waters. The total run time is approximately 9.5 min. The gradient elution started at 0.1% B and was at 0.1% B for 0.54 min, then was increased a few times, to 9.1% B within 5.74 min, to 21.2% B up to 7.74 min, to 59.6% B up to 8.04 min and to 90% B up to 8.05 min, and was maintained at 90% B for 0.59 min then directly returned to the initial percentage for column equilibration during 0.77 min. The signal is detected at 260 nm (2.4 nm resolution, 20 points/sec).

2.2.7 Calculations

Waters (Milford, MA, USA) Empower 3 software is used for data acquisition and data processing. 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. Calibration curve of each determined amino acid is prepared in range from 10 to 500 µmol/L using the internal standard (IS) concentration 250 µmol/L. Concentrations of AAs are calculated from standard curve by using the peak area ratio (peak area of analyte/peak area of IS). The lowest point of the standard curve, 10 µmol/L, is used as the LOQ. The protein content of the samples is estimated based on the sum of amino acid residues determined.

3. Tips and tricks

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

The method is developed for small sample amounts, 5–15 mg of dry sample. Therefore, the sample needs to be properly milled and mixed to achieve homogenous consistency. Additionally, it is recommended to prepare replicate samples to control analytical variability.

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CHAPTER 4

Determination of the extractable protein content of (fermented) plant-based raw materials and foods

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Summary
- Raw materials: Cereals and legumes
- Relevant applications: A wide range of foods, including dairy and meat analogues
- Fermentation-induced changes: Altered protein extractability and solubility due to enzyme-induced cell wall degradation, proteolysis, pH changes and phytic acid breakdown
- Analytical method:
  – Quantification of proteins after centrifugation of aqueous dispersions of plant material

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 2,000 (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%. For example, wheat typically has a protein content of 10–15% (Shewry & Hey, 2015); oat groats, approximately 15–20% (Peterson, 2011); pea, 20–31% (Day, 2013); and faba bean, 22–38% (Mayer Labba et al., 2021).

1.2 Protein solubility and extractability

Plant storage proteins have traditionally been categorised into four groups, i.e. albumins, globulins, prolamins and glutelins, based on their solubility and extractability in various solvents, as proposed by Osborne (1907). According to this classification, which comprises sequential extractions, albumins are extractable in water, globulins in saline solutions, prolamins in concentrated aqueous alcohol solutions, and glutelins in dilute aqueous acid or alkali solutions (Day, 2013; Osborne, 1907). As research has evolved, it has become clear that each protein class, even for a given raw material, comprises a complex mixture of proteins and that there is some overlap among the classes (Day, 2013). The proportion of each protein class in plant-based raw materials varies depending on the plant source as well as the prior processing of the material.

The solubility of proteins is dictated by their surface properties, which are influenced by the quantity and arrangement of hydrophilic and hydrophobic amino acids on the protein surface.

In aqueous media, solubility is hampered if hydrophobic patches remain on the protein surface, facilitating hydrophobic interaction between protein molecules, possibly triggering precipitation. Additionally, medium pH and ionic strength, which impact electrostatic repulsive effects between proteins, as well as temperature, are important factors affecting protein solubility (Lam et al., 2018). The solubility of proteins in aqueous media is often, although not in all cases, of great relevance for their functional properties (e.g. gelling, foaming, emulsifying properties) in liquid and semi-solid food systems. More detailed information about the solubility of plant proteins can be found in the excellent review by Grossmann and McClements (2023).

Equally important as the inherent solubility of a protein is its extractability from the plant tissue. In plants, proteins are commonly enclosed within cellular structures that require disintegration to release

the proteins. Therefore, a protein may be soluble under a given set of conditions, but not extractable. Importantly, processing of plant materials can have an impact on the extractability of plant proteins, for example by making them physically more (or less) available for extraction. At the same time, however, processing can also affect the proteins structurally, e.g. by inducing (partial) denaturation or aggregation, in turn affecting the protein solubility and thus also the extractability.

To determine the extractability or solubility of plant proteins, a typical approach involves dispersing the plant material in an extraction medium (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 variation in the specific methods employed, comparing results obtained from different studies becomes challenging. For example, the quantity of protein present in the supernatant is significantly influenced by the magnitude of the centrifugation force applied to the dispersion. This is the case because some of the protein present in the supernatant may, e.g. as the result of prior processing, not occur in a purely soluble state, but, rather, exist as a larger colloidal structure, such as a fractal aggregate (Wouters & Nicolai, 2024). Such structures can be readily dispersible and even display functional properties but cannot be considered as single solubilised molecules. Therefore, while in the literature the term “protein solubility” is often used, in many cases, it is more accurate to refer to protein extractability.

1.3 How can fermentation affect protein extractability?

Fermentation can influence the extractability of proteins from plant materials. The specific outcome depends on the micro-organisms, substrate and fermentation conditions. Fermentation may cause degradation of cell walls through the activation of carbohydrate-hydrolysing enzymes present in the plant material. Alternatively, cell walls can be broken down by such enzymes produced by starter cultures added to the medium or by micro-organisms present in the raw material (Fan et al., 2024). 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 micro-organisms present in the matrix, which may also enhance the extractability of proteins from the plant tissue (Emkani et al., 2022; Sharma et al., 2020).

Some micro-organisms, such as certain LAB and many filamentous fungi, secrete proteolytic and peptidolytic enzymes that hydrolyse plant proteins into peptides and FAA, 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 et al., 2022). Many plant proteins have a low solubility at acidic pH values, i.e. in proximity of the isoelectric point of many globular plant proteins, including those of pea, faba bean and oats (Arogundade et al., 2006; Li & Xiong, 2021). Therefore, investigations into the impact of fermentation on protein extractability are needed and are of great significance, given the various functionalities extractable proteins may display in food systems.

2. Methods of analysis

2.1 General principle

The evaluation of protein extractability 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. Analysis of the protein content of the supernatant

Protein extractability is often simply expressed as the ratio of the protein concentration in the supernatant to the protein concentration in the initial dispersion. An alternative and more precise method to quantify protein extractability is to divide the total mass of protein recovered in the supernatant by the mass of protein in the initial dispersed powdered sample. However, this method requires accurate collection and weighing of the entire supernatant, which can sometimes be challenging in practice. For example, some authors include a filtration step to prevent “contamination” of the supernatant with material from the residue during decantation. However, this also implies the need for thorough rinsing of the filter so that the entire supernatant can be recovered.

The methods employed in scientific research for protein extractability (or solubility) determination exhibit significant variation in how these different specific steps are executed, posing challenges when attempting to compare results obtained from different studies. Although two standardised AACC (American Association of Cereal Chemists) methods for protein extractability analysis exist, namely the Nitrogen Solubility Index (AACC 46-23-01) and the 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 during the dispersion of the proteinaceous material prior to centrifugation. These methods will not be discussed here further.

Overall, while determining protein extractability may appear straightforward, in reality, determination is more complex due to various factors in the applied procedures that can influence the final outcome. These factors are discussed in detail in what follows. Sample properties that cannot be altered during the analysis phase, such as composition and processing history, are not addressed here.

2.2 Factors affecting analysis outcome

2.2.1 Sample preparation

Extractability analyses are 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. For example, Figure 4.1 shows that in a study on defatted oat wholemeal, reducing the particle size (D50) by ball milling from 34 to 16 µm increased the protein extractability, otherwise measured in a standardised way, significantly (Janssen et al., 2023).

To ensure accurate comparisons of extractability results across different ingredients, it is advisable to standardise the particle size. 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 the sample will go through a 100-mesh (~147 µm) screen. Here, it should be noted that standardisation with regard to particle size should be done with care and with sufficient background knowledge on the raw material under study. For example, if intact cells smaller than the targeted particle size are still present,

protein extractability may still be affected. This is particularly relevant when comparing protein extractabilities for different plant sources with different tissue morphologies. When milling a sample before analysis, it is also 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 or to employ alternative milling techniques, such as cryogenic ball milling.

During analyses of the effect of fermentation on the extractability of proteins from plant materials, the sample of interest may still contain a substantial amount of water. These kinds of samples can be analysed as is (after dilution if necessary) or dried and milled before the extractability analysis. In the latter case, it must be noted that the drying process may have an effect on the extractability of the proteins.

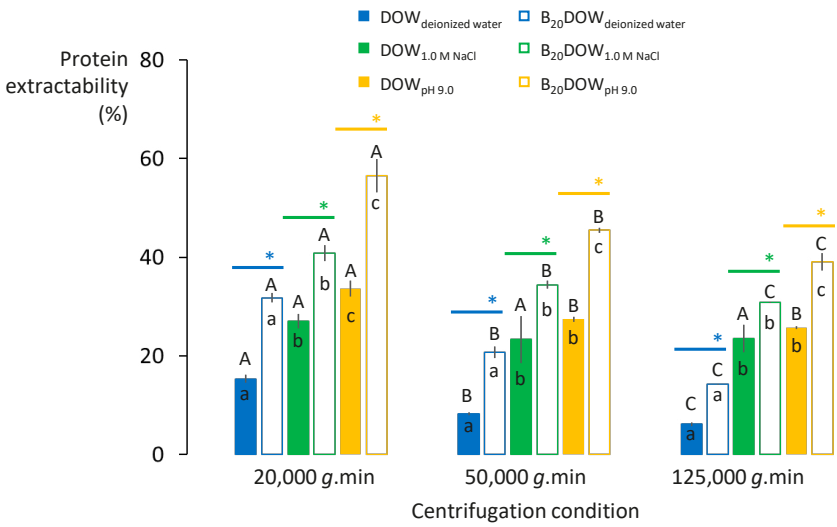

Figure 4.1. Protein extractabilities of defatted oat wholemeal (DOW) or 20 min ball-milled DOW (B_{20}DOW) in deionised water, a 1.0 M NaCl solution and a solution at pH 9.0, after centrifugation at 20,000 *g*.min, 50,000 *g*.min and 125,000 *g*.min. Bars with a different capital letter for a given extraction medium and DOW type or a different lowercase letter for a given centrifugal force and DOW type are significantly different (α = 0.05). Bars marked with an asterisk indicate significant differences (α = 0.05) between DOW and B_{20}DOW extracts for a given extraction medium and centrifugal force. Reproduced from Janssen et al. (2023) with permission from Elsevier.

2.2.2 Dispersion medium

In food research, water (deionised) is the most common solvent used in protein extractability studies. The solubility of proteins is influenced by such factors as pH and the presence of salts. For example, Figure 4.1 shows that significantly different protein extractabilities are obtained for oat wholemeal when the extraction is performed with deionised water, a solution at 1.0M NaCl or a solution at pH 9.0. 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. 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 considered 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, such as magnetic stirring. Additionally, a high viscosity can affect the precipitation behaviour of the proteins during the subsequent centrifugation step. Grossmann and McClements (2023) indicated that to achieve reproducibility, the protein concentration should be kept low, between 0.1–0.5% w/v, which minimises the amount of water trapped in the pellet after centrifugation. Nevertheless, it is important to select a protein concentration that aligns with the intended application.

Temperature is another variable that needs to be controlled during the dispersion phase. Generally, protein solubility increases slightly with increasing temperature, up to a point where the protein may denature and/or aggregate and where the solubility decreases. These effects and the temperatures at which they occur are protein specific (Grossmann & McClements, 2023). 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 enzyme activation, suitable enzyme inhibitors can be added to the dispersion. Sometimes preservatives, such as sodium azide, are also 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 or 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 h. Longer mixing times are typically required when adjusting the pH to allow for it to stabilise 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 protein 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 × *g*. The amount of protein in the supernatant occurring as larger colloidal structures, which may, for example, be present as the result of prior processing, will decrease with an increase in centrifugation force. The extent to which proteins will end up in the precipitate depends on the size distribution of the colloidal structures present. As an example, Figure 4.1 shows that for oat wholemeal, the protein extractability significantly increases when less-intensive (centrifugation force, centrifugation time) centrifugation processes are used. As a point of interest, the authors of this study reported centrifugation conditions with *g*.min units, to take both the centrifugation force and the time into account, as both parameters are relevant in determining the impact of centrifugation (Janssen et al., 2023). 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 × *g* was applied (Runyon et al., 2015).

In addition to the centrifugation force, the way the centrifugation process is halted (with or without braking) can impact the results. Also,

the type of centrifuge rotor (whether fixed angle or swinging buckets), the size of the centrifugation container (or tube) and the volume of the centrifuged sample must be standardised to allow for proper sample comparison.

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 TAA content after acid hydrolysis, 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 methods, such as the Dumas or Kjeldahl methods. 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, FAA, peptides and phospholipids, can be present in the samples (Lam et al., 2018; McClements et al., 2021). In most plant-based materials, however, the contribution of nonprotein nitrogen to the total nitrogen content is typically low. 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 composition of the material. In the amino acid analysis, care must be taken that the amino acids that are degraded during the typically applied acid hydrolysis (tryptophan, cysteine and methionine) 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 instead of protein extractability.

The protein content of the liquid supernatant obtained after centrifugation is often determined using spectrophotometric protein assays, such as the Lowry or Bradford methods (Mæhre 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 of protein from different sources.

To ensure consistency and comparability, it is recommended to employ the same protein assay method, such as the Dumas or Kjeldahl method,

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. Even so, one should take care when interpreting results of samples with heterogeneous protein compositions. For example, when specific subfractions of proteins are preferentially soluble, this may imply that different responses will be obtained in a given assay for the same amount of protein in the supernatant and raw material.

2.3 Example of detailed protocol

From the above, it is clear that a wide range of factors can affect the outcome of protein extractability measurements. Therefore, there is no single protocol that can be deemed ideal for all particular situations, purposes or applications. The important thing is to be aware of the importance of the above-discussed parameters and take them into consideration when utilising a protein extractability protocol. In the following, an example of such a protocol is given, here applied to investigate the impact of varying pH on the protein extractability of different plant-based protein concentrates (faba bean, pea, oat). Another example can be found in a recent publication (Janssen et al., 2023), of which some example results were discussed previously in this chapter (Figure 4.1).

2.3.1 Sample preparation

This protocol is developed for plant-based samples in dry, powdered form. Powdered samples can be analysed as is 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, e.g. by oven drying at 105 °C until constant mass is reached (overnight is usually sufficient).
- Analyse the nitrogen content of the samples with the Dumas method and calculate the protein content if the nitrogen-to-protein conversion factor is known.
- Weigh 1.75 g dry sample (on dry-matter basis) into a 100 mL glass beaker and add deionised water to a total weight of 35 g (to make a 5% w/w dispersion). Prepare dispersions in triplicate.
- Agitate the dispersion using a magnetic stirrer for 30 min, avoiding excessive foam formation.
- Measure the "native" pH of the dispersion.
- Adjust the pH of the dispersion with 1 M HCl or NaOH to the desired pH value (do not add anything to a sample to be measured at native pH) under continuous magnetic stirring and write down the consumed amount of HCl or NaOH.
- Stir for another 30 min and adjust the pH again if needed and write down the consumed amount of HCl or NaOH. Repeat this once more.

  Note: the above steps regarding pH correction apply to the specific situation here described. If one is interested in, for example, the effect of varying ionic strength, dispersion media containing varying levels of salt should be used to prepare the dispersions.

- Pour the entire dispersion from the beaker into a 50 mL centrifuge tube and centrifuge the sample at 10,000 × g for 15 min at 22 °C.
- Collect at least 10 mL of the supernatant with a pipette.
- Analyse the nitrogen content (% w/w) of the supernatants with the Dumas method in duplicate and calculate the protein content if the nitrogen-to-protein conversion factor is known.

2.3.4 Calculations

- Calculate the protein content (% w/w) in the initial dispersion. In the calculations, use as the total weight of the dispersion 35 g + the weight of added HCl or NaOH.
- Protein solubility (%) = 100 * (protein content of supernatant)/(protein content of initial dispersion).

3. Example of results

Results obtained with the above-described method for the here-intended application are typically presented as shown in Figure 4.2, i.e. as protein extractability as function of pH. Figure 4.2 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. In addition, there is a clear effect of pH on the extractability of proteins from these raw materials. More specifically, low solubilities are noted for all protein sources at pH values in proximity to their isoelectric points (~4.5–5.0). More examples of the pH dependence of protein solubility can be found in the review by Ma et al. (2022).

As an alternative example of protein extractability analyses, the previously discussed Figure 4.1 shows the dependence of protein extractabilities of oat wholemeal on extraction medium, centrifugation conditions and prior ball milling treatment. The conditions of the methods used for protein extractability determination can be found in the paper text (Janssen et al., 2023).

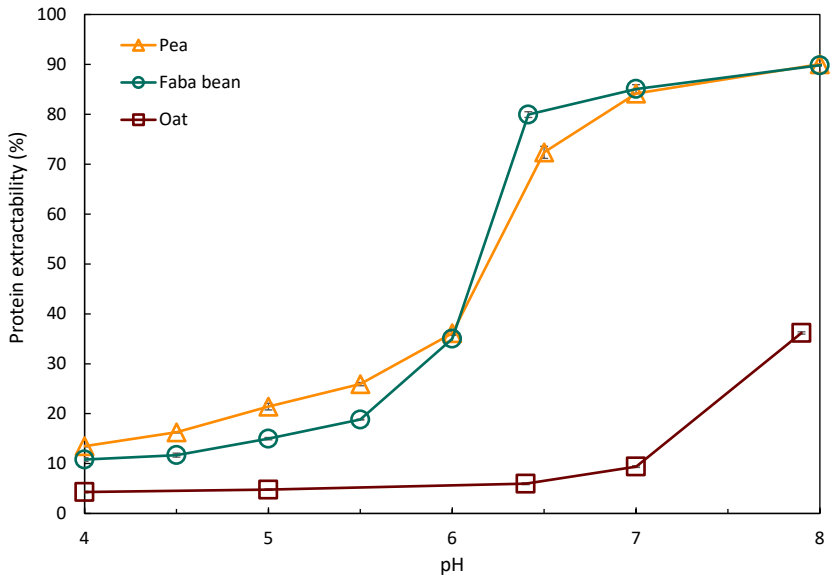

Figure 4.2. Extractability of proteins from pea, faba bean and oat protein concentrates at various pH values. Unpublished data from VTT (Technical Research Centre of Finland).

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CHAPTER 5

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, altered protein digestibility
- Analytical methods:
  - Protein degree of hydrolysis
    - Ortho-pthalaldehyde (OPA) assay
  - Protein apparent MW 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. On a dry-matter basis, wheat and oats contain about 12–15% (Hermans et al., 2021) and 15–20% (Sunilkumar et al., 2017) of protein, 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 thousand 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 et al., 2023).

Oat proteins consist of up to 12% albumins and up to 80% globulins (Klose & Arendt, 2012; Mäkinen et al., 2017). The globulin fraction comprises 3S, 7S and 12S globulins, with the latter as the main fraction (Mäkinen et al., 2017). 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., 2017; Peterson, 1978). Oats have traditionally been used as wholemeal or flakes, but the ongoing shift from animal protein to plant protein–based foods has extended the applicability of oat and its 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, 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 et al., 2023), the physical unavailability of oat proteins as a result of their encapsulation in intact cell wall structures (Janssen et al., 2023; Miller & Fulcher, 2011), and the heat treatment ('kilning') that is typically applied to oat groats to ensure lipase inactivation (Norlander et al., 2024; Runyon et al., 2015).

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

Food fermentation micro-organisms, often LAB, 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 can be expected to inactivate not only 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. Not only lactic acid fermentation, but also fermentation with *Bacillus* species can result in the production of extracellular proteases that exhibit

a wide range of activity and stability across various pH levels and temperatures (Li et al., 2023). In general, the proteases hydrolyse native proteins in a food matrix into lower-molecular weight (MW) peptides and amino acids (Figure 5.1). These peptides can have structures varying in, among other aspects, MW, hydrophobicity and charge density (Wouters et al., 2016). All this can have major consequences along the entire food value chain.

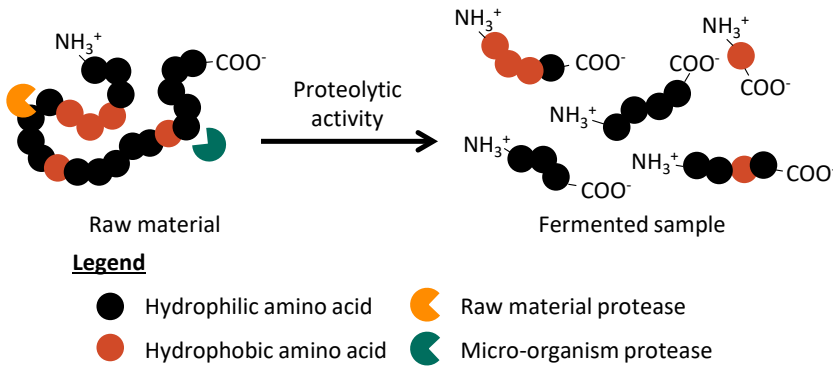

Figure 5.1. Possible impact of food fermentation on the molecular structure of wheat and oat proteins.

As mentioned, wheat gluten and oat proteins have low extractabilities in aqueous media, for several reasons. Enzymatic protein hydrolysis can result in increased protein solubility (Wouters et al., 2016) and, hence, increased protein extractability (Arte et al., 2016). This may lead to a more efficient use of cereal 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 a low protein content (Walther et al., 2022). In addition, partial protein hydrolysis can lead to higher digestibility of the protein (Joye, 2019), thus increasing the bioavailability of EAA. 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). In addition, the gas cell–stabilising 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 a 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 that allow assessing such changes in the molecular structure of proteins.

2. Methods of analysis

2.1 Advantages and disadvantages of the three 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 5.1). Considering all of this, it may be concluded that the ortho-phtaldialdehyde (OPA) assay offers the highest efficiency and accuracy for monitoring fermentation-induced proteolytic changes. Therefore, the pH-stat and trinitrobenzenesulfonic acid (TNBS) methods will not be further discussed here.

Table 5.1. Advantages and disadvantages of analytical methods for determining the degree of protein hydrolysis. Adapted from Rutherfurd, 2010. TNBS = trinitrobenzenesulfonic acid method, OPA = ortho-phtaldialdehyde.

Method	Advantages	Disadvantages
pH-stat	Real-time monitoring No derivatisation	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 derivatisation	No real-time monitoring Reducing agents cannot be used Inaccurate for proline-rich proteins
OPA	Rapid derivatisation 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 hydrolysed, or which types of peptides are released. These factors can, however, significantly affect the nutritional and technological outcome of a proteolytic process. Therefore, as a 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 sulphate 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 MW distribution (based on well-defined protein MW markers). However, it is important to note that the UV intensity at 214 nm, which is often used for protein detection in such measurements, depends on the number of peptide bonds in a protein. Thus, lower MW peptides will generate a lower UV intensity than higher MW 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). 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 analysing proteins based on their MW. However, it also has several drawbacks, as it provides only qualitative information on the protein's apparent MW distribution and is more time consuming than SE-HPLC analysis. Therefore, SDS-PAGE will not be further discussed here.

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 (Figure 5.2). Note that the method described below involves the use of specific column and HPLC types. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems and/or columns, although this would require some optimisation, which is not included within the scope of this chapter.

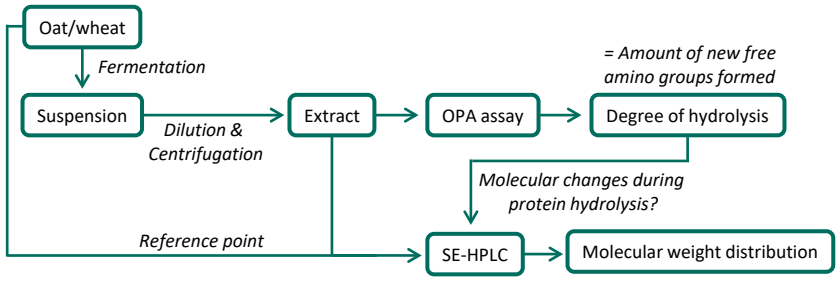

Figure 5.2. Schematic overview of the experimental setup. OPA = ortho-phtaldialdehyde; SE-HPLC = size-exclusion high-performance liquid chromatography.

2.2 Sample preparation

Food fermentation can be performed by inoculating (e.g. at 10^7 CFU/g dm) oat or wheat wholemeal suspensions (e.g. in a 1:5 w:v ratio) followed by an incubation period (e.g. 24 h). These fermented suspensions can then be analysed as is or, if the extractability of the proteins is of interest, further diluted (e.g. to a 1:10 w:v ratio) and then 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 time frame, by inactivating proteases in the system. This can be achieved in several ways. One possibility is to apply a heat shock (e.g. 10 min at 100 °C) prior to determining the DH and protein apparent MW distribution. If such heat treatment results in the formation of insoluble protein aggregates in the extracts, an additional centrifugation step is required to separate the insoluble and soluble protein materials. If the extent to which the heat shock causes protein aggregation and precipitation is deemed too large, 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 to be expected after the fermentation timeframe. In such case, the heat shock can be omitted as well. Finally, if the analyses described below can be performed immediately after the fermentation process, proteases can be inactivated by the addition of a SDS-containing medium (see below).

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 the process under investigation, such as a food fermentation trial. Peptide bonds cleavage liberates terminal NH_2/NH_3^+ groups (Figure 5.1), which, in the presence of a reducing agent (a necessity for this reaction to occur), react with OPA, thereby forming a fluorophore compound that absorbs UV light at 340 nm (Figure 5.3). Complete protein solubilisation, ensuring that all targeted NH_2/NH_3^+ groups are, in fact, measured, is achieved by using media containing SDS and dithiothreitol (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).

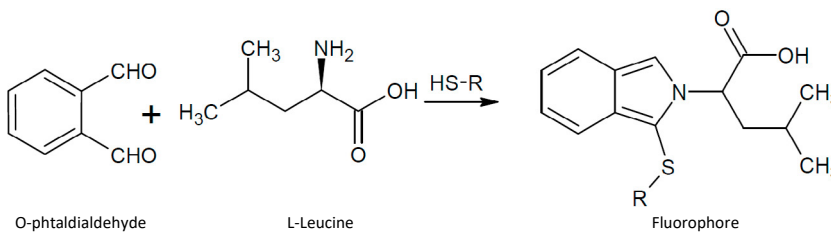

Figure 5.3. Reaction of o-phtaldialdehyde with L-leucine forming a fluorophore compound.

2.3.2 Reagents

- OPA reagent
  - Prepare a 0.10 M disodium borate (Borax) solution containing 1.0% w/v SDS and 0.088% w/v DTT in deionised water. Use only 90% v/v of the total volume.
  - Dissolve 0.080% w/v OPA in 8% v/v aqueous ethanol and add it to the Borax solution.
  - Add deionised water until the desired volume is reached and flush with nitrogen gas.
  - This solution should be prepared fresh.

- Buffer A
  - Prepare a sodium phosphate buffer (0.050 M, pH 6.8) containing 2.0% w/v SDS and 1.0% w/v DTT.
  - This buffer should be prepared fresh and flushed with nitrogen gas.
- L-Leucine stock solution
  - Prepare an L-leucine stock solution (2 mM) in buffer A.

2.3.3 Sample preparation

Samples

- Accurately weigh in triplicate around 2–3 mg of protein (on a dry-matter basis) and note down the weight.
- Add 1.8 mL of buffer A and vortex extensively.
- Shake (30 min at 150 rpm) and centrifuge (10 min at 10,000 *g*).
- Transfer 400 µL of each supernatant to a test tube.

  Note: 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 (buffer A) and the SDS and DTT concentration therein to achieve the same final concentrations as when analysing dry samples.

Calibration curve

- Prepare a dilution series (0 mM, 0.5 mM, 1.0 mM, 1.5 mM and 2 mM, where the 0 mM sample represents a blank) of the L-Leucine stock solution in buffer A.
- Transfer 400 µL of each L-Leucine standard solution to a test tube (in triplicate).

2.3.4 Sample analysis

- Add 3.0 mL OPA reagent to each test tube.
- Incubate for 20 min at room temperature.
- Immediately after exactly 20 min, measure the absorbance at 340 nm.

2.3.5 Calculations

The DH is calculated by comparing the amount of free amino groups between the processed (e.g. fermented) and control (e.g. unfermented) samples, according to the following formula (Spellman et al., 2003).

$$\mathrm{DH} = \frac{100 * n}{N} \; with \; n = \frac{\left(Abs_p - Abs_{np} * \frac{C_p}{C_{np}}\right) - Intercept_{CC}}{Slope_{CC} * C_p}$$

Figure 5.4. Calculation of the degree of protein hydrolysis (DH). n = calculated hydrolysis equivalents (mmol/g); N = total theoretical number of peptide bonds present in a sample (for example 7.81 mmol/g for oat (Wang et al., 2015)); Abs_p = absorbance at 340 nm of processed material; Abs_{np} = absorbance at 340 nm of non-processed material; C_p = protein concentration of processed samples (mg/mL); C_{np} = protein concentration of non-processed samples (mg/mL); CC = calibration curve based on L-leucine (Adler-Nissen, 1986; Spellman et al., 2003).

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 by the elution rate. The absorbance of the eluted proteins is monitored by UV detection at 214 nm (peptide bonds) and/or 280 nm (aromatic amino acids). Apparent MWs are estimated based on the elution times of MW protein standards, analysed 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, so that all proteins are analysed in their unaggregated, molecular and unfolded state. It can be noted that 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 2.0% w/v SDS, 1.0% w/v DTT and 2 M urea (= extraction buffer for dry powders = extraction buffer 1).
- Prepare a sodium phosphate buffer (0.10 M, pH 6.8) containing 4.0% w/v SDS, 2.0% w/v DTT and 4 M urea (= extraction buffer for wet extracts = extraction buffer 2).
- Prepare a sodium phosphate buffer (0.050 M, pH 6.8) containing 0.10% w/v SDS (= elution buffer).

2.4.3 Sample preparation

- Dry powders:
  - Accurately weigh 1.0 mg protein in triplicate in an Eppendorf tube and note down the weight.
  - Add extraction buffer 1 to obtain a solution with a concentration of 1.0 mg protein/mL.

- Wet extracts:
  - Dilute the extract (in triplicate) with deionised water to a concentration of 2.0 mg protein/mL.
  - Dilute an aliquot of the extract (1:1 v:v) in extraction buffer 2.

- 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 deionised water and 0.10 M sodium phosphate buffer containing 2.0% w/v SDS.

- MW protein standards:
  - Potential candidates are phosphorylase B (97 kDa), bovine serum albumin (66.5 kDa), 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). Other standards can

be considered as well, depending on the targeted constituents and MW ranges.
    - Weigh 1.0 mg protein in an Eppendorf tube.
    - Add 1.0 mL extraction buffer 1.

- When working under reducing conditions, always work under nitrogen atmosphere and ensure that the samples are analysed within 24 h after preparation.
- Vortex the Eppendorf tubes extensively.
- Shake the Eppendorf tubes (60 min, 150 rpm) at room temperature.
- Centrifuge the Eppendorf tubes (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

- Analyse an aliquot (*ca.* 10 µL) of the sample with a SE-HPLC system equipped with a BioSep Sec-S2000 column (300 × 7.8 mm, Phenomenex, Torrance, USA) 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 MW protein markers as a function of the logarithm of their corresponding MW (Figure 5.5).
- Use the obtained equation to estimate the MW 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).
- If the aim is to compare protein fractions or estimate the protein extractability, determine the peak area corresponding to specific protein

fractions or the total area under each profile using the HPLC software or alternative data processing software (e.g. Matlab or OriginLab).

- By comparing the area of extracts of fermented samples with the maximum possible area (i.e. all proteins extractable, based on the area obtained for the starting material after extraction with SDS- and DTT-containing medium), an indication for the protein extractability is obtained.

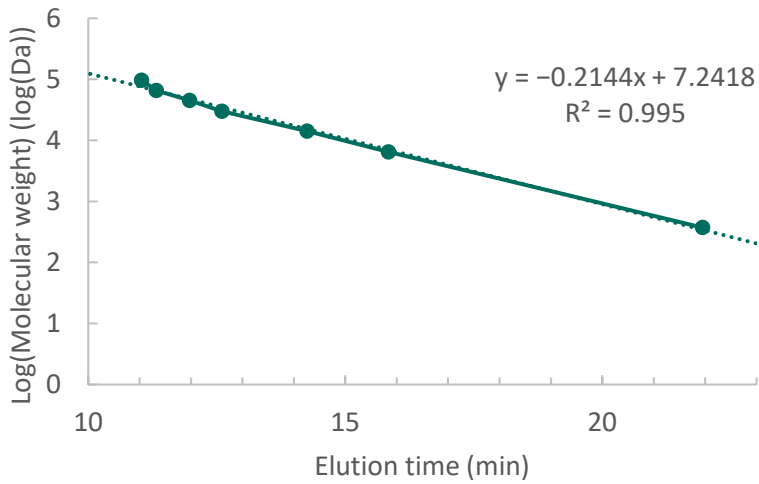

Figure 5.5. Correlation between the logarithm of the molecular mass and respective elution time of phosphorylase B (97 kDa); bovine serum albumin (66 kDa); chicken egg ovalbumin (45 kDa); carbonic anhydrase from bovine erythrocytes (30 kDa); alpha-lactalbumin (14.2 kDa); aprotinin (6.5 kDa); and $(Ala)_5$ (373 Da) protein standards.

3. Example data

3.1 Sample preparation

To illustrate the application of the above-described methodologies, non-kilned oat, fermented non-kilned oat and enzymatically hydrolysed (using alcalase) non-kilned oat were analysed. For the fermented sample, non-kilned wholemeal oat suspensions at a 1:6 w:v ratio were inoculated at 10^7 CFU/g of a *Lactiplantibacillus plantarum* strain and fermented for 24 h at 30 °C. To produce the enzymatically hydrolysed sample, the

exogenous enzyme alcalase (at 2.4 U/mg protein) was added to a non-kilned wholemeal oat suspension at the same 1:6 w:v ratio and incubated in the presence of antibiotics (to prevent spontaneous fermentation) for 24 h at 30 °C. The suspensions were then analysed for their DH (using the OPA assay) and protein MW distribution (via SE-HPLC).

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

The free amino group contents of the fermented and enzymatically treated oat wholemeal suspensions were compared with those of the raw material (i.e., non-kilned oat wholemeal). A calibration curve using L-Leucine was generated as described in sections 2.3.3 and 2.3.4, resulting in the following equation:

$$Abs = 0.6509 * C + 0.1793$$

where Abs represents the absorbance at 340 nm and C represents the amino acid concentration (mmol/L), equivalent to the free amino group concentration. The formula in Figure 5.4 was then used to calculate the number of peptide bonds cleaved (n) and the DH. Relevant details are provided in Table 5.2.

Table 5.2. Calculation of the degree of protein hydrolysis (DH) for fermented and enzymatically treated (alcalase) non-kilned oat. n = the calculated hydrolysis equivalents (mmol/g), Abs_p = the absorbance at 340 nm of processed material, Abs_{np} = the absorbance at 340 nm of non-processed material, C_p = the protein concentration of processed samples (mg/mL), C_{np} = the protein concentration of non-processed samples (mg/mL).

Sample	Abs_p (AU)	Abs_{np} (AU)	C_p (g/mL)	C_{np} (g/mL)	n (mmol/g)	DH (%)
Fermented	1.083	0.745	1.518	1.506	0.155	1.980
Exogenous enzyme	2.207	0.745	1.514	1.506	1.298	16.615

The results indicate that during fermentation, approximately 2% of the peptide bonds were hydrolysed due to the action of endogenous and microbial proteolytic enzymes. In contrast, when the exogenous enzyme alcalase was added, the DH exceeded 16%, meaning that roughly one in six peptide bonds was hydrolysed during the enzymatic treatment. It would be particularly valuable to identify which specific proteins were

hydrolysed during these processes, as e.g. the action of exopeptidases can affect the overall DH of the system without significantly altering the overall protein structure. Therefore, these findings were here combined with SE-HPLC data to better understand the changes in MW distribution.

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

The same samples were also analysed with SE-HPLC, as explained in sections 2.4.2–2.4.5. An extraction buffer under reducing conditions (SDS and DTT) was used to extract the oat proteins. SE-HPLC chromatograms are shown in Figure 5.6.

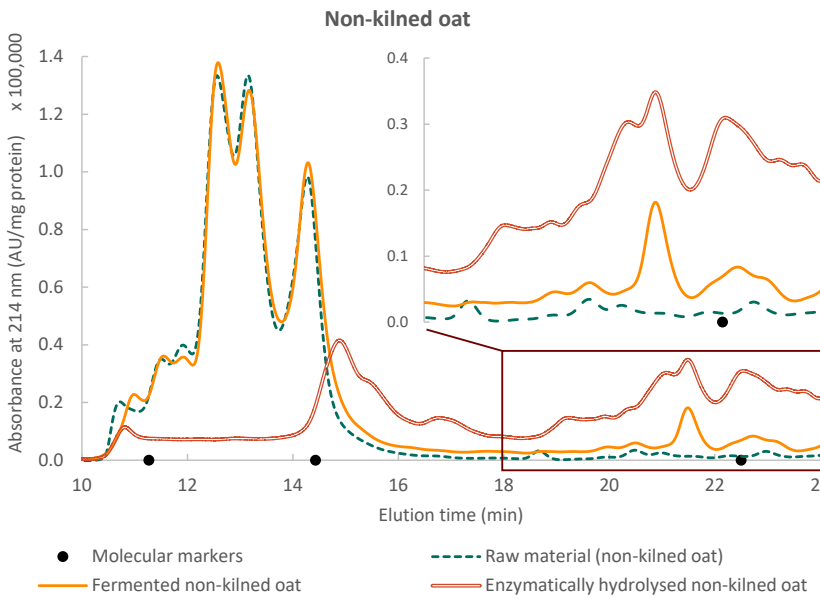

Figure 5.6. Size-exclusion high-performance chromatogram on constant protein basis of 1.0 mg protein/mL of the proteins of defatted non-kilned oat wholemeal (100% extractable proteins); fermented wholemeal suspension (*L. plantarum*, 24 h at 30 °C); and enzymatically (alcalase) incubated wholemeal suspensions at pH 6.4 (24 h at 30 °C). Molecular markers are, respectively, bovine serum albumin (66.5 kDa, 11.3 min); α-lactalbumin (14.2 kDa, 14.4 min); and alanine-5 (373.4 Da, 22.5 min).

The results indicate that during the fermentation of non-kilned oats, the major protein fraction, which elutes between 10.5 and 15.0 min (corresponding to MWs between 100 kDa and 10.5 kDa, based on the calibration curve in Figure 5.5), remained largely unaffected, as can be seen in Figure 5.6. This fraction is hypothesised to consist primarily of oat (12S) globulins and albumins, as discussed in the introduction. Fermentation led to the formation of only few low-MW peptides, which eluted after 18 min, corresponding to MWs below 2.4 kDa. Overall, the action of microbial and oat endogenous enzymes did not result in significant changes in protein MW, as the oat globulins and albumins remained largely intact.

In contrast, when the exogenous proteolytic enzyme alcalase was added, the oat suspension proteins were almost completely degraded to a fraction with a mean MW of approximately 10.5 kDa. Notably, a substantial number of low-MW peptides were formed, most of which eluted within the MW range of 1.5 kDa to 125 Da. It is important to note that protein detection (at 214 nm) was here based on peptide bonds absorbing UV light. The more extensive the hydrolysis, the fewer intact peptide bonds remain, which can lead to an underestimation during protein quantification. This factor should be taken into consideration, especially when analysing samples with high DH values.

4. Other aspects

A similar approach to the one outlined above can also be applied for other raw materials (e.g. legumes), although this would require some optimisation. To determine the DH with the OPA assay, the amount of analysed sample should be optimised 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 may need to be altered according to the type of sample. Therefore it can be useful to follow up the absorbance in function of the time and to choose an incubation time where the absorbance is rather stable and does not decrease again, making it feasible to measure multiple samples in one batch.

For SE-HPLC, the specifications of the separation column must be optimised according to the molecular mass distribution of the analysed proteins. If needed, multiple columns can be combined to obtain a better peak separation in certain MW ranges.

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SECTION 3

Carbohydrates

CHAPTER 6

Analysis of total, resistant and damaged starch content and its fine structure

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Summary

- Raw materials: Wheat, oat, yellow pea, faba bean
- Relevant applications: Bread, plant-based yoghurts, tempeh
- Fermentation-induced changes: Starch hydrolysis by microbial and endogenous amylases, modified starch molecular structure
- Analytical methods:
  - Starch isolation
  - Total starch content
    - Enzymatic degradation (α-amylase and amyloglucosidase) and colourimetric detection
  - Resistant starch content
    - Enzymatic degradation (pancreatic amylase) and colourimetric detection
  - Damaged starch content
    - Enzymatic degradation (fungal α-amylase with specificity for damaged starch and amyloglucosidase) and colourimetric detection
  - MW distribution of starch
    - Size-exclusion high-performance liquid chromatography (SE-HPLC)
  - Amylose/amylopectin ratio
    - Size-exclusion high-performance liquid chromatography (SE-HPLC)
    - Concanavalin A precipitation procedure

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 & Zeeman, 2016). Cereals are classed as high-starch foods, with reported starch content ranges between 60 and 75%. Legumes are also classed as

high-starch foods, with reported starch content ranging between 46–47% for yellow pea and 40–44% for faba bean (Johansson et al., 2022; Thomas & Atwell, 1999). The consumption of starchy foods that induce high glycaemic responses (e.g. white bread) is linked to an increased risk of obesity, cardiovascular disease, metabolic syndrome and type 2 diabetes (Svihus & 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).

1.2 The chemical structure of starch

Starch is a glucose polymer consisting of the two components, amylose and amylopectin, with amylopectin typically being the more predominant or abundant constituent (Figure 6.1A). 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 MW ranging from about 243,000 to 972,000, but typically the MW is < 0.5 million (Thomas & 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 et al., 2009). The MW of amylopectin ranges between 10 to 500 million, making it one of the largest polymers found in nature (Thomas & Atwell, 1999). The starch granule consists of alternating layers of amorphous (amylose and branched segments of amylopectin) and semi-crystalline (double helices of amylopectin) lamella, as depicted in Figure 6.1B. Depending on the crystalline pattern, starch is classified into three different polymorphs: A, B and C type (Dome et al., 2020). The A types, which are more structurally compact, are usually found in cereals, whereas the B types, with a looser crystalline packing, 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 (Wang et al., 2014). The thermal transition temperatures are higher for A-type than for B-type starches; therefore the proportion of the two is a critical factor for legume starches (Wang et al., 2014). The degree of crystallinity and the polymorphic patter can be determined using X-ray diffraction (XRD) techniques (Sarko & Wu, 1978).

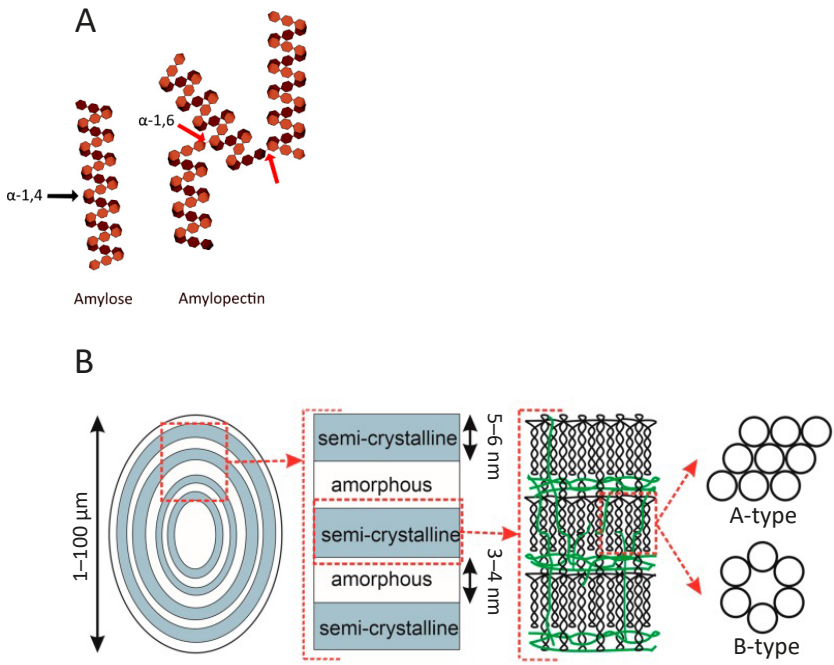

Figure 6.1. (A) General structure of amylose (left) and amylopectin (right), with α-1,4 linkage and α-1,6 linkages indicated by black and red arrows, respectively. (B) Semi-crystalline organisation of a starch granule (Dome et al., 2020).

Starch digestion begins in the mouth, with mastication and α-amylase action. 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 and that thus functions as a fibre. In the colon, the resistant starch is fermented by the gut microbes. Resistant starch has associated health benefits, such as a prebiotic effect, blood sugar control and colonic cancer prevention (Bede & Zaixiang, 2021). Resistant starch is classified into five categories:

- Physically inaccessible starch (RS1)
- Native starch (RS2)
- Retrograded starch (RS3)
- Chemically modified starch (RS4)
- V-complex starch (RS5)

Legumes tend to contain more resistant starch than cereals, with for example Faba bean having 13.1% resistant starch in the hulls and 6.7% in the cotyledon, compared with 0.5% in wheat (Nilsson et al., 2022). Such factors as variety, preparation or cooking method, storage temperature and duration, and serving temperature can affect the resistant starch content (Patterson et al., 2020).

1.3 How can fermentation affect starch?

Starch is essential in grain-based fermentations, as micro-organisms metabolise fermentable sugars, such as glucose and maltose, to produce organic acids, alcohol and CO_2. Grain-based products typically have a low fermentable sugar content, necessitating additional starch hydrolysis to sustain microbial activity (Katina & Poutanen, 2013).

Milling can cause mechanical damage to the starch granules (Donald, 2004), enhancing their hydrolysis susceptibility by endogenous amylases, which is vital for providing fermentable sugars during during processes such as breadmaking (Wang et al., 2020). Wheat and oat flours contain 5–15% and 1–10% damaged starch, respectively (Barak et al., 2014; Hüttner et al., 2010).

Fermentable sugars are provided via the hydrolysis of damaged starch by endogenous and microbial amylases (Leman et al., 2006)). Such micro-organisms as *L. amylovorus, L. plantarum* and *L. fermentum* (Padmavathi et al., 2018) have been reported to exhibit amylase activity, while *Aspergillus sp*. and *Rhizopus sp*. have the ability to convert native starch to glucose without prior gelatinisation (Sun et al., 2010). The extent of starch hydrolysis during fermentation of grain-based flours impacts the starch's molecular structure, such as the MW distribution, amylose/amylopectin ratio and relative crystallinity (Zhao et al., 2019). Such changes may affect starch gelatinisation and retrogradation behaviour during and after a heat treatment, which has implications for the application the starch is present in.

Not only starch hydrolysis, but also 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, consequently reducing starch bioavailability (Östman et al., 2002). Numerous studies have suggested that the interaction of starch and lactic acid causes reduced starch digestibility in sourdough bread (Poutanen et al., 2009). However, underlying mechanisms remain to be elucidated.

In the production of protein-rich plant-based yoghurts from Faba bean, the high thickening power of starch is undesirable because it interferes with the protein-based emulsion gel. Therefore, the starch needs to be removed or gelatinised and pretreated with α-amylase before the introduction of lactic bacteria (Jiang et al., 2020).

Traditional tempeh is produced using dehulled soybeans that are soaked, boiled and dried prior to the introduction of *Rhizopus* ssp. sporangiospores to induce fermentation (Ahnan-Winarno et al., 2021). These preprocessing steps of soaking and boiling the bean beforehand will partly gelatinise the starch granule and increase amylose susceptibility for the fungal α-amylase.

The changes in starch composition during the pretreatments and after fermentation that occur during tempeh production from raw materials other than soy, such as peas, oat and Faba bean, are not well characterised (Ashenafi & Busse, 1991; Cai et al., 2014; Eklund-Jonsson et al., 2006; Nassar et al., 2008; Polanowska et al., 2020). Van der Riet et al. (1987) reported a reduction in total starch of soybean after fermentation with *Rhizopus oligosporus;* the starch reduction benefited from prolonged incubation with the spores. A similar reduction in starch content 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 the quality of wheat bread made with regular baking yeast (Huang et al., 2019). The addition of tempeh flour improved dough handling properties and increased bread volume compared with the control doughs/bread made solely with wheat or the addition of unfermented soy protein. The addition of tempeh flour also decreased moisture migration and water loss of the breadcrumbs, which reduces starch recrystallisation during storage (retrogradation) and increases the softness of the baked product. According to Huang et al. (2019), the presence of fungal amylases in the tempeh flour results in an increase in bread volume. The fungal amylases catalysed the hydrolysis of starch to low-MW components, such as fermentable sugars, which are more readily consumed by the yeast, thus generating a larger amount of gas.

2. Methods of analysis

2.1 Sample preparation

Based on the type of sample and the analytical method, different sample preparations are required. Figure 6.2 provides a schematic overview of the type of sample preparations and experimental methods for determining the content, structure and relevant physicochemical properties of starch.

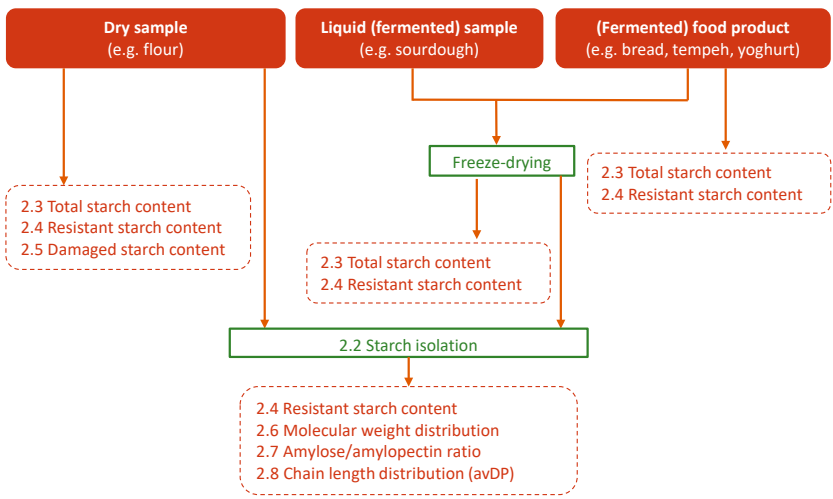

Figure 6.2. Schematic overview of the sample preparation and analytical methods required for analysing the content, structure and physicochemical properties of starch.

2.2 Starch isolation

2.2.1 Wheat-based flours

Starch isolation from wheat-based flours involves the formation of a dough and subsequent washing of the dough to separate the starch from the gluten. The procedure is adapted from Frederix et al. (2003).

- Weigh 250 g flour (on 14% moisture basis).
- Mix flour to a dough in a stand mixer bowl with deionised water (150 mL) for 4 min (position 2 on the mixer's speed dial, use dough hook).

- 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 1,000 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 ca. 70 Hz vibration speed.
- Wash for approximately 2 min with 1,000 mL of tap water. Repeat three times.
- Recover the gluten fraction from each sieve, put them on metal plates and freeze them in liquid nitrogen and lyophilise.
- Collect all wash water (containing starch) and centrifuge at 2,000 × *g* for 10 min.
- Wash the pellet with ethanol.
- Collect the pellet on filter paper and dry overnight.

2.2.2 Oats

Compared with starch extraction in other cereals, starch extraction in oats is rather complicated due to the strong association with proteins and β-glucans (Hoover & Vasanthan, 1992).

- 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 5,000 × g for 15 min.
- Remove the supernatant and suspend the pellet in in excess of 0.02% NaOH, let stand for 1 h and remove the supernatant. Repeat this procedure six times.
- Suspend the sediment in distilled water and vacuum-filtrate the solution with a Büchner 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.

2.2.3 Pulses

Starch isolation from certain pulses can be challenging because of the presence of highly hydrated fine fibre fractions and insoluble protein. The method described for pulse starch isolation is based on the papers of Li et al. (2020) and Nilsson et al. (2022). For pulses that have a low lipid content, such as faba bean and pea, samples do not need to be washed with ethanol for further lipid removal.

- Dissolve flour in 0.02% sodium hydroxide (NaOH) at a ratio of 1:10 for 24 h with constant stirring at room temperature, then an additional 24 h without stirring at 4 °C. Different sorts of legume flour yield different slurry viscosities; therefore the intensity of the stirring needs to be adjusted to suit each type of legume flour. The most important criterion is that the stirring be moderate and constant.
- Centrifuge the slurry at 3700 × *g* for 5 min.
- Remove the supernatant and replace it with distilled water and centrifuge once more at 3700 × *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 500 mL of distilled water in a kitchen blender until fully dispersed. In the study by Nilsson et al. (2022), the kitchen blender (Wilfa, XPLODE Vital, Hagan, Norway) was used on the “smoothie” setting, and the mixing was repeated once.
- Filtrate the homogenised solution with a Büchner funnel with a 70 µm nylon filter or cheesecloth.
- To maximise starch extraction, mix the filtercake up to 10 times with distilled water in the kitchen blender and filter as before.
- For optimal starch recovery, leave the filtrate standing without agitation overnight at 4 °C.
- Scrape away the brown top layer of the starch and let it dry for 48 h at 40 °C.

2.3 Analysis of the total starch content via enzymatic degradation and colourimetric detection

2.3.1 General principle

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 the glucose oxidase/peroxidase (GOPOD) assay.

2.3.2 Method

The analysis is conducted according to the Megazyme protocol K-TSTA (procedure A), based on AOAC Method 996.11 and AACC Method 76-13.01, with following modifications:

- Use small glass centrifuge 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. If raw material is scarce, using smaller amounts can save the amount of raw material needed; heating, mixing and diluting samples is also easier with smaller amounts.
- Place round-bottomed screwcap tubes in a heating block at 110 °C instead of using the boiling water bath. The use of a heating block means heating is more controlled and minimises the risk of splashing or water getting into the test tubes and contaminating the samples.
- 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, min in the 50 °C water bath. This step assures that the test tubes cool down as they should.

2.3.3 Tips

Preferably use glass test tubes rather than plastic test tubes for the analysis because glass conducts heat better than plastic and heating the samples to the right temperatures for the correct period is critical to performing the experiments.

2.4 Analysis of resistant starch content via enzymatic degradation and colourimetric detection

2.4.1 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.

2.4.2 Method

The complete method is described in the K-RSTAR procedure of Megazyme and is based on AOAC Method 2002.02 and AACC Method 32-40.01.

2.4.3 Tips

For efficiency for samples with unknown resistant starch content or an expected high content of resistant starch, such as legumes, it is advisable to use the resistant starch kit to measure both the resistant starch and total starch content.

2.5 Analysis of damaged starch content via enzymatic degradation and colourimetric detection

2.5.1 General principle

Measuring damaged starch is based on the use of a fungal α-amylase that 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.

2.5.2 Method

The complete procedure is described in the K-SDAM procedure of Megazyme, and is based on AACC Method 76-31.01 and ICC (International Association for Cereal Science and Technology) Method No. 164.

2.6 Analysis of starch molecular weight distribution via size-exclusion chromatography

2.6.1 General principle

Isolated starch granules are suspended in a buffer containing dimethylsulfoxide (DMSO) to completely solubilise the starch and lithium boride (LiBr) to minimise intermolecular hydrogen bonds of the starch molecules. Afterwards, amylose and amylopectin molecules are separated based on their molecular size using SE-HPLC. Note that the method described below involves the use of specific column types. These represent the conditions used by the authors. It may well be possible to perform a similar analysis with other columns, although this would require some optimisation, which is not included within the scope of this chapter.

2.6.2 Method

- Weigh 20.0 mg of isolated starch granules in a 10.0 mL round-bottomed glass screwcap 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 does not go below 30 °C, otherwise another heating step may be necessary before analysis.)
- Transfer 1 mL of the supernatant to an HPLC vial.
- Column specifications: Agilent GRAM 3000Å (8 × 300 mm, particle size 10 µm, separation range 5 to 5,000 kDa) and GRAM 30Å (8 × 300 mm, particle size 10 µm, separation range 0.1 to 10 kDa) (Agilent, Santa Clara, CA, USA).
- 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

2.6.3 Interpretation of results

Figure 6.3 shows a typical MW distribution of wheat and pea starch using SE-HPLC. The hydrodynamic volume of molecules influences the time required for the molecular species to flow through the column. Hydrodynamic volume is a parameter that is proportional to the product of the intrinsic viscosity [η] and the molar mass M of the product, and that correlates to the shape of the molecule and its spherical volume.

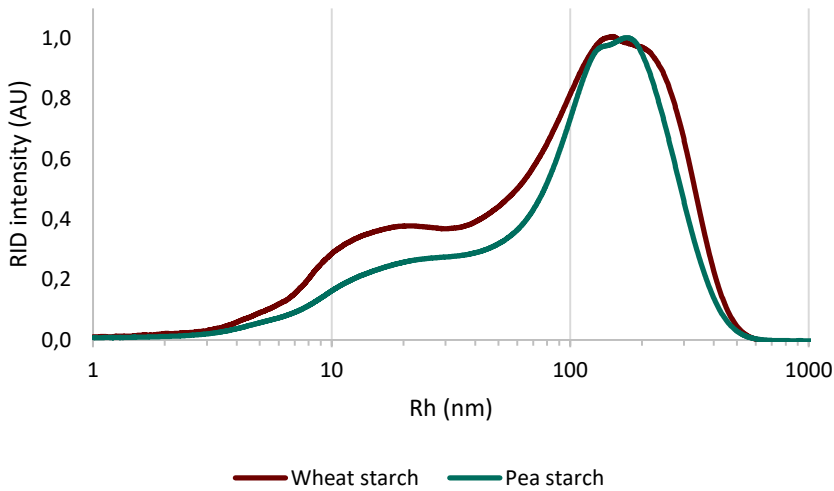

Figure 6.3. HPSEC distribution of wheat and pea starch. Rh = hydrodynamic volume; RID = refractive index detector; AU = arbitrary units.

2.7 Analysis of amylose/amylopectin ratio via size-exclusion chromatography

2.7.1 General principle

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

2.7.2 Method

- Weigh 30.0 mg of isolated starch granules in a 10.0 mL glass tube with red screwcap.
- Add 4.5 mL milli Q water 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 gelatinised 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, Bray, Ireland) and incubate the sample for 24 h at 40 °C.
- Add another 60 µL of isoamylase solution and incubate the sample for 24 h at 40 °C.
- Neutralise the suspension by the addition of 480 µL 0.1 M NaOH.
- Heat the samples for 2 h at 80 °C in a water bath.
- Centrifuge at 4,000 × *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 2.6.

2.7.3 Interpretation and analysis of results

An example of a typical HPLC profile obtained using the above-mentioned procedure is shown in Figure 6.4. Amylose/amylopectin ratio is obtained by dividing the total area of the amylose peak (100–10,000) by the amylopectin peak (1–100).

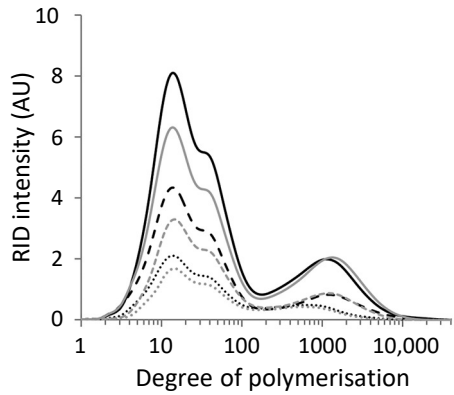

Figure 6.4. HPSEC profiles of different debranched starches showing amylopectin (degree of polymerisation [DP] 1–100) and amylose (DP 100–10,000) in terms of average degree of polymerisation. Adapted from Reyniers et al., 2019.

2.8 Analysis of amylose/amylopectin ratio via concanavalin A precipitation

2.8.1 General principle

The principle is described in the Megazyme (Bray, Ireland) kit Amylose/Amylopectin Assay Protocol (K-AMYL). It is based on a concanavalin A precipitation method and is recommended for cereal-based products. However, it has also successfully been used to analyse the amylopectin:amylose ratio in faba bean starch (Nilsson et al., 2022).

2.8.2 Method

The complete procedure is described in the K-AMYL procedure of Megazyme, and is a modification of a concanavalin A method by Yun and Matheson (1990) that includes an ethanol pretreatment step modified from Morrison and Laignet (1983).

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CHAPTER 7

Analysis of mono- and disaccharides, fructans, and raffinose-family oligosaccharides

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Summary

- Raw materials: Cereal- and legume-derived flours, protein isolates, starch isolates
- Relevant applications: Solid (breads and other baked products) foods, semi-solid (yoghurt-type, creamy filling–type, etc.) foods, liquid (milk-type) foods and their preparations
- Fermentation-induced changes: Utilisation of carbohydrates for metabolism of lactic acid bacteria and yeasts, reduction in FODMAP and raffinose family oligosaccharides content
- Analytical methods:
  - Content of mono- and disaccharides
    - High-performance liquid chromatography (HPLC)
  - Quantification of fructans
    - Enzymatic degradation and colourimetric detection
  - Quantification of raffinose family oligosaccharides
    - Enzymatic degradation and colourimetric detection

1. Introduction

1.1 Carbohydrates

Carbohydrates are the macronutrients found abundantly in cereals and legumes. They are chemically classified based on the molecular size determined by the DP, linkage type and individual monomers, as detailed in Table 7.1. However, Englyst and Englyst (2005) suggested an alternative 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 7.1. Classification of carbohydrates. Adapted from Cummings and Stephen, 2007.

Class	Degree of polymerisation	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	Fructo- and galacto-oligosaccharides (raffinose, stachyose, verbascose, etc.), 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, e.g. cellulose, resistant starch and fructans. These nondigestible 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 subject 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 DF, 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 and branching), it is also influenced by the type of monosaccharides. For instance, fructose absorption is slow in the presence of an excess of glucose in the small intestinal lumen, and polyols are only passively absorbed (Gibson & Halmos, 2020). The prolonged presence of carbohydrates in the small intestine 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 behaviour is observed for the disaccharides, such as lactose, in cases 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 diarrhoea. Such carbohydrates are collectively termed fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs). FODMAPs include slowly absorbed monosaccharides (e.g. fructose and polyols), 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 (Bellini et al., 2020; Simões et al., 2022). RFOs are galactosyl derivatives of sucrose, which include 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). A recent study by Ispiryan et al. (2020) on the FODMAP levels in commercial cereal products and their gluten-free alternatives revealed that fructans are predominant in wheat-derived products (1.19–1.88 g/100 g DM), wherein fermentation was able to degrade these FODMAPs with levels as low as 0.14 g/100 g DM. Moreover, oat-based products were found to contain very low levels of galacto-oligosaccharides (GOS) (up to 0.20 g/100 g DM), whereas seeds and flours derived from legumes contain up to 10% of GOS, with stachyose being the most prominent. Therefore, efforts are being made continuously to reduce the content of indigestible carbohydrates and accelerate the metabolism of other carbohydrates by virtue of enzymatic digestion using enzymes secreted by beneficial micro-organisms during fermentation.

1.2 Effect of fermentation on carbohydrates

Carbohydrates act as a substrate for the growth of micro-organisms (e.g. LAB and yeasts) during the fermentation process. These micro-organisms metabolise 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 glucose concentration due to the starch-hydrolysing effect of microbial maltase and α-amylase during early stages of fermentation, and the released glucose is further available for metabolism of starter micro-organisms (Osman, 2011). The microbial enzymes secreted during the fermentation process have been shown to degrade FODMAPs, including fructans and RFOs (Gänzle, 2014). Further, 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 & Gänzle, 2018). Legumes and the flours or isolates derived from them are abundant in GOS (or RFOs) that are reduced by the action of α-galactosidase secreted by LAB or other endogenous enzymes during fermentation (Curiel et al., 2015; Galli et al., 2019; Nkhata et al., 2018). Interestingly, *in vitro* colonic 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). Moreover, the metabolic availability of NDCs to the gut microbiota has been shown to be enhanced as a result of the 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 Brief overview of methods available for quantitative determination of carbohydrates

The quantity of simple/soluble carbohydrates, including glucose, fructose and sucrose, can be determined using refractometric or colourimetric methods. 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 using suitable columns and detection methods, including refractive index detection (RID),

evaporative light-scattering detection (ELSD) and pulsed amperometric detection (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 and fats, that require 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 HPAEC-PAD (Ispiryan et al., 2019). However, a lack of suitable standards leads to semiquantitative measurements requiring alternate methods (Muir et al., 2007). Therefore, enzymatic hydrolysis is the preferred method for quantification of FODMAPs.

Combinations 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, 2022). Commercial kits are now available with optimised protocols based on enzymatic digestion for the quantification of mono-, di-, and oligosaccharides, including specific FODMAPs (fructans) and RFOs (raffinose/α-galactose) (Megazyme, Bray, Ireland).

2. Methods of analysis

2.1 Quantification of mono- and disaccharides via 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 analysing carbohydrates, but with less sensitivity compared with 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. Note that the method described below involves the use of specific column types. These represent the conditions used by the authors. It may well be possible to perform a similar analysis with other columns, although this would require some optimisation, which is not included within the scope of this chapter.

2.1.2 Reagents

- Perchloric acid
- Acetonitrile
- Mono- and disaccharides

2.1.3 Sample preparation

- Transform raw and fermented samples into powder using a freeze-drier.
- Extract 2 g of samples with 20 mL of water/perchloric acid (95/5, v/v) and sonicate (amplitude 60) with a macro-probe for 1 min (2 cycles, 30 sec/cycle, 5 min interval between cycles) in an ice bath.

  Note: Perchloric acid is a useful extraction solvent for HPLC because it denatures enzymes, solubilises the analytes, removes interfering substances and is compatible with the HPLC system. The simplicity and effectiveness of extraction with 5% perchloric acid makes it a common choice for preparing samples for HPLC analysis of labile compounds.

- Put the samples under stirring conditions in an orbital shaker at a speed of 200 rpm at room temperature for 1 h.
- Keep the suspension at 4 °C overnight.
- Centrifuge for 10 min at 11,180 × *g*.
- Filter the extracts using 0.22 µm filters and store at –20 °C.

2.1.4 Sample analysis

- Run the extracts through HPLC analysis equipped with a Spherisorb column (Waters, Millford, MA, USA) and a Perkin Elmer 200a refractive index detector (Perkin Elmer, Shelton, CT, USA).
- Set the flow rate at 1 mL min^{-1}, using acetonitrile 80% as mobile phase.
- Set the column temperature at 32 ^{0}C.
- Generate a standard curve using solutions of known mono- and disaccharide concentrations (0.1–15 g/L).
- Calculate the mono- and disaccharide concentrations using the respective standard curves, taking into account any dilution of the sample.

2.1.5 Interpretation of results

Based on the criteria – such as molecular size, polarity and refractive index – the standards in the mixture are separated by the HPLC system at distinct time intervals, as indicated by peaks of varying sizes, which are typically proportional to their concentrations (Figure 7.1). Analysing samples alongside standards allows for the identification of peak overlaps, thereby confirming the presence of specific standards in the extract under investigation. The area under these peaks is then used for generating calibration curves to quantify the concentration of each compound in the extract.

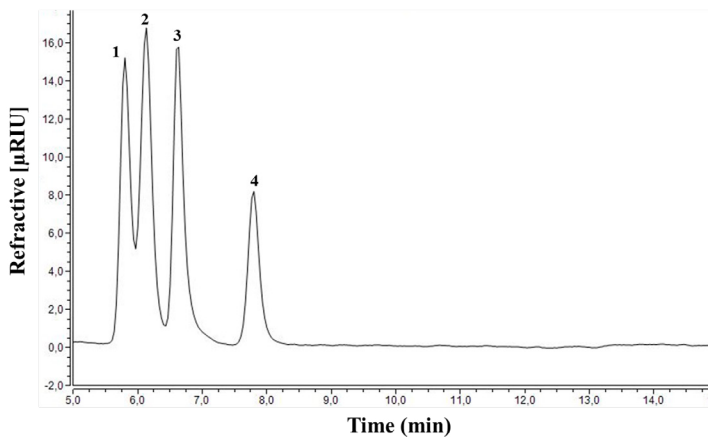

Figure 7.1. Separation of sugars in a standard mix (10 g/L) by HPLC equipped with a Perkin Elmer 200a refractive index (RI) detector. Peak assignments: 1 = fructose; 2 = glucose; 3 = mannitol; 4 = sucrose.

2.2 Quantification of fructans via enzymatic assay

2.2.1 General principle

Fructans vary in molecular structure and MW 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, in contrast to the 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 optimised using multiple enzymes releasing monosaccharides that can be measured colourimetrically (K-FRUC; Megazyme, Bray, Ireland). Sucrose is first hydrolysed by the action of sucrase (Figure 7.2A). Starch and maltodextrins are hydrolysed to maltose and maltotriose by pullulanase and β-amylase followed by their hydrolysis to D-glucose by maltase (Figure 7.2B). Furthermore, D-glucose and D-fructose are reduced by sodium borohydride to the corresponding sugar alcohols, i.e., D-sorbitol and D-mannitol, respectively (Figure 7.2C). It is important to note that in hydrolysed inulin preparations, during this reaction, the D-fructosyl residue at the reducing end of fructo-oligosaccharides (FOS) is also reduced to the sugar alcohol (Figure 7.2C). The reduced FOS are then hydrolysed by exo- and endo-inulinase and endo-levanase to D-glucose and D-fructose (Figure 7.2D) that can be measured using the p-hydroxybenzoic acid hydrazide (PAHBAH) reducing sugar method (Figure 7.2E).

(A) $Sucrose + H_2O \xrightarrow{sucrase\ (pH\ 6.5,\ 30\ °C)} D\text{–}glucose + D\text{–}fructose$

(B) $Starch + maltodextrins \xrightarrow[pH\ 6.5,\ 30\ °C]{pullalanase,\beta\text{–}amylase} maltose + maltotriose \xrightarrow{maltase} D\text{–}glucose$

(C) $D\text{–}glucose + D\text{–}fructose \xrightarrow[40\ °C]{sodium\ borohydride} D\text{–}sorbitol + D\text{–}mannitol + reduced\ FOS$

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

(E) $D\text{–}glucose + D\text{–}fructose \xrightarrow[100\ °C,\ 6\ min]{PAHBAH\ reagent} PAHBAH\ colour\ complex$

Figure 7.2. Reaction mechanisms of the enzymatic assay to quantify fructans. (A) Sucrose hydrolysed by the action of sucrase. (B) Starch and maltodextrins hydrolysed to maltose and maltotriose by pullulanase and β-amylase followed by their hydrolysis to D-glucose by maltase. (C) D-glucose and D-fructose reduced by sodium borohydride to the corresponding sugar alcohols, i.e., D-sorbitol and D-mannitol, respectively. (D) The reduced FOS hydrolysed by exo- and endo-inulinase and endo-levanase to D-glucose and D-fructose that can be measured using (E) the p-hydroxybenzoic acid hydrazide (PAHBAH) reducing sugar method.

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 hydrolysing with α-galactosidase to avoid the overestimation of fructans, and RFOs are therefore measured separately. The quantification of fructans in flours derived from cereals or legumes and in the baked goods (e.g. sourdough bread) prepared with such flours has been standardised using the Megazyme (Bray, Ireland) kit based on this enzymatic hydrolysis method. The procedure was followed as per the kit instructions, and any modifications to adapt to the sample type has been mentioned in the next sections.

2.2.2 Reagents

- Sodium maleate buffer (100 mM, pH 6.5): This buffer was not provided with the kit; however, it was prepared as per kit instructions and is stable for up to 3 months at 4 °C.
- Sodium acetate buffer (100 mM, pH 4.5): This buffer was not provided with the kit; however, it was prepared as per kit instructions and is stable for up to 3 months at 4 °C.
- Enzyme solution A: Prepare a solution containing 50 mg of bovine serum albumin (provided in the kit) in 100 mL of sodium maleate buffer (see the recipe above). 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 (see the recipe above) to the vial containing freeze-dried fructanase powder (provided in the kit), 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 a tablet of sodium borohydride (CAS 16940-66-2, Merck KGaA, Darmstadt, Germany), weighing 1 g, in 10 mL of 50 mM NaOH by vortexing. Dilute the solution further (1:10) with 50 mM NaOH to obtain the final concentration of 10 mg/mL. This solution is stable for only 4–5 h at room temperature and hence should be prepared fresh on the day of the experiment.
- PAHBAH reducing sugar assay reagent: Prepare solutions A and B as per kit instructions.
  Solution A: This solution is stable for approx. 2 years at room temperature.
  Solution B: 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.
  Note: Calculate the quantity of PAHBAH working reagent based on 5 mL/tube before the preparation.

2.2.3 Sample preparation

Baked products are subjected to freeze-drying followed by grinding to obtain a powdered form. Weigh 400 mg (for samples containing 0–10% fructans) or 100 mg (for samples containing 10–40% 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. 20,000 × *g*) 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 controls should be included and analysed concurrently. The controls include powders of inulin, levan and sucrose, as well as a standard solution of D-fructose (1.5 mg/mL). Although the powders are processed for estimation as per the sample preparation, D-fructose control is only prepared when the samples reach the step of PAHBAH working reagent addition. The preparation of reagent blanks and D-fructose controls is mentioned as follows:
  - 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).
- 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.

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 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 effervescence 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, which 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 colour complex fades with time.

2.2.7 Calculations

The equation in Figure 7.3 is used for calculating the percentage of fructans.

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

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

Figure 7.3. The equation to calculate the amount of fructans.

2.3 Quantification of raffinose-family oligosaccharides via enzymatic assay

2.3.1 General principle

The quantification of RFOs is based on the principle of stepwise enzymatic action of multiple enzymes (Figure 7.4) that converts α-galactosides, including raffinose, stachyose, verbascose and galactinol, into D-galactose with the release of nicotinamide adenine dinucleotide (NADH). The released NADH is measured by the increase in absorbance at 340 nm using a commercial kit (K-RAFGA; Megazyme, Wicklow, 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 analysed.

(A) $$raffinose + stachyose + verbascose + H_2O \xrightarrow{\propto-galactosidase} D-galactose + sucrose$$

(B) $$\propto -D-galactose \xrightarrow{galactose\ mutarotase} \beta-D-galactose$$

(C) $$\beta-D-galactose + NAD^+ \xrightarrow{\beta-galactose\ dehydrogenase} D-galactonic\ acid + NADH + H^+$$

Figure 7.4. Reaction mechanisms of the enzymatic assay to quantify raffinose-family oligosaccharides.

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

2.3.2 Reagents

- Solution I: a buffer containing sodium azide (0.02% w/v) at pH 8.6.
- NAD^+ solution: Dissolve NAD^+ powder provided with the kit in 12.2 mL of distilled water and store at –20 °C until further use.
- Combined suspension of D-galactose dehydrogenase and galactose mutarotase stored at 4 °C.
- α-Galactosidase solution: Dissolve the lyophilised 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.
- Raffinose control solution (positive control): Weigh 0.5 g of raffinose control powder 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 (a freeze-dried ground sample in the 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 sec followed by centrifugation

at 1,500 × *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, four different wells are considered: 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 Table 7.2.

Table 7.2. Reagent volumes to be added for the enzymatic treatment with α-galactosidase to release free D-galactose in the negative control (blank) and sample extracts (samples). RFO = raffinose-family oligosaccharides.

	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) (Figure 7.5A).
- The values of ΔA should be a minimum of 0.1 units to achieve efficient results.
- The concentrations of individual RFOs and free D-galactose can be calculated using the formula in Figure 7.5B.
- 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 shown in Figure 7.5C.
- The concentration of raffinose control powder should be in the range of 0.2 to 1.25 g/L.

A) $\Delta A = (A_2 - A_1)_{sample} - (A2 - A1)_{blank}$

B) $C = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \text{ (g/L)}$

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 × mol–1 × cm–1)
d = light path (cm)
v = sample volume (mL)

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

Figure 7.5. The equations needed to calculate the amount of raffinose-family oligosaccharides or free galactose.

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 L 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 8

Analysis of the content, extractability and fine structure of arabinoxylan

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Summary

- Raw materials: Wheat, oat (whole meal, bran)
- Relevant applications: Dough, bread, meat and dairy alternatives, digesta
- Fermentation-induced changes: Solubilisation, debranching, release of antioxidants (e.g. ferulic acid)
- Analytical methods:
  - Total arabinoxylan content and extractability
    - Gas chromatography (GC)
  - Degree of arabinose substitution
    - Gas chromatography (GC)
  - Average degree of polymerisation of arabinoxylan
    - Gas chromatography (GC)
  - Arabinoxylan molecular mass distribution
    - Size-exclusion high-performance liquid chromatography (SE-HPLC)
  - Substitution pattern of arabinoxylan
    - Liquid-state 1H nuclear magnetic resonance (NMR) spectroscopy

1. Introduction

1.1 What is arabinoxylan?

Arabinoxylan (AX) is one of the major non-starch polysaccharides of cereals and represents an important source of 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 (Figure 8.1). 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 the formation of diferulic acid bridges (Wang et al., 2020). A distinction is generally made between AX molecules that are water-extractable (WE-AX) and those that are water-unextractable (WU-AX). WU-AX are not extractable in water because they have very high MWs 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 great structural heterogeneity, with molecules varying considerably in MW, degree of substitution (as assessed by the ratio of arabinose to xylose), and substitution pattern (Dervilly et al., 2000).

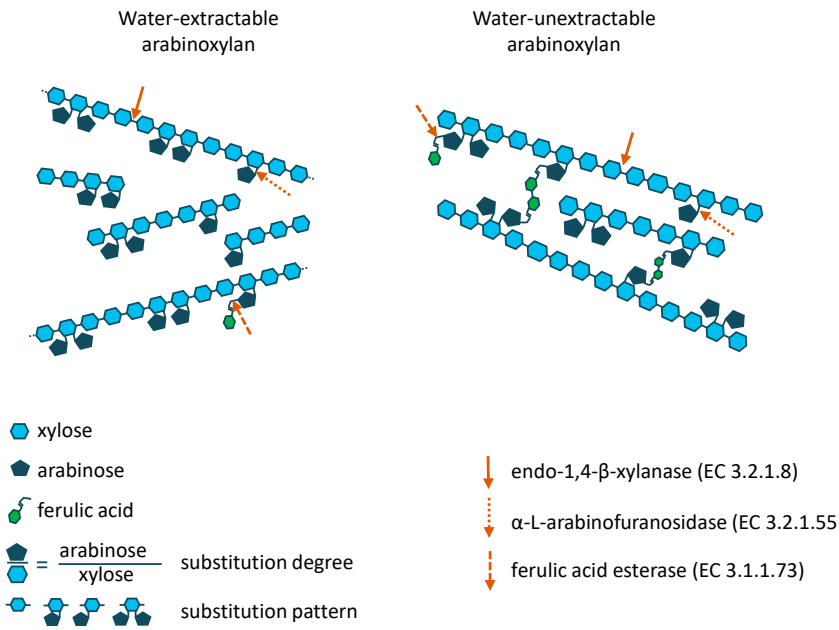

Figure 8.1. The general structure of cereal arabinoxylan and the cleavage sites for the main degrading enzymes.

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 endogenous grain enzyme activity, enzyme activity from micro-organisms populating the outer grain kernel layers or enzyme activity from starter cultures added for fermentation (Dornez et al., 2009). Furthermore, micro-organisms can use arabinose and xylose released by enzymes as a carbon source for their metabolism, which would result in a decrease in the AX content (Gobbetti et al., 2000).

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 depolymerisation of the xylose backbone, as it cleaves the internal β1-4-D-xylosidic bonds. The bonds selected for hydrolysis depend on the chain length, degree of branching and presence of substituents. Many micro-organisms produce xylanases, and individual micro-organisms can express a multiplicity of xylanases with different characteristics. Another backbone hydrolysing enzyme is β-xylosidase (1,4-β-D-xyloside xylohydrolase; EC 3.2.1.37), which does not act on AX but hydrolyses 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 (Bajpai, 2014; Ergün & Çalık, 2016; 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 solubilised. 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. It was also shown that during sourdough fermentation, AX is solubilised and that, depending on the fermentation type, the MW of AX can decrease or remain unaffected (Maina et al., 2021).

1.3 Implications of arabinoxylan structure for 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 MW 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, as well as in liquid food systems, where viscosity is a key quality parameter. Furthermore, the properties of hydrogels are related to molecular mass and ferulic acid content (He et al., 2021).

The bioactive properties of AX also largely depend on its structure. The extent and rate of colonic fermentation is higher for soluble fibres than for insoluble fibres (Verspreet et al., 2016). Particularly AX with a low MW and high solubility, such as AX oligosaccharides (AXOS), are linked to prebiotic properties (Courtin et al., 2008). 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 (Chen et al., 2019; He et al., 2021).

2. Methods of analysis

2.1 Sample preparation

Figure 8.2 provides a schematic overview of the sample preparation and experimental methods needed for analysing the content and fine structure of AX.

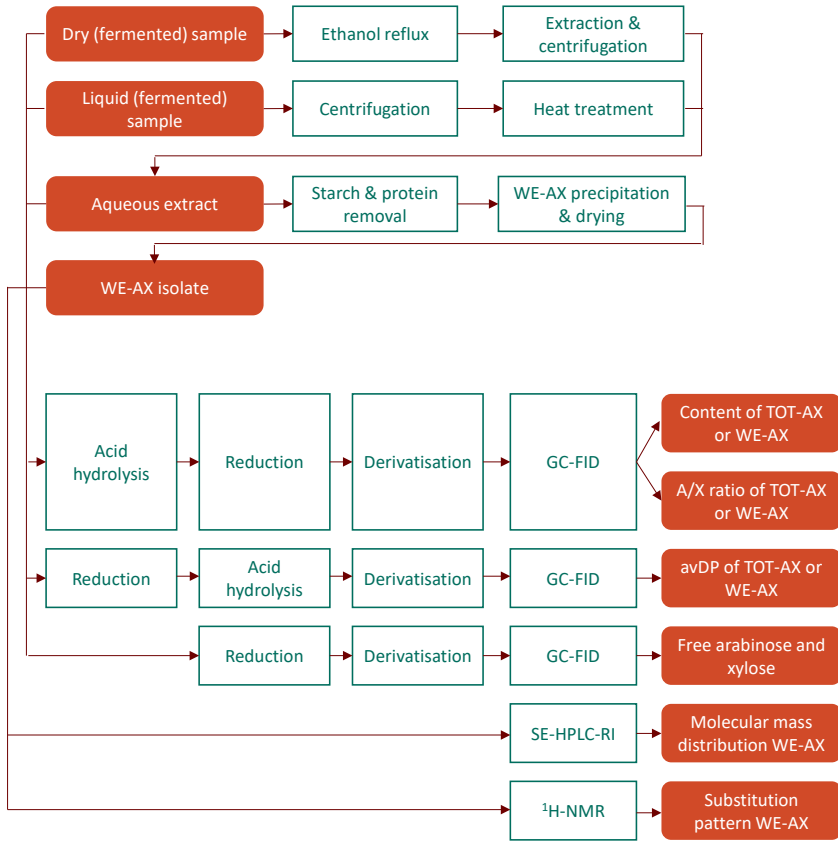

Figure 8.2. Schematic overview of the sample preparation and analytical methods required for analysing the content and fine structure of cereal arabinoxylan (AX). WE = water-extractable; TOT = total; GC-FID = gas chromatography with flame ionisation detector; SE-HPLC-RI = size-exclusion high-performance liquid chromatography with refractive index detector; avDP = average degree of polymerisation; A/X ratio = degree of arabinose substitution; ^{1}H-NMR = proton nuclear magnetic resonance.

2.1.1 Inactivation of endogenous enzymes and aqueous extraction

The determination of the content, degrees of substitution and DP can be done on dry or liquid (fermented) samples as is and on their extracts to analyse WE-AX. It may be important to inactivate cereal endogenous and micro-organism-associated xylanases to avoid enzymatic activity during the analytical procedures. For instance, samples fermented for short time periods (e.g. 2 h) should be deactivated, as they may still contain a lot of residual enzyme activity. This heat treatment could be omitted for samples that are fermented for longer times (e.g. 24 h). However, the need for this inactivation procedure should be evaluated case by case.

Enzyme inactivation:

- Dry samples: reflux in 80% v/v ethanol (1:10 sample to ethanol ratio, 1 h)
- Liquid samples: heat treatment for 5–10 min at 100 °C is advised

The amount of arabinoxylan that is extracted in water depends on the extraction procedure (ratio of solids to water, time of extraction, centrifugation settings and extraction repetitions). Also the enzyme inactivation can affect extractability. Therefore, if only low enzyme activities are expected, the enzyme inactivation step can be performed after the aqueous extraction. To avoid enzyme activity during the extraction procedure, it can be performed at low temperatures.

Aqueous extraction:

- The procedure may vary depending on the type of sample, but it should remain consistent across different samples if you intend to make comparisons.
- Suggested method for wheat flour:
  - Weigh accurately 2 g flour in a centrifuge tube
  - Add 20 mL water
  - Centrifuge (10 min, 1,000–1,500 *g*, 7 °C)
  - Filter the samples over paper

2.1.2 Isolation of water-extractable arabinoxylan

For SE-HPLC and proton nuclear magnetic resonance (^{1}H-NMR) analyses of WE-AX, high-purity AX is required. To achieve this high purity, the WE-AX in the aqueous extract of wheat wholemeal or bran can be isolated following the procedure described in De Man et al. (2021). In this procedure, starch and proteins are eliminated by amylase and heat treatment, respectively. Afterwards, WE-AX is precipitated in 65% v/v ethanol.

- Before proceeding with the isolation procedure, make sure the extract is diluted to 1:10 flour to water ratio.
- Treat the aqueous extract with α-amylase (*Bacillus licheniformis*, type XII-A, saline solution, ≥500 units/mg protein): Add the required amount of enzyme solution to obtain 3.8 U/mL in the extract.
- Incubate for 30 min at 90 °C under continuous shaking.
- Let cool to room temperature and centrifuge (3,000 *g*, 15 min).
- Gradually add a total of 35 volumes of ethanol to 65 volumes of supernatant over 30 min under continuous stirring.
- Continue stirring for an additional 30 min.
- Store the mixture overnight at 4 °C.
- Centrifuge this mixture at 10,000 *g* for 30 min at 4 °C.
- Dry the obtained pellet under a fume hood overnight.
- Redissolve the dried pellet in 100 mL water, flash freeze with liquid nitrogen and freeze-dry.

For isolation of WE-AX from grains other than wheat, it is possible that the procedure will have to be optimised to remove other water-extractable components, such as β-glucan.

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

2.2.1 General principle

The gas-chromatography method for quantifying the total monosaccharides is based on the protocol described by Englyst and Cummings (1984). First, AX is degraded into monosaccharides through acid hydrolysis. These monosaccharides 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 derivatised to alditol peracetates with acetic anhydride using a catalyst (1-methylimidazole) (Englyst & Cummings, 1984). The volatile alditol peracetates are transferred to a test tube, dried to remove residual water and subsequently analysed with gas chromatography with flame ionisation detector (GC-FID). 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.

To determine the avDP, the reducing end xylose content is 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 avDP of WE-AX and TOT-AX.

To quantify free sugars, the acid hydrolysis step is omitted. Note that the HPAEC-IPAD method, which works without reduction and derivatisation, can be used as an alternative method (Alyassin et al., 2020). However, quantification of reducing ends is not possible, thus no data on the avDP can be obtained.

Note that the method described below involves the use of specific column and GC types. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems and/or columns, although this would require some optimisation, which is not included within the scope of this chapter.

2.2.2 Reagents

- 50% (v/v) saturated benzoic acid (purity >99.5%) solution
- IS 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 distilled H_2O (d H_2O)
- NH_4OH solution of 25%
- 2.0 M NH_3 (purity >25%) in dH_2O
- $NaBH_4$ (purity >96%)
- 7.5 M KOH (purity >86%) in dH_2O
- 2.0 M and 4.0 M trifluoroacetic acid (TFA) (purity >99%) in dH_2O
- 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 Procedure

Below, the procedures to determine the concentration of total and water-extractable monosaccharides and to quantify reducing and free sugars are described. As illustrated in Figure 8.2, the procedures primarily differ in the sequence of the reaction steps.

Total and water-extractable monosaccharides content	Reducing and free sugar content
(1) Acid hydrolysis *Aqueous extracts:* – Transfer 2.5 mL aqueous extract (see section 2.1.1) or calibration solution to a 12 mL glass centrifuge tube with screwcap (in triplicate). – Add 2.5 mL TFA 4.0 M to the supernatant and calibration solution. – Add 5.0 mL TFA 2.0 M to 12 mL glass centrifuge tube with 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. Dry samples: – Transfer 10 to 20 mg of accurately weighed sample to a 12 mL glass centrifuge tube with screwcap (in triplicate). In the case of WE-AX isolates, use around 15 mg. – Add 5.0 mL TFA 2.0 M to the samples. – Add 2.5 mL TFA 4.0 M to 2.5 mL of the calibration solution. – For the blanks, add 5.0 mL TFA 2.0 M. – 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.	(1) Reduction *Aqueous extracts:* – Add 2.5 mL aqueous extract (in triplicate) or calibration solution into a 12 mL glass tube with screwcap. – Add 500 µl IS solution and vortex. *Dry samples:* – Weigh 40 to 50 mg sample into a 12 mL glass tube with screwcap (in triplicate). – Add 2.5 mL dH_2O and 500 µl IS and vortex. *Blanks:* Use 3.0 mL of deionised water. – Place the tubes in ice water, add 50 µl 25% NH_3 and vortex. *(Verify if the pH is alkaline with a pH indicator strip. If it is not, add more NH_3.)* – Add 8 droplets of 2-octanol to avoid foaming in the subsequent steps. – Add 200 µl sodium borohydride solution (= 200 mg sodium borohydride in 1 mL 2.0 M NH_3) and vortex. *(Add 2.0 M NH_3 just before using the solution. Sodium borohydride is very toxic and explosive.)* – Close the tubes but do not tighten the screwcaps 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.

<table>
<tr><th>Total and water-extractable monosaccharides content</th><th>Reducing and free sugar content</th></tr>
<tr><td>(2) Reduction
– Transfer 3.00 mL of cooled hydrolysate or calibration solution or blank samples into a 12 mL glass tube with screwcap, add 1.0 mL IS solution (not in blank samples!) and vortex.
– Place the tubes in ice water, add 1.0 mL 25% NH_3 and vortex. (Verify if the pH is alkaline with a pH indicator strip. If it is not, 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 of 2-octanol to avoid foaming in the subsequent steps.
– Add 200 µl sodium borohydride solution (= 200 mg sodium borohydride in 1 mL 2M NH_3) and vortex. (Add 2.0 M NH_3 just before using the solution. Sodium borohydride is very toxic and explosive.)
– Close the tubes but do not tighten the screwcaps 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. (When performing derivatisation on the same day, reduction samples can be left at room temperature. When performing it on a different day, it is better to freeze the reduction samples.)</td><td>(2) Acid hydrolysis (skip this step for free sugars!)
– Transfer 2.5 mL of the reduction solution to a 12 mL glass tube with screwcap.
– 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.</td></tr>
<tr><td colspan="2">(3) Derivatisation
– Transfer 500 µl into a 50 mL glass tube with screwcap.
– Add 500 µl 1-methylimidazole and 5.00 mL acetic anhydride and then vortex. Wait at least 10 min.
– Add 1,000 µl absolute ethanol and then vortex. Wait at least 5 min.
– Add 10 mL deionised water and then 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 another 5.0 mL 7.5 M KOH.
– Mix the tubes by turning them upside down. Wait for at least 30 min.
– Add a small amount of anhydride sodium sulphate 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.</td></tr>
<tr><td colspan="2">(4) GC
– 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; Supelco, Bellefonte, USA) in an Agilent 6890 Series Chromatograph equipped with autosampler and splitter injection port (injection volume: 1.0 µL; split ratio: 1:20; Agilent, Santa Clara, USA). Detection is with a flame ionisation detector. The carrier gas is helium with a flow of 1.1 mL/min. Separation is at 225 °C, while injection and detection are at 270 °C.</td></tr>
</table>

2.2.4 Calculations

- For each type of monosaccharide (MS), a correction factor (CF) is used to account for losses during acid hydrolysis under the conditions used. A CF is determined as the ratio of the normalised peak area of a MS of a non-hydrolysed calibration solution to the normalised peak area of a MS of an equal amount of hydrolysed calibration solution:
  CF = (peak area $MS_{non\text{-}hydrolysed}$/peak area IS)/(peak area $MS_{hydrolysed}$/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 IS to normalise all GC chromatogram peak areas. A RF is calculated as follows:
  RF = [(mg MS/mg IS)/(peak area MS/peak area IS)] × CF
- The MS concentrations in a sample are calculated from their GC peak areas and corresponding RF as follows:
  [MS] (%) = (peak area MS/peak area IS) × RF × (mg IS/mg dry sample) × 100
- The levels of WE-AX, TOT-AX and WU-AX and their A/X ratios and number average degrees of polymerisation are calculated as follows:

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

- with $B = 0.7 \times [Gal]_{WE}$, a CF 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, emmer wheat, einkorn wheat and spelt. No correction is used for flour from oat, bran and wholemeal samples because in these

samples the galactose present cannot be solely attributed to arabinogalactan peptide.
- For calculation of avDP, samples should be corrected for free arabinose and xylose content by subtracting these from $[ara]_{TOT}$, $[xyl]_{TOT}$ or $[ara]_{WE}$ and $[xyl]_{WE}$ where relevant (e.g. in the case of fermented samples).

2.2.5 Interpretation of data

After the run, a profile such as that shown in Figure 8.3 should be obtained. The order of elution – from left to right – is arabinose – xylose – allose – mannose – galactose – glucose.

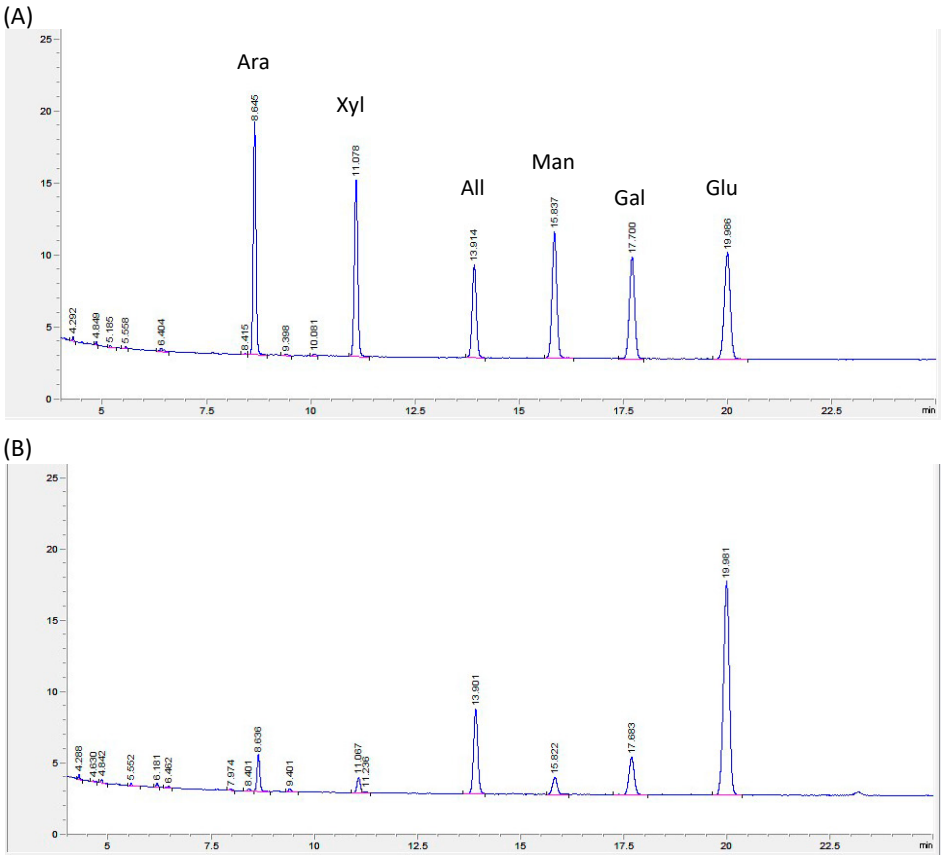

Figure 8.3. Gas chromatogram of (A) a calibration solution and (B) aqueous extract of oat wholemeal.

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

2.3.1 General principle

The apparent molecular mass distribution of WE-AX is determined with SE-HPLC. 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 longer retention times, while large particles will not enter the beads and therefore will reach the column end faster.

Liquid-state ^{1}H-NMR spectroscopy allows characterising branching patterns in AX by assessing H-1 peaks of xylose and arabinose residues (Hoffmann et al., 1992). Spectroscopic measurements are based on the interactions between atomic nuclei and an external magnetic field. These allow detailed information on the molecular structure of target constituents to be obtained (De Man, 2022). Hoffmann et al. (1992) provided a detailed identification of 1H resonances in wheat endosperm WE-AX by combining ^{1}H-NMR spectroscopy with methylation analysis and monosaccharide identification (Figure 8.4).

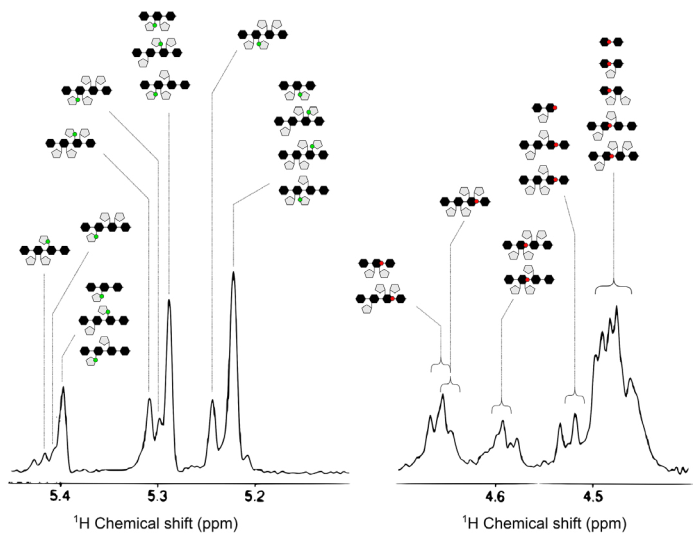

Figure 8.4. Detailed regions of a ^{1}H-NMR spectrum of wheat endosperm water-extractable arabinoxylan, illustrating the H-1 spectral resonances of arabinofuranosyl (δ 5.2–5.4 ppm; green dots) and xylopyranosyl (δ 4.4–4.7 ppm; red dots) residues. Reproduced from De Man (2022) with permission.

Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.3.2 SE-HPLC

This method is based on the method described by De Man (2022).

- Dissolve 5.0 mg of AX isolate in 1.0 mL 0.3% NaCl.
- Inject 50.0 µl of the sample (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 × 6 mm). Elution (0.5 mL/min) with 0.3% NaCl is 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, 1.32×10^3, 6.2×10^3, 10×10^3, 23×10^3, 48.8×10^3, 133×10^3, 200×10^3, 348×10^3, 805×10^3, and 1600×10^3 g/mole; 1.0 mg/ml in 0.3% w/v NaCl) are injected for calibration purposes.

An example of a typical profile is shown in Figure 8.5. Note that absolute quantification of MW is not possible since pullulan was used for calibration. Pullulan has a different relationship between hydrodynamic size and MW compared with AX. Therefore, this method can only be used for comparative analysis of different samples; it cannot provide absolute MW values.

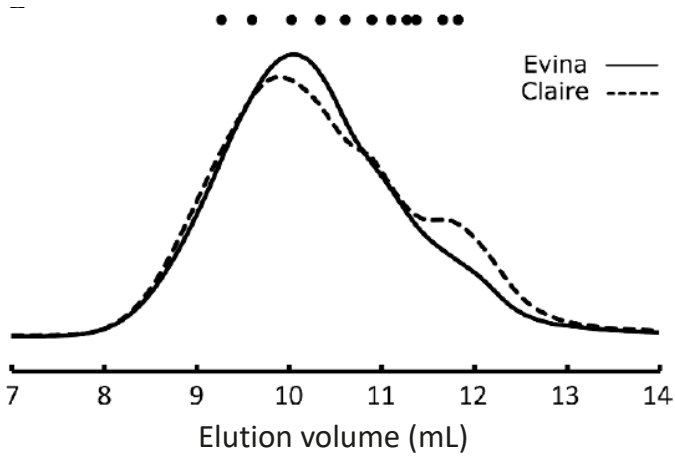

Figure 8.5. Size-exclusion high performance liquid chromatography profile of isolated water-extractable arabinoxylan from two wheat flours (Evina and Claire). Elution volumes of pullulan standards of different molecular weights (342, 1.32 × 10^3, 6.20 × 10^3, 10 × 10^3, 23 × 10^3, 48.8 × 10^3, 133 × 10^3, 200 × 10^3, 348 × 10^3, 805 × 10^3, and 1600 × 10^3 g/mol) are indicated as black dots. Reproduced from De Man (2022) with permission.

2.3.3 Liquid-state ^{1}H nuclear NMR spectroscopy

This method is based on the method described by De Man et al. (2021).

- Exchange 10.0 mg of isolated AX 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, should be 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 (Bruker, Ettlingen, Germany). ^{1}H NMR spectra should be referenced at 0 ppm, using sodium trimethylsilylpropanesulfonate (DSS) (0.0173 ppm) as a secondary reference. Spectra can be obtained with 64 scans, 20 ppm sweep width, 24,192 complex data points, and active suppression of residual solvent at 4.363 ppm [HDO (90% H_2O,

10% D_2O) shift at 333 K]. Exponential line broadening (0.3 Hz), automatic phase correction, baseline correction, and integration can be 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).

The ratio of di-substituted to mono-substituted xylose (D/M) can be calculated as shown in Figure 8.6A. Combining this calculation with the A/X ratio of a given sample allows calculation of the relative proportions of unsubstituted, mono-substituted and di-substituted xylose (Figure 8.6B–D).

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

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

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

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

Figure 8.6. Equations used to calculate (A) the ratio of di-substituted to mono-substituted xylose (D/M) with the area of the ^{1}H NMR spectrum assigned to O-2 di-substitution (O2di), O-3 di-substitution (O3di) and O-3 mono-substitution (Mono); (B) the relative proportion of mono-substituted xylose (mXyl); (C) the relative proportion of di-substituted xylose (dXyl); and (D) the relative proportion of unsubstituted xylose (uXyl). A/X is the ratio of arabinose to xylose, which can be measured with GC.

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CHAPTER 9

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

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Summary

- Raw materials: oat, barley, rye, wheat
- Relevant applications: dough, bread, dairy and meat alternatives, digesta
- Fermentation-induced changes: MW reduction, solubilisation
- Analytical methods:
  - Enzymatic assay kit + colourimetry:
    - Total cereal β-glucan content
    - Extractability
  - Lichenase treatment + high-performance anion-exchange chromatography-coupled pulsed amperometric detector (HPAEC-PAD):
    - DP3/DP4 ratio for total vs. water-extractable β-glucan
  - β-Glucan isolation + size-exclusion chromatography with refractive index detection (SEC-RID) (and low-angle light-scattering/right-angle light-scattering (LALS/RALS) or multi-angle light scattering (MALS)):
    - Weight-average (MW_w) and number-average MW (MW_n), dispersity ($Đ = MW_w/MW_n$)

1. Introduction

1.1 Structural features, functionalities and extraction of cereal β-glucan

Cereals are the main source of dietary fibres in the human diet (EFSA, 2010). The two major cereal dietary fibres are arabinoxylans (see Chapter 8) and mixed-linkage (1→3,1→4)-β-D-glucans (BG) (Torbica et al., 2022). BG is a high-MW, partially water-soluble, linear homopolysaccharide composed

mainly of cellotriosyl (degree of polymerisation 3 (DP3)) and cellotetraosyl (DP4) units (and ≤10% DP5–9), linked through β-(1→3)-bonds (Figure 9.1). This fibre is most abundantly found in oat and barley (content of 3–7% and 5–11%, respectively, see Table 9.1), 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) and wheat (3.0–4.5 (Table 9.1; 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 rate increases from wheat BG > barley BG > oat BG when comparing materials of the same MW_w and at the same concentration (Lazaridou et al., 2004). An alternative function of MW_w and concentration is the ability of BG to form viscous aqueous solutions due to its long, flexible chains – the higher the MW_w, the higher the viscosity (for MW_w ranges, see Table 9.1) (Regand et al., 2009; Wolever et al., 2010). Note that the reported MW_w ranges for barley and oat BG are very broad (400–2500 kDa; Table 9.1) – which may be partially an artefact of the processing steps the raw material went through and the way BG was extracted.

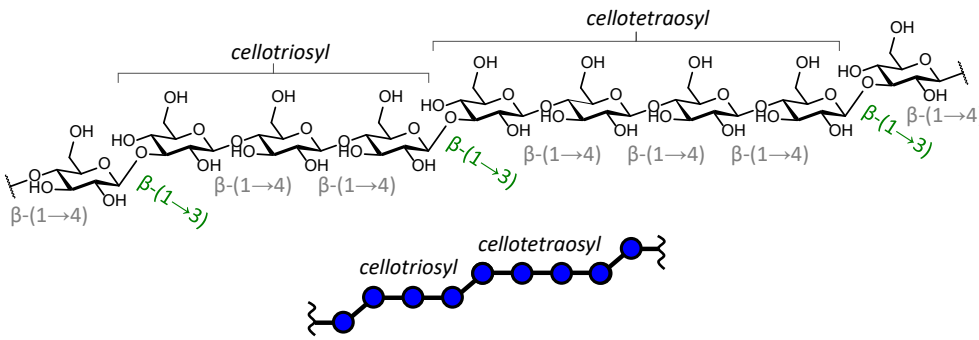

Figure 9.1. Chemical structure of mixed-linkage cereal β-glucan (BG) comprised mainly of cellotriosyl (DP3) and cellotetraosyl units (DP4) β-(1→3)-linked as indicated in the figure above (the ≤10% DP5–9 domains are not shown, for the sake of simplicity). The bottom figure is the symbolic representation 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 glucose units.

Table 9.1. Cereal β-glucan (BG) content, weight-average molecular weight (MW_w), 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 [%] (w/w)	MW_w [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

BG viscosity has been associated with various technological and health-promoting properties (Daou & Zhang, 2012; Lazaridou & Biliaderis, 2007). Among various health-promoting properties, the most recognised 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) positive effects on immunomodulatory properties (Estrada et al., 1997), with indications that it acts favourably against certain cancers of the digestive system (Choromanska et al., 2015; 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 type 2 diabetes (Patel, 2015). It should be noted that other positive effects of BG are less dependent on its viscosity or high MW. Notably, BG can display prebiotic effects (Schmidt, 2022). BG can be a fermentable substrate for 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 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).

Cereal BG exist as high-MW polymers in the cereal cell walls, tangled together physically or linked to other components chemically (Lazaridou & Biliaderis, 2007). This may 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-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-glucanases (Gangopadhyay et al., 2015). Extraction of BG from barley at pH 8.0 led to lowered activity of β-glucanases and, consequently, lowered extraction of high-MW BG polymers.

The solubilisation of BG from cereals is important to leverage 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 solubilisation of BG under physiologically relevant conditions (*in vitro* digestion at 37 °C) is rather low from oat bran that was previously heat-treated for enzyme deactivation, giving rise to an extractability of 13–28%, as reported by Beer et al., (1997), with a high MW_w of 1–2 MDa. Hence, there is a lot of potential to increase extractability through fermentative processes.

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 LAB (*Lactiplantibacillus plantarum*), on the physicochemical properties of BG in oat sourdough. These authors verified that the total BG content decreased (by ~13%) after 12 h of fermentation, which was deemed to be caused by LAB growth, since oat endogenous β-glucanases were deactivated by heating before fermentation – although this claim of LAB consuming BG was not further investigated (e.g. by testing for β-glucanase and β-glucosidase activity of the LAB). The authors also observed moderate changes in BG solubility and MW_w fluctuations, which led to the hypothesis that there are two processes during fermentation that influence the amount of extractable/soluble BG and its MW_w: on the 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_w high, and on the other hand, the degradation and consumption of already soluble BG as food for the micro-organisms (during propagation of LAB), reducing both the MW_w and the content of soluble and hence also the total BG. Depending on the relative rate of these two processes – namely enzymatic solubilisation vs. digestion of dissolved BG – soluble BG content and MW_w can be increasing or decreasing during the course of fermentation.

In another sourdough fermentation study, also with a *Lactiplantibacillus plantarum* strain, Rieder et al., (2012) observed the MW_w reduction as a function of the barley flour and oat bran sourdough's fermentation time, with a 80–90% and ~23% relative decrease in MW_w after 18 h, respectively. In the case of the heat-treated oat, this maximum 23% relative reduction in MW_w can be attributed to the fermentation process itself. This is in contrast with the untreated barley flours, with assumed high endogenous β-glucanase activity, where it is harder to distinguish what effect the endogenous enzymes had compared with the added LAB.

An important factor that determines fermentation and the influence of fermentation on the BG structure is the type of micro-organisms involved during fermentation (Bai et al., 2021; He et al., 2022). He et al., (2022) studied the effect of five different LABs (*Lactiplantibacillus plantarum, L. acidophilus, L. casei, L. bulgaricus* and *Streptococcus thermophilus*) on oat fermentation using a whole-oats preparation that was roasted, milled, liquefied at 1:4 solids/water (m/v), saccharified and homogenised. The authors verified that the highest content in BG was retained when *S. thermophilus* was used in the fermentation process and that, on the other hand, there was a decrease of ~30% in total BG content when *L. casei* was used. While the used BG quantification method may raise doubts whether the BG content actually decreased and was not, instead, an artefact of the method's ethanol precipitation step, whereby low-MW BG may be lost, it is still clear that something indeed changed with BG during the fermentation.

All in all, this shows that endogenous enzymes (when not previously deactivated) and fermentation can modify cereal BG properties (i.e. MW_w, solubility and total content). These molecular changes may alter the functional, nutritional and sensorial properties of foods (Singh et al., 2012). However, the exact mechanism has not yet been elucidated, i.e. which microbial enzymes of certain LABs exactly are responsible for such effects. This could be elucidated by demonstrating extracellular β-glucanase activity.

In addition, to the best of our knowledge, no studies have investigated the influence of fermentation on the DP3/DP4 ratio of BG. Although it is expected that this ratio will not be significantly affected by such enzymatic hydrolysis, depending on which parts of BG are solubilised via enzymes, the DP3/DP4 ratio of the water-extractable fraction may change. Hence, analytical procedures for characterising fermentation-induced changes in the molecular structure of BG are described here, including proposed modifications to established methods.

2. Methods of analysis

The main methods and sample preparations described in this chapter are (see Figure 9.2 for the overview):

- The quantification of total cereal BG content in fermented samples via the Megazyme (Wicklow, Ireland) (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 and MW-distribution analysis by size-exclusion chromatography coupled to a refractive index detector, low-angle/right-angle light-scattering, and viscosity detectors (SEC-RID-LALS/RALS-VISC).

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 9.3). These are then fully hydrolysed to glucose with β-glucosidase in the BG quantification method.

Equipment

- Analytical scale (0.1 mg accuracy)
- Adjustable volume pipettes (20–200 µL, 100–1,000 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 (Sunnyvale, USA) CarboPac PA1 (4 mm × 250 mm) with a CarboPac PA1 guard column (3 mm × 50 mm)

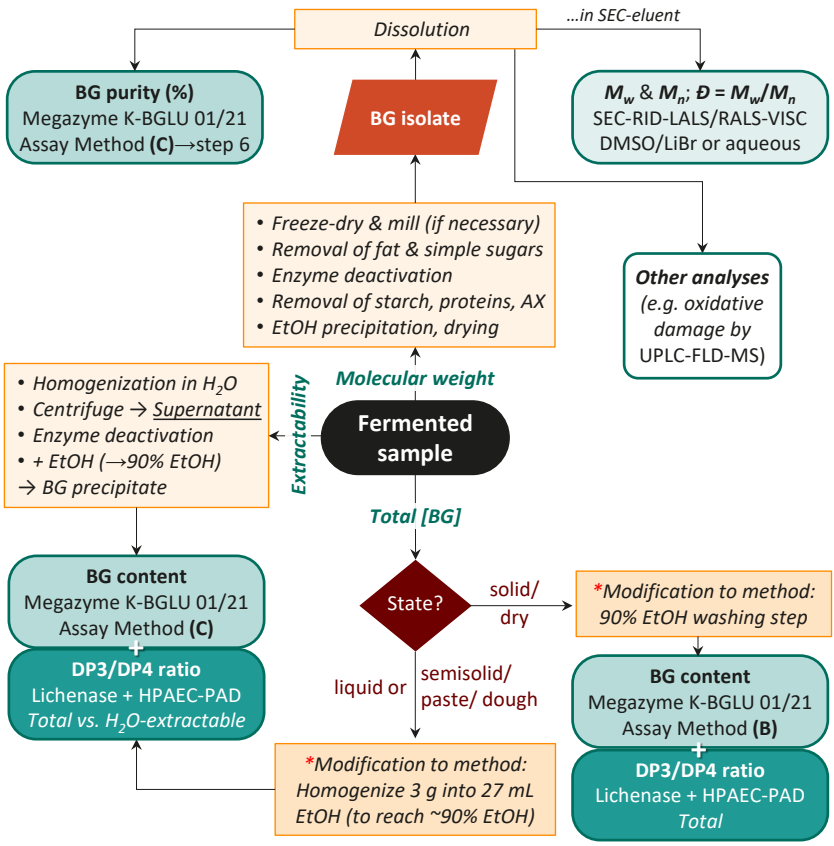

Figure 9.2. Schematic overview of sample preparation steps and analytical methods for cereal β-D-glucan (BG), starting with the fermented sample in the middle. * = modifications to the standard Megazyme K-BGLU method (Version 01/21) for BG quantification, introduced to minimise loss of low-MW BG while still allowing removal of glucose that could interfere with the assay.

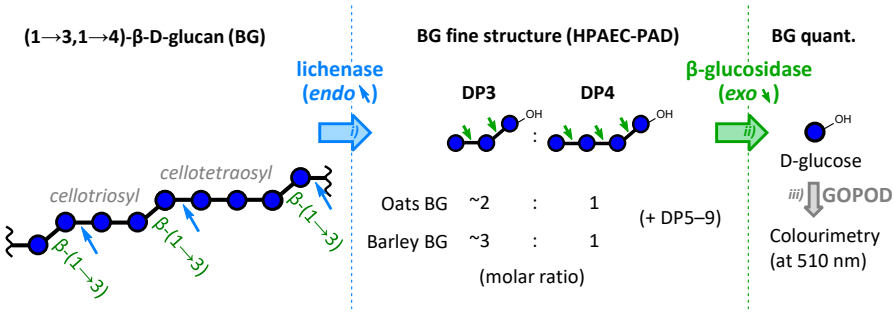

Figure 9.3. Symbolic representation of the BG polysaccharide (on the left), most commonly found in oat and barley, as well as the impact the enzyme lichenase has on it, important for BG quantification and fine structure analysis. Lichenase (Step i) hydrolyses BG specifically at the β-(1→4) linkage of a β-(1→3)-linked glucose unit (indicated with ↖), releasing oligosaccharides with DP3 and DP4 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 hydrolyses these oligosaccharides to glucose, which can be quantified by means of an assay using glucose oxidase + peroxidase and 4-aminoantipyrine (GOPOD reagent; Step iii).

2.1 Quantification of BG via colourimetric detection using the Megazyme method

The colourimetric 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 quantification in 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 fibre 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, yoghurt 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 and 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 hydrolyses BG specifically at the β-(1→4) linkage of the C3-substituted glucose units in the chain (Figure 9.3 Step i). Hence, cellulose, for instance, which is also a type of β-glucan, but one that is perfectly linear, with only β-(1→4) linkages, is not hydrolysed, 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. These enzymatically released BG oligomers (mostly DP3 and DP4, with 5–10% DP5–9) are water-soluble and, hence, remain in the supernatant after centrifugation, where they are subsequently hydrolysed by the added β-glucosidase to glucose (Figure 9.3 Step ii). The last step is treatment with the GOPOD reagent, which is a reagent mixture that enzymatically oxidises glucose, producing gluconic acid and H_2O_2, with the latter reacting with 4-aminoantipyrine to produce the purple-pink colour 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 di-/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) and (C)) may be necessary to remove free sugars and, hence, for reasons of simplicity, comparability and uniformity, it is suggested here that this washing step 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 solubilised when mixed in a 1:20 ratio of solids to water, during 2 h 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 its 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 isolates, described in section 2.3.2.), what has to be taken into account experimentally when comparing the effect of fermentation on BG solubilisation, is the fact that fermentations typically take place for several hours, leading to solubilised BG just due to this contact time with water, irrespective of the fermentation (in addition to effects from endogenous enzymes, which can be eliminated by prior deactivation of the raw materials). Hence, a proper control, to be able to assess what effect the fermentation alone has on the BG solubilisation (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 add 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 methods

The main modifications of the original Megazyme methods (A), (B), and (C) are summarised here, largely to minimise the loss of low-MW_w BG in the case of enzymatically pretreated/fermented samples:

- For samples with high BG content (oat bran concentrate, BG isolates): Use 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 higher ethanol concentrations, namely 90% (v/v) aq. EtOH instead of only the 50% used in Method (B) (section 2.1.4).
- For semi-solid samples: Add 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 procedures 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 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, together with 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 content (oat bran concentrate, BG isolates) 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 BG content indicated on the bottle. Measure at least one of them with each analytical run.

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 for instant mixing, cap tube immediately, and vortex mix until fully dispersed (if necessary, shake manually until all lumps disappear).[1]
(4) Right away, place the tube with dispersed sample 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 and allow to equilibrate for 5 min.

[1] The original Megazyme Method (A) uses 0.2 mL aq. EtOH and 4 mL phosphate buffer; however, experience has shown 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 content.

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

(6) Add 200 µL lichenase solution (50 U/mL in 20 mM phosphate buffer; 10 U), then cap and vortex the mix. Incubate for 1 h at 50 °C in shaking water bath, with vigorous vortex mixing every 15–20 min (or continuous stirring if a continuous stirring device is available).
(7) Add 5.0 mL sodium acetate buffer (200 mM, pH 4.0), then vortex mix vigorously.
(8) Keep tubes at room temperature for 5 min, then centrifuge (10 min at >3,000 × *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 the solution happen to land on the tube wall instead of on the tube bottom, centrifuge the tube 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 equation shown in Figure 9.4, using the exact sample weights *W* (~100 mg), dilution factor *D* = 1, and final volume FV = 10.6 mL for all samples.

$$BG\ content\ \left[\frac{g}{100g}\right]"as\ is" = (A_{sample} - A_{blank}) \times \frac{100}{W \times A_{(100\ \mu g\ Glc)}} \times FV \times D \times 0.9$$

Figure 9.4. Equation for β-glucan (BG) content "as is" calculation, with A_{sample} = sample absorbance average after β-glucosidase treatment (from tubes #1 and #2, see Step (9) of Method (A)); A_{blank} = absorbance of the blank (from tube #3); $A_{(100\ \mu g\ Glc)}$ = absorbance average of the 100 µg glucose (Glc) calibration standard; W = exact sample weight "as is" [mg] (often ~100 mg); FV = final total volume [mL] before taking aliquots for β-glucosidase treatment (e.g. 10.6 mL for Method (A)); D = dilution factor = 1, unless otherwise noted in the respective method section; in cases where $A_{sample} > A_{(100\ \mu g\ Glc)}$ (meaning: outside of calibrated range), dilution of the solution prior to incubation with β-glucosidase and re-analysis is required; 0.9 = 162/180 = transforming the total glucose content to total BG content.

Method (A'): For oat bran concentrates or BG isolates 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 vigorous manual shaking and vortex mixing every ½ h. For BG isolates, 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), then vortex mix vigorously. Then, transfer entire contents, including any solids, to a new 50 mL Falcon tube with the help of 30 mL sodium acetate buffer (50 mM, pH 4.0), and stir for 10 min. Then continue with the regular Step (8) from Method (A).

For the calculation of the BG content, with the equation from Figure 9.4, use the exact sample weights *W* (in mg), dilution factor *D* = 1, and final volume *FV* = 40.8 mL.

[3] For BG isolates that are precious, where one does not want to use 3 × 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 one could 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 and controls

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

<table>
<tr><td>For total BG content – adapted Method (B)</td><td>For extractable BG (solid/dry samples)</td></tr>
<tr><td>(1) Accurately weigh ~100 mg sample in Falcon tubes (15 mL size) in triplicate.</td><td>(1) Accurately weigh ~200 mg sample in Falcon tubes (15 mL size) in triplicate.</td></tr>
<tr><td colspan="2">(2) Deactivate enzymes (and reduce free glucose amounts) 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 >3,000 × 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, and centrifuge for 10 min at >3,000 × g. Discard the supernatant.</td></tr>
<tr><td>(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 equation from Figure 9.4, using the exact sample weights W (~100 mg), dilution factor D = 1, and final volume FV = 10.4 mL.</td><td>(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, mixing manually every ½ h.
(6) Centrifuge for 15 min at >3,000 × 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) and 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 equation from Figure 9.4, using the exact sample weights W (~200 mg), dilution factor D = 2.8, and final volume FV = 7.15 mL.[4]</td></tr>
</table>

[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 and controls

Herein, the suggested procedure is presented to determine total and extractable BG of fermented semi-solid/liquid samples (such as plant-based yoghurt 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 triplicate. (2) Next, deactivate enzymes (and reduce free glucose amounts) 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 >3,000 × 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 >3,000 × g. Discard the supernatant.	(1) Accurately weigh ~3.0 g sample in Falcon tubes (15 mL size) in triplicate. (2) Add the appropriate amount of water to each replicate to reach a solids/water ratio of 1:20 – in this 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 content).[5] Mix until properly dispersed. (3) Incubate at 40 °C for 2 h in a shaking water bath, then mix manually every ½ h. (4) Centrifuge for 30 min at >3,000 × 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 abs. ethanol to reach ~90% (v/v) EtOH. Vortex mix. Centrifuge (>3,000 × *g*, 15 min), and decant 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 equation from Figure 9.4, using the exact sample weights *W* (~3,000 mg), Dilutionfactor *D* = 1, and final volume *FV* = 10.2 mL.	(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), then 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 equation from Figure 9.4, using the exact sample weights *W* (~3,000 mg), Dilutionfactor *D* = 6.3,[8] and final volume *FV* = 7.15 mL.

[5] Calculation: For a 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 × g is available, centrifugation time may be reduced to 15 min.

[7] If this solution needs to 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. Calculate the correct dilution factor from the actually used added water amounts in Step (2).

2.1.6 Calculations

In general, for optimal comparability between samples, total BG content should be given on a dry-matter basis, by using the moisture content of the sample (determined e.g. by drying an aliquot of the sample to constant weight at 105 °C in an oven, usually overnight):

$$Content\ (dry\ matter\ basis) = Content\ "as\ is" \times \frac{100}{100 - moisture\ content\ (\%\ w/w)}$$

Figure 9.5. Equation for expressing content on a dry-matter basis.

The BG extractability is calculated using the following formula:

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

Figure 9.6. Equation for β-glucan (BG) extractability calculation.

However, since the total BG content may also change during fermentation (consumption of BG through micro-organisms), 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 actual detected total BG content. In any case, the calculation used just needs to be clearly defined when reporting results.

2.2 Fine structure analysis of DP3/DP4 via HPAEC-PAD

2.2.1 Method principle and explanation of the various steps

The β-glucan fine structure, defined by the molar DP3/DP4 ratio (cellotriosyl-to-cellotetraosyl ratio), can be determined for different β-glucan populations in the sample. Here, we focus on two key populations:

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 section 2.1.1). To measure the total population, one takes advantage of the fact that lichenase is also able to hydrolyse 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, can 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.

Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.2.2 DP3/DP4 ratio for total BG

(1) Depending on the sample, follow the first four steps of the 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), then 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 a continuous stirring 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, >3,000 × *g*), take supernatant and dilute it 20× with water, filter (0.45 µm), and transfer 1,000 µL into HPLC vials (1.5 mL).

(5) Analyse by HPAEC-PAD, continuing with section 2.2.4.

2.2.3 DP3/DP4 ratio for water-extractable BG

(1) Follow the first ten steps of the extractable BG-procedure from section 2.1.4 (or first nine steps of section 2.1.5 in case of semisolid/liquid sample), up to and including the 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, >3,000 × *g*), take supernatant and dilute it 20× with water, filter (0.45 µm), and transfer 1,000 µL into HPLC vials (1.5 mL).
(4) Analyse by HPAEC-PAD, continuing with section 2.24.

2.2.4 HPAEC-PAD conditions

Two general procedures are commonly used for HPAEC-PAD, depending on the available specific column setup (see Table 9.2).

Table 9.2. Summary of two general HPAEC-PAD procedures used for fine structure analysis of BG.

Column setup	Eluents	Reference
CarboPac PA1 analytical (4 mm × 250 mm) plus guard (3 mm × 25 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 mm × mm 250 mm) plus guard (4 mm × 50 mm) columns from (Dionex)	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 in Table 9.2. Glucotriose (O-BGTRIB) and glucotetraose (O-BGTETB) are recommended to be used as external standards to quantify the cereal BG DP3 and DP4 (up to 100 µM and 50 µM, respectively), and e.g. xylotriose (O-XTR) as an IS[9] (added e.g. at 25 µM level to all samples and standards; all oligosaccharides are from Megazyme, Bray, 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

The following procedure for the extraction and isolation of cereal BG necessary for MW-distribution analysis via size-exclusion chromatography 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 MW for samples that underwent enzymatic pretreatments or fermentation, such as freeze-dried plant-based yoghurt (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 arabinoxylan (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® (Novozymes A/S, Bagsværd, Denmark) at 90–95 °C, where the high temperature presumably allows for the extraction of also higher MW 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 solubilised 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 and 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 solubilisation 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 gelatinisation 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 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* 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 dispersion: 50 U/mL in 0.5 M NaOAc (pH 4.0)
  To have enough for 10 samples, disperse 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 × 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 ½ h.
(3) Centrifuge for 15 min at >3,000 × 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 >3,000 × 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 >3,000 × 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 >3,000 × 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® 300 L to each sample.
(18) Incubate at 80 °C for 1 h in shaking water bath.
(19) Heat in boiling water bath (covered with aluminium foil) for 30 min to precipitate proteins.
(20) Cool the tubes by placing them in cold water.
(21) Centrifuge for 15 min at >3,000 × 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 → 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 >3,000 × 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 the inside temperature reaches 80 °C (which should be after ~4 min).
(32) Cool in cold water.
(33) Centrifuge for 15 min at 4,000 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 > 3,000 × g.
(38) Transfer and wash pellet with ~15 mL EtOH abs. into tared 15 mL Falcon tube (use 3 mL plastic pipettes; keep Nalgene bottle and pipette for 2nd washing round).
(39) Centrifuge for 5 min at > 3,000 × 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 extracted amount of BG isolates. Store BG isolates in desiccator in the dark.

2.3.3 Dissolution of BG isolates and purity determination

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

(1) If necessary, grind BG isolate into a powder using a mortar and pestle.
(2) Accurately weigh ~30 mg BG isolate 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 aluminium 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 triplicate (record exact amounts). Keep the rest in the fridge (e.g. to be used for size-exclusion chromatography analysis 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 with the equation from Figure 9.4, use sample weight W = 500 mg (use exact amounts), Dilutionfactor D = 1, and final volume FV = 10.2 mL.

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

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

Figure 9.7. Equation for β-glucan (BG) purity calculation of BG isolates.

[10] Hence, instead of e.g. 3 × 25 mg BG isolates = 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 (3 × 500 mg), leaving the rest for SEC analysis after dilution with the SEC eluent.

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

2.4 Molecular weight distribution analysis

The HPSEC 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 2.3.2), to have other co-extracted substances removed as much as possible (proteins, AX, starch). This is due to the reliance on RI and light-scattering detectors (LALS/RALS or multi-angle light-scattering) that would give a signal for most eluting substances. Using a simple water extract of sample material instead, other co-extracted soluble fibres, starch and proteins would overlap in the elugram, not allowing the characterisation 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 minimise 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 isolates

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 analysed 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 an RI detector (VE 3580, Viscotek).

Separation occurs in two PLgel MIXEDA LS 20 µm analytical columns (7.5 mm × 300 mm; Agilent Technologies, Santa Clara, CA, USA) maintained at 40 °C and a PLgel MIXED 20 µm guard column (7.5 mm × 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_w) 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 isolates 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 size-exclusion chromatography recovery), but also more accurate results for low-MW BG, which have a tendency for visible aggregation in the size-exclusion chromatography elugram.

2.4.1.3 Limitation

- Needs BG isolate (elimination of other soluble polysaccharides and proteins).
- Does not represent the behaviour 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 isolates

2.4.2.1 Description

For aqueous size-exclusion chromatography 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 MW (MW_w) and dispersity (Đ) of cereal BG isolates diluted in size-exclusion chromatography eluent to 1 mg/mL are determined by HPSEC (OMNISEC, Malvern Panalytical, Malvern, UK) 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 × 300 mm, Viscotek, parent organisation: Malvern Panalytical, Malvern, UK). OMNISEC RESOLVE detector compartment is equipped with a low and right-angle laser light-scattering detector (LALS/RALS), an 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 MW_w determination, a calibration is performed using narrow-MW-distribution polyethyleneoxide (PEO-24K) standard using the RI increment (dn/dc) value of 0.146 mL/g for cereal β-glucan, and checking the calibration with the injection of a dextran standard with known MW_w and MW_n.

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 isolate (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 size-exclusion chromatography system) – lower size-exclusion chromatography 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. Other aspects

- The water content of the solid samples should be determined to express the BG content on a dry-matter basis.
- Always analyse 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 characterisation – 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 10

Analysis of exopolysaccharides in fermented cereal- and pulse-based materials

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Summary

- Raw materials: Cereal- and pulse-derived flours, protein isolates, starch isolates
- Relevant applications: Dough, bread, meat alternatives and dairy alternatives
- Fermentation-induced changes: Micro-organisms as natural factories of exopolysaccharides
- Analytical methods:
  - Quantitative characterisation of exopolysaccharides
    - Phenol–sulphuric acid assay
  - Quantification of dextran
    - Enzyme-assisted method using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

1. Introduction

1.1 What are exopolysaccharides?

Polysaccharides, widespread in multiple organisms, are high-MW polymeric carbohydrate structures that exhibit chemical heterogeneity and physiological properties and biological activities that are structure and source related (Chen & Huang, 2018). Polysaccharides are made of monosaccharide units connected by glycosidic linkages. When these polymers are adhered to the outer cell surface, or when they are fully expelled to the surroundings, they are termed exopolysaccharides (EPS) (Angelin & Kavitha, 2020). EPS are extracellular macromolecules made up of carbohydrates (sugar residues), substituted with proteins, DNA, phospholipids and non-carbohydrate substituents, such as acetate, glycerol, pyruvate, sulphate, carboxylate, succinate and phosphates (Freitas et

al., 2011). In general, EPS may be homopolysaccharides (HoPS) or heteropolysaccharides (HePS). HoPS and HePS may differ not only in their fundamental subunits, but also in their structure, chain length, degree of branching and glycosidic bonds. HoPS are mainly composed of only a single monomer of glucose or fructose units, which commonly form glucans (dextran, mutan, alternan and reuteran), and fructans (levan-type and inulin-type polysaccharides), respectively (Ryan et al., 2015). On the other hand, the constituent repeating units of HePS (multiple monomers) 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 MW greater than 10^6 Da, whereas HePS range in MW from 10^4 to 6.0×10^6 Da (Angelin & Kavitha, 2020). Figure 10.1 shows a schematic classification of EPS.

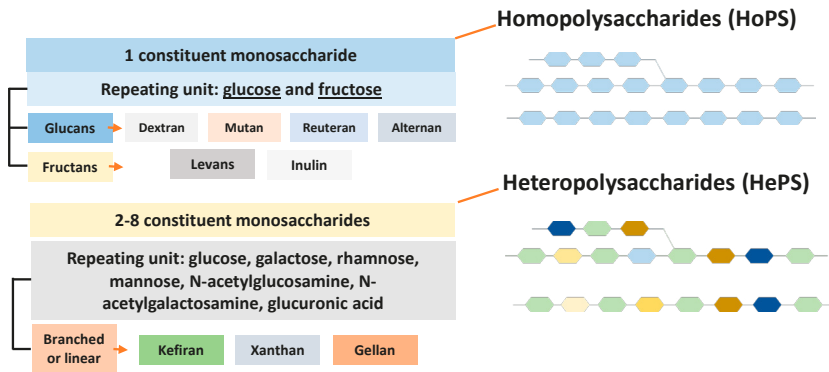

Figure 10.1. Schematic representation showing classification and characterisation of exopolysaccharides.

1.2 Natural factories of exopolysaccharides

Natural polymers, rather than synthetic polymers, have garnered a lot of interest among scientific communities over the past few decades owing to their advantageous therapeutic potential. Remarkably, various micro-organisms, such as LAB and yeasts, are well known to produce EPS with a 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 recognised as EPS producers (Rahbar Saadat et al., 2021). The production of EPS is not exclusively species dependent and, in some instances, is strain dependent. Generally, micro-organisms synthesise EPS to cope with harsh and adverse growth circumstances, such as dehydration, osmotic stress and pathogenic microbes. 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 that 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 and pH changes, reactive oxygen species, and antibiotics compared with individual microbial cells (Daba et al., 2021).

Although EPS is synthesised throughout the exponential and stationary phases, peak synthesis occurs only in the late exponential phase (Freitas et al., 2011). The level of EPS produced differs depending on the strain, medium composition and growth conditions, such as pH, temperature and carbon/nitrogen ratios. All enzymes involved in the production of EPS are regulated by specific genes (Badel et al., 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) polymerising and exporting (Korcz & Varga, 2021). From a biochemical standpoint, EPS are synthesised in two ways: intra- and extracellular. HoPS are typically produced by extracellular simple processes 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 polymerisation process (Korcz & Varga, 2021). With the aid of these enzymes,

the sugar residues are split into monomeric units outside the cell and assembled extracellularly into a polymer (Angelin & Kavitha, 2020). In contrast, 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 a group of hydrophobic enzymes, such as flippase, permease or ATP-binding cassette (ABC) transporters (De Vuyst et al., 2001).

1.3 Applications of exopolysaccharides

Nowadays, the contributions of EPS produced from micro-organisms as biotechnological tools in food, pharmaceutical and medical applications have been extensively documented. EPS are popular for their shelf life–extending properties and their technofunctional-enhancing properties in the dairy and baking industries and in the production of other 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, stabilisers, thickeners and gelling agents; to retain moisture, influence rheology and firmness, and reduce syneresis; and to improve texture, sensory and mouthfeel characteristics (Daba et al., 2021). Low dough machinability; unfavourable texture, volume or rheology; and a limited shelf life, are commonly encountered challenges during bread making, and these can be overcome by the use of EPS instead of expensive hydrocolloidal polysaccharides (Das et al., 2015). EPS have proven to be a reliable molecule for numerous drugs, and for therapeutic and medical treatments in curing inflammation and other disorders. Several EPS produced from natural sources have been reported as a good substitute to certain synthetic anticancer drugs (Wang et al., 2014). In fact, these molecules are able to impede tumour cell 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). In addition, EPS can trigger a nonspecific reaction against viral and bacterial infections and against inflammation. Therefore,

EPS have a high potential to be used as immunomodulating drugs that activate the immune system to fight infectious diseases (Nik Ubaidillah et al., 2015). In addition, EPS treatment has a relevant anti-diabetic activity through inhibiting α-glucosidase and α-amylase enzymes under *in vitro* conditions. *In vivo* experiments revealed that EPS extracted from kefir grains have good cholesterol-reducing effects, decreasing very-low-density lipoprotein cholesterol concentrations (Lim et al., 2017).

2. Methods of analysis

2.1 Techniques for characterisation of exopolysaccharides

To evaluate the potential applications of EPS, it is crucial to study their structure and composition. The primary step in EPS characterisation is optimising extraction and purification procedures. The features of the EPS and the desired outcome of the extraction should be considered during the optimisation of 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 resins are examples of chemical methods that may contaminate the extracted products (Comte et al., 2006). Physical methods, including centrifugation, ultrasonication, and heating, mostly reduce contamination but achieve lower efficiencies when compared with chemical methods (Liang et al., 2010). A subsequent 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 deproteinisation, and membrane techniques (ultrafiltration, diafiltration and gel filtration) (Barcelos et al., 2020). To quantify and further analyse the composition and structure of EPS, colourimetric methods, such as the phenol–sulphuric acid method, HPLC, GC, SEC, ion-exclusion chromatography (shortened to IEC), Fourier-transform infrared (shortened to FTIR) spectroscopy, and NMR spectroscopy, are commonly used (Loeffler et al., 2020). This chapter is focused on description of the main methods involved in the characterisation of the evolution of the composition of microbial EPS during cereals-based fermentation processes.

Quantification of EPS can be done using total carbohydrate analysis or using specific analysis of monosaccharides released from the EPS after depolymerisation. For the latter, it is common to use methanolysis,

acid hydrolysis or enzyme hydrolysis. Methanolysis and acid hydrolysis are nonspecific and, therefore, yield monosaccharides from any other polysaccharides that are present in the extract. With enzyme hydrolysis, the EPS can be specifically degraded and the release of monosaccharides quantified to determine the amount of EPS produced. In this chapter, two methods are described: one based on total carbohydrate analysis using the phenol–sulphuric acid assay, and another that has been developed specifically for dextran analysis. The semiquantitative method is based on specific enzymatic hydrolysis followed by analysis of glucose using a chromatographic method. The same approach can be used for quantification of other EPS if appropriate enzymes are available commercially.

2.2 Quantification of exopolysaccharides via the phenol–sulphuric acid assay

2.2.1 Sample preparation

Typically, recovery begins with precipitation, most commonly using ethanol, which reduces EPS solubility and causes precipitation. However, ethanol alone is insufficient, as residual lactose may co-precipitate with EPS, leading to inaccurately high values. Therefore, additional steps, such as protein precipitation, dialysis to remove low-molecular-mass carbohydrates, and drying, are essential, resulting in significantly higher yields of purified EPS (Sørensen et al., 2022).

- Transform raw and fermented samples into powder using a freeze-drier.
- Add 20 mL of chilled absolute ethanol to 20 g of the dried samples, then incubate for 12 h at 4 °C.
- Discard the supernatant and save the pellets after centrifugation at 12,000 × *g* for 20 min at 4 °C.
- Suspend the pellets containing EPS in 5 mL of distilled water with gentle heating at lower than 50 °C, then centrifuge at 6,000 × *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 at 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-drier for at least 24 h.
- Resuspend the EPS in distilled water for further analysis.

2.2.2 Phenol–sulphuric acid assay

The simplest method for quantifying EPS is weighing the dried powder, but this is imprecise due to the inclusion of impurities. A more commonly used method is the colourimetric phenol–sulphuric acid assay, which measures carbohydrate content by producing an orange-yellow colour upon reaction with simple sugars, oligosaccharides, polysaccharides, and their derivatives (Sørensen et al., 2022).

- Mix 20 µL of EPS extract with 20 µL of 5% phenol in the 96 wells plate.
- Add 100 µL of sulphuric acid (95–98% v/v) and incubate for 10 min at 25 °C.
- Analyse the colourimetric reaction by measuring the absorbances at 492 nm.
- Generate a standard curve using solutions of known glucose concentrations (0.01–5 mg/mL).
- Calculate the EPS concentration based on comparison with the standard curve, taking into account any dilution of the sample.

2.3 Enzyme-assisted method for quantification of dextran via HPAEC-PAD

2.3.1 General principle

The enzyme-assisted method can be applied for the analysis of pure dextran; however, it can also be used as a semiquantitative method for the analysis of dextran in 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. (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 to glucose that is quantified via HPAEC-PAD. The method requires the action of two enzymes: dextranase and α-glucosidase. Dextranase catalyses the hydrolysis of α-(1→6) linkages in dextran, releasing glucose, a homologous series of isomaltooligosaccharides and branched isomaltooligosaccharides, while α-glucosidase hydrolyses linear isomaltooligosaccharides to final glucose. The amount of branched isomaltooligosaccharides formed depends on the degree of branching in the original dextran. These branched isomaltooligosaccharides are resistant to further hydrolysis and are usually not fully hydrolysed to glucose by α-glucosidase. For this reason, the method is only suitable for dextrans with a low degree of branching. An evaluation of the enzyme-resistant branched oligosaccharides can be found in Maina et al. (2011).

Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.3.2 Materials

- Aqueous ethanol (50% v/v) (Sigma-Aldrich, Darmstadt, Germany)
- 0.05 M Sodium citrate (Sigma-Aldrich) 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°C
- 2-Deoxy-D-galactose (ISD, 5.025 mg/mL)
- D-glucose (Merck KGaA, Darmstadt, Germany)
- Commercial dextran (*Leuconostoc* spp., Sigma-Aldrich)
- Transglucosidase 16670 nkat/ml (α-glucosidase from *Aspergillus niger*, E-TRNGL, Megazyme, Bray, Ireland)
- Dextranase, 195 808 nkat/ml (dextranase from *Chaetomium erraticum, D0443*; Sigma-Aldrich)
- HPAEC-PAD: CarboPac PA1 column (250 × 4 mm i.d.; Dionex, Sunnyvale, CA, USA) Carbopac PA-1 Guard column (4 × 50 mm; Dionex) Waters 2465 pulsed amperometric detector (Waters, Milford, MA, USA)
- External calibration standard. Prepare glucose standard solutions (Table 10.1)

Table 10.1. Glucose standard final concentrations.

Standard solution	Concentration (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

2.3.3 Enzyme dosage and enzymatic activity verification

Before performing the analysis on actual samples, the activity of dextranase and α-glucosidase should be determined from the manufacture specification or analysed with a suitable method. The appropriate dosage of the enzyme for the assay is dextranase (10,000 nkat/g dextran) and α-glucosidase (1,000 nkat/g dextran). A control sample (pure dextran 3–5% branching) should always be prepared for each sample set to verify the activity of the enzymes.

- Dilute both enzymes 1:100 in sodium citrate buffer (pH 5.5).
- Disolve pure dextran (e.g. 5 mg/mL) in sodium citrate buffer (pH 5.5).
- Transfer 500 µL of dextran solution into an Eppendorf.
- Calculate the appropriate amount of enzyme needed to ensure a final activity dextranase (10,000 nkat/g) and α-glucosidase (1,000 nkat/g). E.g. when using enzymes listed in section 2.3.2, in a 500 µL sample, add 13 µL of the diluted dextranase and 15 µL of the diluted α-glucosidase.
- Hydrolyse the solution for 48 h at 30 °C with constant shaking.
- 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 CF 0.90. Recovery should be between 65–70%. Lower recovery may indicate that the enzyme did not function properly or the dextran analysed as a high degree of branching.

2.3.4 Sample analysis

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

- Weigh approx. 100 mg (99–102 mg) of freeze-dried and powdered sample into a 15 mL centrifuge tube. Prepare four 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, prepare two control tubes weighing 10 mg dry weight (DW) of commercial pure dextran (by *Leuconostoc* spp.) + 90 mg of fermented sample (one EPS sample is used) and analyse 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 that the solution is dispersed.
- Place the samples in boiling water for 5 min.
- Vortex the sample and add another 3 mL of aqueous ethanol. Vigorously vortex until an even dispersion is formed. All aggregates should be broken down to ensure that free sugars and oligosaccharides are completely dissolved in the solution.
- Centrifuge the mixture at 11,500 × *g* 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, resuspend 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 a further 3 min.
- Allow the solutions to cool down to room temperature before adding the enzymes.
- Dilute enzymes 1:100 to buffer immediately before using them. When using the same enzymes listed in section 2.3.2, add 25 µL dextranase (dextranase 10,000 nkat/g) and 30 µL transglucosidase (transglucosidase 1,000 nkat/g) to the B sample and adjust the volume to 5 mL and add only 30 µL of transglucosidase for the A sample.

- Hydrolyse the samples for 48 h at 30°C 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 11,500 × *g* and collect the supernatants.
- Use the supernatants for glucose analysis with HPAEC-PAD. Specifically, take 500 µL of supernatants and centrifuge with Amicon (Merck Millipore, Darmstadt, Germany) centrifuge filters. Dilute the samples before injecting into HPAEC-PAD. (For instance, pure dextran samples are diluted 20 times, to ensure the glucose analyses is within the standard range.)
- The amount of dextran is calculated as the sum of anhydro glucose using a CF of 0.90.
- Standard curve is built in the HPLC software, followed by the integration of peaks to obtain the amount of glucose detected in samples.

2.3.5 Calculations

Below is the general equation applied to find dextran content (% DW):

- Dextran content (% DW) = (glc in B – glc in A) mg/mL × 5 mL × 0.9 × 100 / CF/ 100 mg

  Note: 0.9 is for correction of anhydro glucose; CF is based on the recovery rate

  First calculate the CF from the pure dextran tubes amounts, taking into account the dilution. The concentration of glucose in mg/mL is multiplied by 5 mL and corrected with a factor of 0.9 (correction for anhydro glucose) to obtain the amount of glucose mg/100mg released from pure dextran. The CF (recovery rate expressed as a percentage) is then determined by dividing the amount of glucose obtained (mg) for the pure sample by 10 mg (10 mg was the amount of pure dextran weighed in the control tube, DW). The average of the two replicated samples with pure dextran is used as the CF in the equation above.

  After the CF is determined, actual samples results can be calculated. First calculate the amount of glucose in mg/mL from the HPAEC-PAD data, taking into account the dilution and multiplying by 5 mL to obtain the amount of glucose (mg). To calculate dextran (mg) in samples, subtract glucose in A from each B replicate value and multiply the result by 0.9. The obtained value is then divided by the CF and by the sample weight (≈100 mg dry weight) to express the dextran content as % DW.

2.3.6 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 utilised are efficient to cause the breakdown of dextran with few branches. The method is therefore not suitable for highly branched dextrans present in a sample (Katina et al., 2009).

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SECTION 4

Minor grain constituents

CHAPTER 11

Analysis of the content of phytic acid, inositol phosphates and minerals

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Summary

- Raw materials: Cereals (wheat, oat), pulses (faba bean, yellow pea)
- Relevant applications: Dough, bread, meat and dairy alternatives, in vitro digesta
- Fermentation-induced changes: Degradation of phytate, increased mineral bio-accessibility
- Analytical methods:
  - Approximation of phytate content
    - Colourimetric determination of total phosphorus content before and after enzymatic hydrolysis
  - Quantification of inositol phosphates
    - High-pressure ion chromatography (HPIC)
  - Determination of mineral concentration
    - Inductively coupled plasma mass spectrometry (ICP-MS)

1. Introduction

1.1 What is phytic acid and how does it chelate minerals?

Phytic acid (myoinositol 1,2,3,4,5,6-hexakisphosphate; IP6) is the major component to store phosphorus in cereals and pulses and typically comprises 85% of the total phosphorus content (Lemmens et al., 2018; Oatway et al., 2001). The phytic acid concentration of cereals and pulses depends on the growth conditions (climate, soil, year) and is on average between 0.2 to 3.4% dry matter (dm), with measured concentrations varying from 0.39–1.35% dm for wheat, 0.42–1.16% dm for oat and 0.22–1.22% dm for peas (Schlemmer et al., 2009; Steiner et al., 2007). Phytic acid is present in cereals mainly as its salt form, phytin, which is stored in globoids within the protein storage vacuoles. In cereals it can be mainly found

in the aleurone layer and the scutellum cells of the germ (De Brier et al., 2016; Skoglund et al., 2009). In legumes, phytin is also found in protein bodies, present in the endosperm or the cotyledon (Schlemmer et al., 2009). In Figure 11.1A, the chemical structure of a fully protonated IP6 is shown. The number of protons present depends on the pH of the system. For example, the fully protonated IP6 with 12 protons only exists at extremely low pH (Figure 11.1B) (Oatway et al., 2001; Wang & Guo, 2021). Under physiological conditions, phytic acid has a strong negative charge, which leads to chelation of cations (Figure 11.1C) and the formation of an insoluble salt, phytate. Phytate confines Potassium (K), Magnesium (Mg), Calcium (Ca), Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn), Sodium (Na) and Sulphur (S) (De Brier et al., 2016; Lemmens et al., 2018; Madsen & Brinch-Pedersen, 2019; Oatway et al., 2001). 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 (also known as bio-accessibility), as they precipitate at neutral pH in the intestine, after being solubilised under the acidic conditions of the stomach (Lemmens et al., 2018; McKie and McCleary, 2016; Schlemmer et al., 2009). In plant-based diets, the antinutritional effect of phytate is considered the most important for inhibiting the absorption of Fe^{2+} and Zn^{2+} and, hence, reducing bioavailability (Egli et al., 2004; Hambidge et al., 2004; Lim et al., 2013). Apart from the chelation of minerals, IP6 can interact with cationic amino acids or form cationic bridges with negatively charged proteins (Wang & Guo, 2021). Electrostatic interactions, hydrogen bonding or esterification reactions can occur between phytic acid and polysaccharides. Thanks to the interaction with digestion enzymes, phytic acid may influence the amino acid and carbohydrate metabolism (Figure 11.1C) (Brouns, 2022; Oatway et al., 2001; Wang & Guo, 2021).

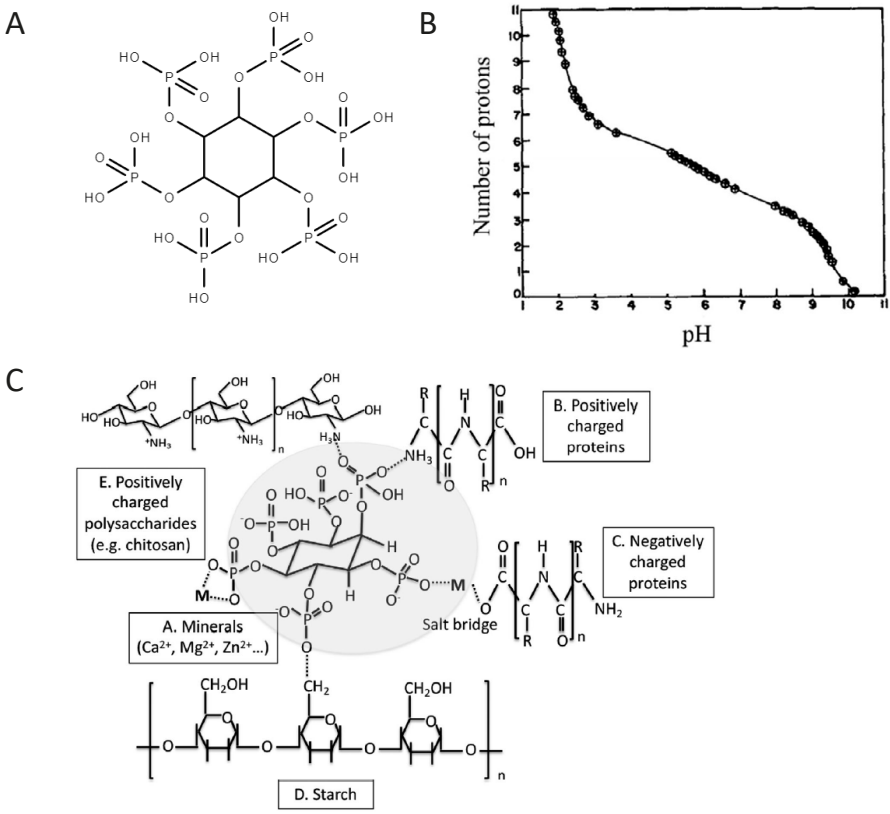

Figure 11.1. (A) Molecular structure of fully protonated phytic acid, with 12 dissociable hydroxyl (–OH) groups. (B) Number of protons bound to a phytic acid molecule in function of the pH of the solution. (C) possible interactions of negatively charged phytate with (a) minerals (b and c) proteins (d) starch and (e) charged polysaccharides. Adapted from Wang and Guo, 2021, and made with ChemSpider (Royal Society of Chemistry, London, UK).

1.2 How can fermentation affect phytic acid and its chelation of minerals?

Cereal fermentation has proven to be a powerful tool to achieve dephytination during food processing (Madsen & Brinch-Pedersen, 2019). The molecular structure of phytic acid and its chelation of 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 lower-inositol phosphates (IP5, IP4, IP3, IP2, and IP1) (Bohn et al., 2007; Greiner et al., 2002; Konietzny & Greiner, 2002). Phytase activity is mostly associated with the outer layers of cereal kernels, and a higher phytase activity is described for bran compared with the starchy endosperm (Steiner et al., 2007). Lower-inositol phosphates have a decreased chelating potential, thus improving micronutrient bio-accessibility. However, humans and monogastric animals lack sufficient phytase activity in their digestive tract to absorb the micronutrients present as phytates in food and feed, respectively (Madsen& Brinch-Pedersen, 2019; Schlemmer et al., 2009). Therefore, the full potential of cereals as a nutrient-rich resource is not completely exploited if phytate is present in the food matrix, and opportunities for exploitation 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, have significant potential as stable and potent enzymes both in feed and in food (Dionisio et al., 2011; Faba-Rodriguez et al., 2022; Madsen & Brinch-Pedersen, 2019). 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 show a higher phytase activity in wheat (2886 ± 645 U/kg dm) compared with oats (496 ± 35 U/kg dm) and peas (262 ± 73 U/kg dm). One unit of phytase activity was equivalent to the amount of enzyme that liberates 1 µmol of orthophosphate from 100 µmol of sodium phytate at 37 °C and pH 5.0 per minute. However, it should be noted that considerable differences are found in phytase activities reported in the literature, which can be attributed to variations in analytical methods used (e.g. the extraction procedure) (Steiner et al., 2007). As lower endogenous phytase activities are described for legumes and oats, the contribution of microbial or

exogenous phytases is relevant to facilitate phytate degradation in such food products (Frias et al., 2003; Steiner et al., 2007; Verni et al., 2017).

In addition, the acidification that takes place during fermentation can affect the appearance of phytate and its interaction with minerals. First, as shown in Figure 11.1B, more protons will be present at lower pH, decreasing the chelating capacity of phytic acid in a food/feed product. However, this will probably not affect the chelating capacity *in vivo*, since the pH in the stomach and small intestine will not be affected. Second, acidification is suggested to accelerate phytase activity, and thus phytic acid hydrolysis, as pH optima range between 4.5 and 6.0 (Konietzny & Greiner, 2002; Lemmens et al., 2019). More specifically, the optimum pH of endogenous phytases in cereals and pulses ranges between 5.0 and 6.0 in wheat and barley and is described to be 6.0 in rye and 5.0 in oat and faba bean (Konietzny & Greiner, 2002). Thus, fermentation often creates optimal conditions for endogenous phytase activity.

Finally, phytate can be degraded during fermentation by the growth of phytase-positive bacteria, which express extracellular phytase activity (Reale et al., 2004). Microbial phytases have been identified as HAP and are commercially used for feed supplementation. Earlier research on sourdough described that phytic acid was degraded more extensively after 12 h of fermentation in wheat-based doughs inoculated with *Lactiplantibacillus plantarum, Levilactobacillus brevis*, and *Lactobacillus curvatus,* compared with dough inoculated with *Saccharomyces cerevisiae* (Reale et al., 2004). However, after 36 h of fermentation, a yeast-inoculated sample showed the highest phytic acid breakdown (Reale et al., 2004). Verni et al. (2017) studied the phytase activity expressed by different LAB strains, isolated from faba bean fermentation through the traditional backslopping method, and determined a wide distribution, ranging from 0 to 0.958 U (amount of phytase that released 1nmol of phosphate from sodium phytate per min under the assay conditions). They attributed the highest activity to strains of *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*; strains of *Lactococcus lactis*, *Latilactobacillus sakei*, *Weisella koreensis*, *W. cibaria* and *Enterococcus casseliflavus* did not express significant phytase activity towards sodium phytate.

Noteworthy is that an improved mineral bio-accessibility thanks to the breakdown of phytic acid does not necessarily facilitate the absorption of divalent cations in the small intestine. Whereas mineral bio-accessibility is commonly evaluated using *in vitro* digestion procedures, the absorption

of the minerals, and thus mineral bioavailability, can only be evaluated with *in vivo* studies. However, *in vitro* digestions can be complemented by human colon carcinoma cell lines (i.e. Caco-2 cells) to obtain an estimation of the mineral bioavailability (Verhoeckx et al., 2015). A recent review by Brouns (2022) discusses that the claims that a reduction in phytic acid per definition leads to a measurable improvement in mineral status require nuancing. Therefore, phytic acid hydrolysis may contribute to an increased mineral bio-accessibility but may be ineffective in enhancing mineral bioavailability and thus reducing the prevalence of micronutrient deficiencies (Brouns, 2022).

2. Methods of analysis

Investigating phytic acid and its degradation products has received considerable attention during the last decades. Recently, Marolt and Kolar (2021) provided a systematic and chronological review of analytical methods for quantifying these compounds. For an overview of advantages and disadvantages of existing techniques, we refer the interested reader to this manuscript. In this chapter, we focus on methods that are frequently used and that are suited to determine low phytic acid concentrations present in cereal- and legume-based samples. Therefore, methods based on the quantification of total phosphorus determination using colourimetry, the separation of inositol phosphates with high-pressure ion chromatography (HPIC), and the assessment of the mineral concentration with inductively coupled plasma mass spectrometry (ICP-MS) are described and discussed below (Figure 11.2).

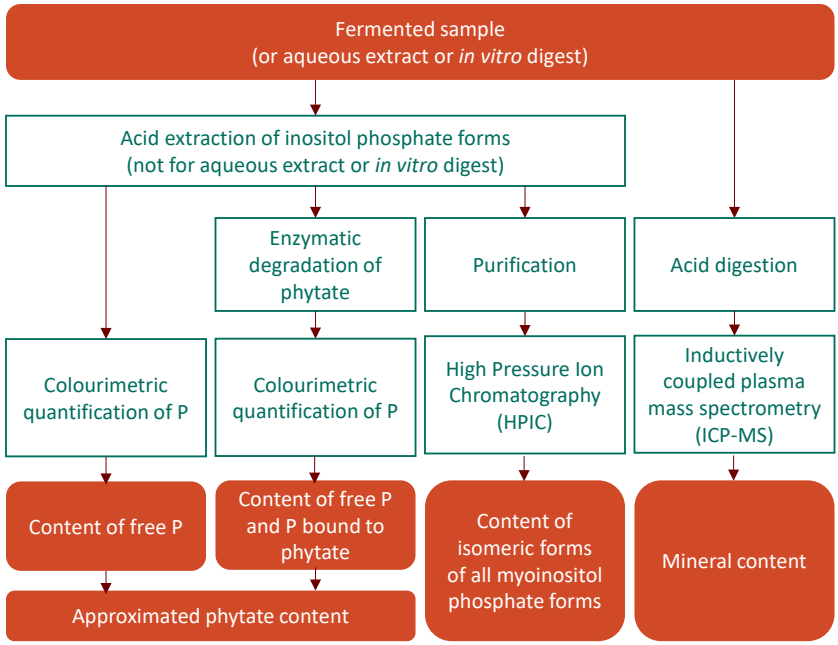

Figure 11.2. Schematic overview of sample preparation steps and analytical methods described in this chapter.

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

The first method is a simple, high-throughput procedure to determine the phytic acid content, 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. Therefore, all phosphate groups are released from all myoinositol phosphate forms present in the sample and, 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 (Bray, Ireland).

2.1.1 General principle

The general principle of this method relies on the enzymatic dephosphorylation of all myoinositol phosphate forms in the sample. First, the acid-soluble compounds are extracted from the sample during an overnight incubation. Phytic acid and lower-myoinositol phosphate forms (IP1–5) are obtained in the supernatant. Neutralising 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 myoinositol phosphate forms (IP6, 5, 4, 3, 2) to IP1 (Figure 11.3A). Second, a pH 10.4 buffer and an alkaline phosphatase solution are added to release the final phosphate group from each inositol in the sample (Figure 11.3B). To ensure the total enzymatic degradation, samples are incubated at 40 °C for 1.5 h, in deviation from the proposed protocol by Megazyme, which applies 10 min. The enzymatic degradation is stopped by adding TCA (50% w/v), and remaining impurities are eliminated by centrifugation.

Free phosphate in the sample is determined by adding the colour reagent. During incubation with this colour reagent, the free phosphate 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 ascorbic acid in sulphuric acid (Figure 11.3C). The amount of free phosphate is proportional to the amount of molybdenum blue and quantified by analysis of the absorbance at 655 nm, making use of a calibration curve of known phosphorus concentrations.

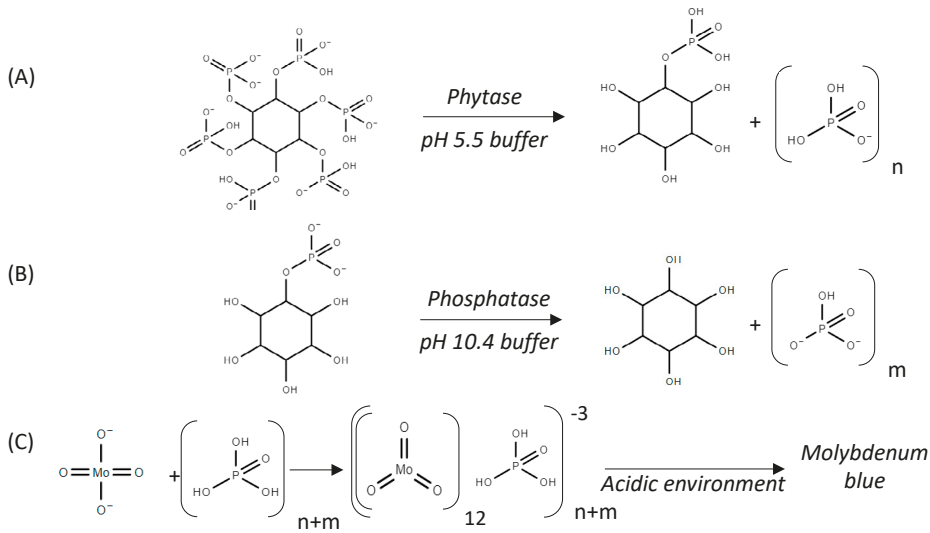

Figure 11.3. Reaction mechanism of the enzymatic degradation of phytic acid and subsequent colorimetric phosphorus determination. (A) Phytic acid and lower-inositol phosphate forms are hydrolysed into myoinositol and inorganic phosphate by phytase in an acidic environment. (B) Alkaline phosphatase hydrolyses myoinositol phosphate into myoinositol and inorganic phosphate in alkaline conditions. (C) Inorganic phosphate in combination with ammonium molybdate reacts to 12-molybdophosphoric acid, which is reduced to molybdenum blue in an acidic environment. The charges on the molecules are indicative but vary due to solvent interactions. Figure made with ChemSpider (Royal Society of Chemistry, London, UK).

Comparing total phosphate released during enzymatic treatment with free phosphate groups in the original sample gives the concentration of phosphorus bound to myoinositol in the sample. This method is suitable for assessing the phytic acid concentration in unfermented and unprocessed samples, assuming that all phosphate groups originate from phytic acid. Fermented samples contain multiple lower forms of inositol phosphate, which will lead to an overestimation of phytic acid. A second drawback could be that alkaline phosphatase releases phosphate from monophosphate esters other than myoinositol phosphate. A high accuracy was obtained for cereal flours and pulses using this method, while the influence of monophosphate esters is hypothesised to be neglectable (McKie & McCleary, 2016).

2.1.2 Reagents

- Buffers:
  - pH 5.5 (200 mM sodium acetate)
  - pH 10.4 (400 mM glycine, 4 mM magnesium chloride, 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: five parts of 1% (w/v) ascorbic acid in sulphuric acid (1 M) + one part of ammonium molybdate (5% w/v)
- Extraction solvent: hydrochloric acid (0.66 M)
- Neutralising solvent: sodium hydroxide (0.75 M)
- Acidic solvent: TCA (50% w/v)

2.1.3 Sample preparation

1. Accurately weigh 1 g* ground 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, no acid extraction is needed. The phosphate concentration in the sample should be within the linear range of the analysis.
2. Incubate overnight (16 h) on a shaking rack (150 rpm) at room temperature.
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

To determine the free and total phosphate concentration, respectively, use two new 1.5 mL Eppendorf tubes for each sample, labelling them with A and B.

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 myoinositol phosphate:
   - 600 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 h.
3. Add the following solutions to the Eppendorf tubes:

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

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

4. Vortex each Eppendorf tube and incubate in a 40 °C water bath for 1.5 h.
5. Stop the reaction by adding 300 µL TCA (50% w/v).
6. Centrifuge the tubes (13,000 rpm, 10 min) and transfer 1.0 mL of the supernatant to a new Eppendorf tube.
7. Prepare the calibration curve in 1.5 mL Eppendorf tubes (Table 11.1).

Table 11.1. Amounts of water and phosphorus standard needed to make a calibration curve

	0 µg	2.5 µg	5 µg	7.5 µg	10 µg
Water (µL)	1,000	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 colour reagent to all the Eppendorf tubes (samples and calibration curve), vortex, and incubate in a 40 °C water bath for 1 h.

9. Vortex, transfer the solution to a cuvette (1 cm light path, 1.5 mL) and 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 h after the incubation.

2.1.5 Calculations

1. Determine the linear ($R^2 > 0.99$) absorbance curve of the phosphorus standards ($Absorbance_{655\ nm}$/µg phosphorus), 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 equation in Figure 11.4A.
3. Expressing the phosphorus ratio (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 31 g/mol)/660 g/mol)], the phytic acid concentration can be calculated with the equation in Figure 11.4 B.

One should consider that these assumptions only hold for non-processed food and that an overestimation of the phytic acid concentration will result when using this calculation for fermented samples.

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

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

Figure 11.4. (A) The equation to calculate the concentration of phosphorus in a sample. $V_{initial}$ = original sample extract volume (mL), which is 20 mL if using this procedure; F = dilution factor, which is 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, which is 1 mL using this procedure. (B) The equation to estimate the concentration of phytate in a sample.

2.2 Quantification of different inositol phosphates via HPIC

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 phytic acid by Phillipy and Johnston (1985). The separation described below, to determine the profile of phytic acid and lower-inositol phosphate forms (IP5–IP2) in wheat and a compound diet (barley, wheat, soybean meal, calcium carbonate, amino acids, sodium chloride and vitamin/mineral premix), was developed and validated by Blaabjerg et al. (2010). The analysis uses a gradient HPIC method and was later adjusted with minor modifications to perform the analysis on legume samples by Wainaina et al. (2022). However, not all isomers are commercially available, and therefore, some peaks should be identified based on in-house reference standard solutions or based on separation sequences reported in the literature.

2.2.1 General principle

Measuring the hydrolysis products from phytic acid with HPIC requires well-controlled experimental conditions for the extended purification of the sample. The acid extraction, to release phytic acid from chelates 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 with previously described methods using gradients of hydrochloric acid. 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. The colour can be measured by UV detection at a wavelength of 290 nm. The detection limit reported by Blaabjerg et al. (2010) is 0.9–4.4 mg/L. Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with

other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.2.2 Reagents

- Hydrochloric acid
- Methanesulfonic acid
- Iron (III) nitrate
- Perchloric acid
- External standard:
  - D-myoinositol-1,4-diphosphate (sodium salt) (IP2)
  - D-myoinositol-1,4,5-triphosphate (sodium salt) (IP3)
  - D-myoinositol-1,3,4,5-tetraphosphate (sodium salt) (IP4)
  - D-myoinositol-1,3,4,5,6- pentaphosphate (sodium salt) (IP5)
  - Phytic acid sodium salt hydrate (IP6)

These standards are commercially available. 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 also be present in the (fermented) samples.

2.2.3 Procedure

2.2.3.1 Sample preparation

1. Freeze-dry and then grind cereals, pulses and fermented materials to a fine powder with a particle size smaller than 125 μm using a ball mill.
2. Extract the inositol phosphate forms by incubating 0.5 g sample into 20 mL of 0.5 M HCl overnight (16 h) at room temperature while stirring/shaking. 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. Evaporate an aliquot of the supernatant (1.0 mL) to dryness at 50 °C to eliminate the extraction solvent (HCl), which could harm the HPIC equipment.

5. Dissolve the dried extract in 1.0 mL of the eluent used for separation (water–methane sulfonic acid solution (95:5 (v/v)), vortex, and filter using a 0.45 µm membrane. This eluent, compared with using HCl, results in a more horizontal baseline, which improves the specificity of quantification.

2.2.3.2 Analysis

A Dionex (Sunnyvale, CA, USA) BioLC system equipped with an AS50 autosampler, GS50 gradient pump, and AD25 ultraviolet-visible (UV/Vis) detector is used to separate the inositol phosphate forms in the sample. An aliquot (50 µL) of the sample is injected into a Dionex CarboPac PA1 analytical column (50 × 4 mm (length × diameter (l×d)), 10 µm particle size) with a Dionex CarboPac PA1 guard column (50 × 4 mm (l × d), 10 µm particle size) and a gradient of two mobile phases (A) 1.5 M methanosulfonic acid and (B) Milli-Q water at a flow rate of 0.6 mL/min. The elution programme applied starts with increasing the solvent A concentration from 5 to 28% over 15 min, followed by an increase to 85% over 10 min, which is maintained for 25 min. The separation is conducted on a column temperature of 30 °C.

Detection and quantification of inositol phosphate forms can be performed with a postcolumn derivatisation reaction with a colouring reagent (0.1% iron(III)nitrate in 2.0% perchloric acid). Using a UV detector at a wavelength of 290 nm, chromatograms can be generated. Here, IPs-Fe chelates are formed in a Dionex mixing tee and a knitted Dionex reaction coil (750 µL).

2.2.4 Interpretation of results

Identification and quantification can be done by analysing individual, external inositol phosphate standards (IP2–IP6). Figure 11.5 shows a chromatogram of a reference sample (Marolt & Kolar, 2021).

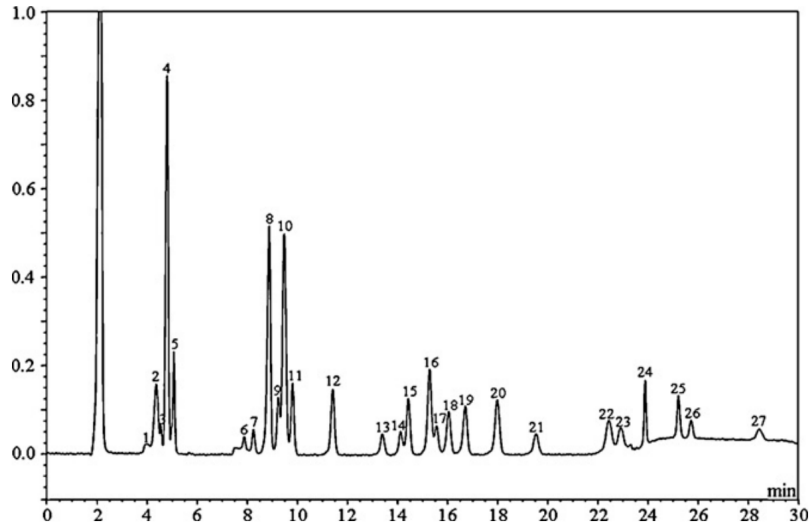

Figure 11.5. Chromatogram of a reference sample analysed with HPIC and gradient elution with methanesulfonic acid, on a CarboPac PA1 column, showing the separation of the peak of IP6 and the multiple isomeric forms of the lower-inositol phosphates. Peak numbers correspond to the following isomers of inositol phosphates: (1–5) InsP2; (6) Ins(1,3,5)P3; (7) Ins(2,4,6)P3; (8–11) InsP3; (12) DL-Ins(1,5,6)P3; (13) DL-Ins(4,5,6)P3; (14) Ins(1,2,3,5) P4; (15) DL-Ins(1,2,4,6)P4; (16) DL-Ins(1,2,3,4)P4; (17) Ins(1,3,4,6)P4; (18) DL-Ins(1,2,4,5)P4; (19) DL-Ins(1,3,4,5)P4; (20) DL-Ins(1,2,5,6)P4; (21) Ins(2,4,5,6)P4; (22) DL-Ins(1,4,5,6)P4; (23) Ins(1,2,3,4,6)P5; (24) DL-Ins(1,2,3,4,5)P5; (25) DL-Ins(1,2,4,5,6)P5; (26) Ins(1,3,4,5,6)P5; (27) InsP6 (Marolt & Kolar, 2021).

2.3 Determination of mineral concentration with ICP-MS

ICP-MS is used to determine elemental concentrations in acid digests of (fermented) food. Additionally, it is also often used to analyse *in vitro* digests of food samples, in order to assess mineral bio-accessibility of the food samples.

Prior to ICP-MS analysis, it is essential to decompose the samples with appropriate methods to reduce the amount of carbon residue in the digest. The sample digestion is a critical step. Dry-ashing methods followed by evaporation or carbonisation or by wet digestion in open vessels are time consuming and pose a high risk of analyte losses. Therefore closed-vessel acid digestions in microwave oven systems are recommended. These closed-vessel methods not only provide faster and more efficient sample

digestion, but they limit the risks of sample contamination and analyte losses through volatilisation (Nardi et al., 2009). Note that the method described below involves the use of specific pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.3.1 General principle

ICP-MS instruments are a very precise instrument for multi-element analysis, with LOQs generally ranging between 0.1 and 1.0 µg/L depending on the mineral of interest (Al-Rimawi et al., 2013). Approximately 80 elements can be measured with ICP-MS (Al-Hakkani, 2019). After acid digestion (see section 2.3.3), the sample is diluted up to the desired concentration. Through the use of a peristaltic pump, the sample is introduced into the nebuliser system, whereupon the solution is converted into an aerosol in the spray chamber. Next, the aerosol is introduced into an argon plasma (composed of ions and electrons with a high ionisation energy) and the elements are ionised (Al-Hakkani, 2019). Subsequently, the MS separates the different ions based on their mass to load ratio using a quadrupole mass filter or a magnetic sector and detects the ions with an electron multiplier. The data are stored through a computer interface (Al-Hakkani, 2019).

2.3.2 Reagents

- Ultra-high-purity acids (HNO_3 and HCl) are used for the destruction of the food sample, both to prepare standards for ICP-MS measurements and to acidify *in vitro* digest samples.
- Milli-Q water (18.2 MΩ) is used to dilute acid and *in vitro* digested samples and to prepare blanks and standards for ICP-MS measurement.
- A reference sample with certified element composition (e.g. NIST1567; National Institute of Standards and Technology, Gaithersburg, MD, USA) is used to evaluate the mineral recovery after acid digestion. Certified reference samples for evaluating the performance of the ICP-MS (e.g. selected reaction monitoring [SRM] 1643f Trace Element in Water from NIST and Reference Material for Measurement of Elements in Surface Waters SPS-SW2 Batch 137 from Spectrapure Standards, Oslo, Norway) are used.

2.3.3 Procedure

2.3.3.1 Sample preparation

Food samples are 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 an open digestion block (120–150 °C) or microwave digestion system (e.g. MARS 6; CEM Corporation, Matthews, NC, USA). During microwave digestion, the temperature is gradually increased from room temperature to 180 °C over 25 min, then held constant at this temperature for 20 min and gradually decreased to 65 °C over 25 min. Next, the digested samples are diluted to appropriate mineral concentrations using Milli-Q water (18.2 MΩ) before analysis. Blank samples and reference samples are included during each acid digestion. The elemental recoveries for the chosen reference sample should preferably range between 90 and 110%.

All solutions, including sample solutions, for ICP-MS analyses should contain 1% HNO3 (v/v).

It is important to note that, as the chloride ion interferes with vanadium and arsenic determination, HCl is not recommended during the ICP-MS sample preparation when analysing those elements. The same holds for the *in vitro* digestion when vanadium and arsenic bio-accessibility is assessed (Al-hakkani, 2019). Alternatively, collision cell technology or dynamic reaction cell can be added to the ICP-MS or kinetic energy discrimination mode can be used to reduce the interference (Al-hakkani, 2019).

2.3.3.2 Sample analysis

Multi-element analysis can be performed in the acid digests (total elemental content) or in *in vitro* digests (bio-accessible elemental content) using an ICP-MS (e.g. Agilent 7700; Agilent Technologies, Santa Clara, CA, USA) 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. The use of He gas in the collision cell helps to reduce interference formation, such as AgO (Al-Hakkani, 2019; Wilschefski & Baxter, 2019). An IS containing

germanium, indium, lithium, scandium or thorium needs to be added during the ICP-MS measurement to correct for variations in operating conditions of the ICP-MS (Al-Hakkani, 2019). The calibration for element quantification used to convert counts per second to concentrations is performed using multi-element certified standard solutions (e.g. Certipur®; Merck KGaA, Darmstadt, Germany). Instrument calibration need to be validated using certified reference solutions (e.g. SRM 1643f Trace Elements in Water, TM-28.4 Trace Elements in Water; National Institute of Standards and Technology, Gaithersburg, MD, USA; High-Purity Standards, Charleston, SC, USA, respectively).

2.4 Other techniques used in the literature

Phytic acid content and phytase activity levels have been studied with various other analytical methods, such as ion-exchange chromatography, complexometric titration and NMR techniques (Reale et al., 2004).

NMR analysis requires stepwise extraction of phytic acid with hydrochloric acid or EDTA from a dough or a 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 by 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 in order to interpret the results of complex systems, such as foods, correctly.

3. Tips and tricks

- Phytic acid concentration and the ratio of free phosphate over total phosphate in the sample are generally expressed on the dry-matter weight of the sample. This facilitates comparison between different studies, and new research should respect this convention of expression 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.

- All phytic acid must be extracted from the sample during the acid hydrolysis step. Large variations within the sample set could be due to the coarseness of the samples. It should be considered that extraction is facilitated by smaller particle sizes and that milling the sample could improve the accuracy of the measurement.
- Avoid metal containers, as phytic acid can remain on the surface because of its high chelating properties.

4. References

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CHAPTER 12

Analysis of saponins and tannins in fermented faba bean–based raw materials

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Summary

- Raw materials: Faba bean flour, protein concentrate, protein isolate
- Relevant applications: Dairy alternatives, meat alternatives
- Fermentation-induced changes: Expected degradation
- Analytical methods:
  - Determination of triterpenoid saponins
    - ultra-high-performance liquid chromatography photodiode array detector (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. Legumes and cereals are just a few examples of plant-protein sources that include saponins (Price et al., 1987). Tannins are mainly nonvolatile secondary metabolites that protect plants from predators and environmental aggressions (Khanbabaee & Van Ree, 2001). They can be found in dark chocolate, wine, tea and coffee (Soares et al., 2020), all of which are frequently characterised as having astringent properties. Indeed, both these classes of compounds can create sensory challenges in protein-rich and plant-based ingredients, contributing also to bitter taste and acrid flavour (Drewnowski & Gomez-Carneros, 2000; Tuccillo et al., 2022; Wang et al., 2022). However, saponins have emerged as commercially demanded compounds with expanding applications in the food, cosmetics and pharmaceutical sectors because of their physicochemical (surfactant) properties and their biological activity (Güçlü-Üstündağ & Mazza, 2007). While saponins were once considered

to be antinutrients, they are now being extensively researched for their antioxidant, hypocholesterolemic and anticancer properties (Shi et al., 2004). Tannins are applied in the food industry, e.g. to clarify wines and beers (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 words *sapon* (soap) and *in* (one of) (Koh & Tay, 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: triterpenoid and steroidal forms (Figure 12.1). In triterpenoid aglycones, three terpene units form the structure, while in steroidal aglycones, a sterol group characterises the composition. As a result, saponins fall into the categories of triterpenoid 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 also categorised based on the number of sugar side chains linked to them. Monodesmosidic saponins possess a single simple sugar chain at one position of aglycone. In the majority of monodesmosidic sapogenins, saccharide chain is attached by an ether linkage to the C3 hydroxy group (Augustin et al., 2011). On the other hand, bidesmosidic saponins are characterised by having two simple sugar chains. In triterpenoid saponins, such second sugar chains are mainly linked by an ester linkage to the C28 carboxy group (Augustin et al., 2011). The extensive number of saponin configurations results from the differences in aglycone structures and the characteristics of side chains (Koh & Tay, 2012). The categorisation of saponins can also be subdivided based on the specific plant source being examined. For instance, legumes contain a rich variety of soyasaponins, which are triterpenoid glycosides grouped into categories A, B and E on the basis of their aglycone structures and linked sugar chains. In addition to saccharide side chains, group B soyasaponins may have a side group, such as 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) moiety, at the C-22 position (Heng et al., 2006).

The known predominant soyasaponin forms in faba bean are βG and B (Shen, 2020). Soyasaponin βG is DDMP conjugated group B soyasaponin, and soyasaponin B is the corresponding non-DDMP conjugated group B saponin (Heng et al., 2006; Hu et al., 2002; Shen, 2020). Soyasaponin βG can be converted to soyasaponin B (Heng et al., 2006) when heated and it has shown instability at acidic pH (Heng et al., 2006), and thus possibly is also decomposed during fermentation. Interestingly, soyasaponin βG is found to be more bitter than soyasaponin B (Shen, 2020). Its decomposition may have positive impacts on flavour during fermentation.

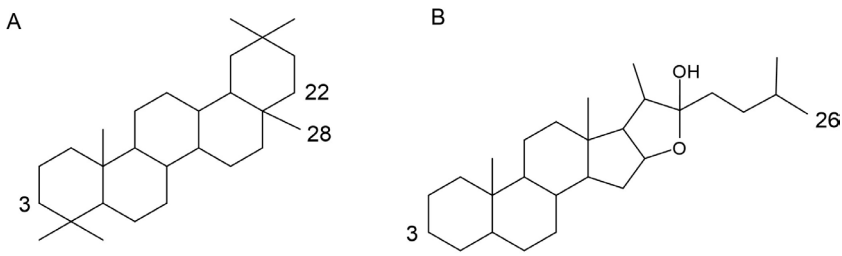

Figure 12.1. Examples of simplified structures of (A) triterpenoid and (B) steroidal saponins (aglycone form). Modified from Kregiel, 2017.

1.2 What are tannins?

According to Bate-Smith (1954), tannins are polyphenolic compounds that have a unique capacity to precipitate and agglomerate proteins. Condensed and hydrolysable tannins are the two main groups of tannins (Soares et al., 2020). In this chapter, we will focus on condensed tannins, which are classified as polymerised flavonoid tannins. They are constructed from flavan-3-ol subunits, which are characterised based on hydroxylation pattern, stereochemistry, functional groups and internflavan-3-ol-lingages (Zeller, 2019). Catechin, epicatechin, gallocatechin and epigallocatechin are four main subunits of condensed tannins. Both catechin and epicatechin and gallocatechin and epigallocatechin differ only in their stereochemistry of hydroxyl group at the C3 position (Figure 12.2). These subunits connect into chains of various length, most commonly through the C4–C8 interflavanic linkages (Figure 12.3) (Zeller, 2019). Two other types of linkages are less common than the 4,8-B-type linkage. A single covalent bond can be formed between C4 and C6 of adjacent

subunits, or two single bonds can be formed between adjacent flavan-3-ol subunits. Condensed tannins have a wide diversity in plant species due to flavan-3-ol subunits, distinct types of interflavan linkages and DP (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 depends on the protein concentration that exceeds the threshold. 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 et al., 1988). In higher protein concentrations, the tannins cross-link to various proteins, creating a hydrophobic surface layer and causing precipitation of the protein (Spencer et al., 1988).

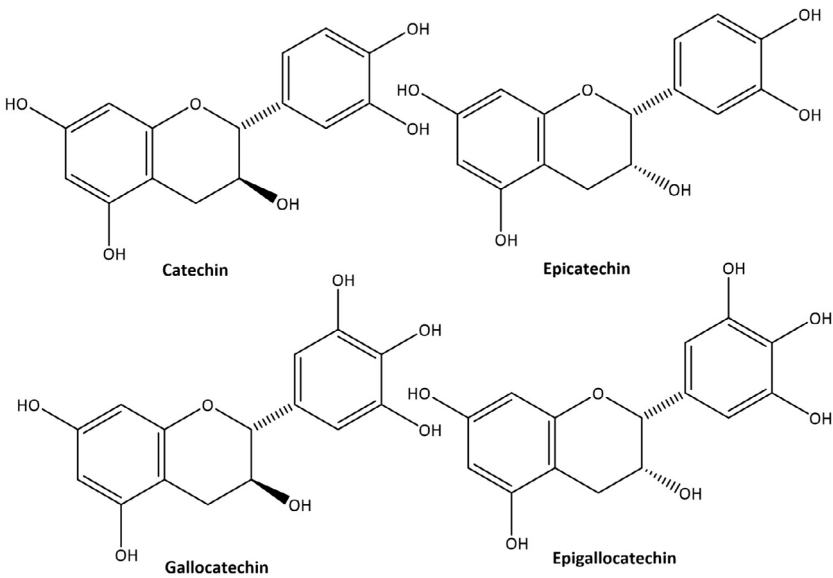

Figure 12.2. Flavan-3-ol subunits of condensed tannins. Modified from Zeller, 2019.

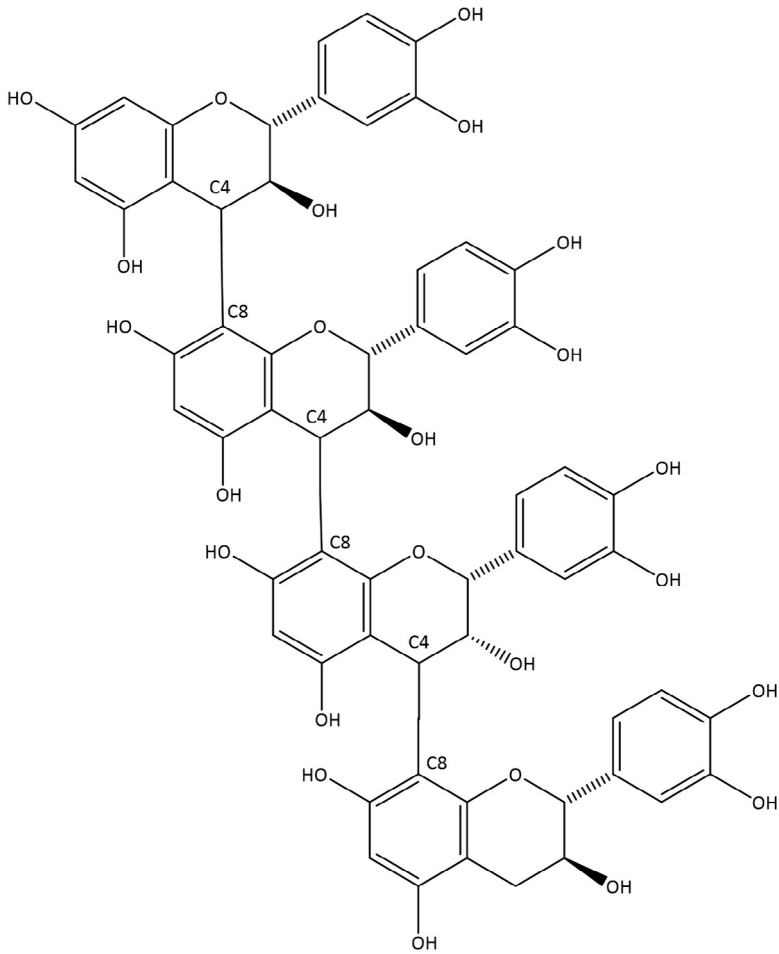

Figure 12.3. The structure of condensed tannins, showing the most prevalent type of interflavan 4,8-type linkage between adjacent flavan-3-ol subunits. Modified from Zeller, 2019.

1.3 How can fermentation affect saponins and tannins?

Saponins showed a significant function in immune system regulation, reducing cholesterol and showing antitumor and antiviral activity (Singh et al., 2017). According to the study by Navarro del Hierro et al. (2018) and several other studies, saponins are characterised by low bioavailability. However, microbiota were found to hydrolyse saponins to secondary glycosides or sapogenins that showed higher bioavailability and bioactivity than their precursors (Yang et al., 2015). Certain micro-organisms have the ability to break down saponins by their glycosidases, 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 hydrolyse saponins (Kim, 2018; Navarro del Hierro et al., 2019). Thus, fermentation can reduce the 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 gelatinisation.

Fermentation can have various effects on tannins, depending on the specific context. In the case of lactic acid 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 the tannins themselves (Svanberg et al., 1993). Several lactobacilli having tannase activity were isolated from fermented foods. and they include *Lactobacillus plantarum, L. paraplantarum* and *L. pentosus* (Osawa et al., 2000). This enzymatic functionality is a shared phenotypic trait among these three species.

2. Methods of analysis

2.1 Sample preparation

Sample preparation for tannin 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. For unfermented materials, such as faba bean flour, the sample can be directly used for analysis, without extensive preprocessing. 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, such as 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 soyasaponins B and βG via UHPLC-PDA

2.2.1 General principle

This analysis focuses on determining the content of soyasaponins B and βG (mg/g) in faba bean samples. The procedure involves extracting the triterpenoid saponins using aqueous MeOH, followed by chromatographic separation and quantification using ultra-high-performance liquid chromatography with a photodiode array detector (UHPLC-PDA). Calibration curves are constructed to relate detected signals to the saponin concentrations. The method described is based on Tuccillo et al. (2022). Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.2.2 Reagents

Prepare the following reagents:

- 80% aqueous MeOH solution
- Water (solvent A) and acetonitrile (solvent B), each with 0.025% trifluoroacetic acid

2.2.3 Extraction

- Weigh exactly 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 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 min. This step enhances the extraction of soyasaponins from the flour.
- After shaking, centrifuge the tubes at 4,000 rpm for 5 min. 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 step 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 min.
- Centrifuge the tubes again and collect the supernatant as before.
- Fill each volumetric flask to the mark using the methanol–water solution. This step standardises the volume of the extracted solution.
- To remove any particulate matter, filter the samples using a 0.2 μm filter to 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, such as faba bean flours, for subsequent UHPLC-PDA analysis, enabling accurate quantification of these compounds.

2.2.4 Standard curve preparation

A six-point calibration curve for soyasaponin Bb is created by diluting stock solution (50 μg/mL in methanol) 1:5 and 1:10 (v/v) in 80% aqueous methanol. The dilutions were injected 5–20 μL to obtain a calibration range of 25–200 ng/injection. Soyasaponin βG was quantified using standard curve of Bb.

2.2.5 UHPLC-PDA

Chromatographic separation is achieved using an Acquity Waters UPLC system (Waters, Milford, MA, USA) equipped with an HSS [high-strength silica] T3 C18 column (1.8 μm, 2.1 × 150 mm) and a photodiode array detector 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 column temperature is 30 °C and 20–30 μL injections are made.

2.2.6 Quantification

Soyasaponin B is identified based on retention time and UV spectrum (maximum at 210 nm). Soyasaponin βG is identified by its maximum absorption at 294 nm according to Hu et al. (2002). Utilise the six-point calibration curve created for soyasaponin B at 210 nm to calculate concentrations of both soyasaponins B and βG in the samples.

2.3 Total condensed tannins assay

2.3.1 General principle

The analysis aims to estimate condensed tannins in a faba bean 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 vanillin reagent reacts with subunits of condensed tannin polymer, forming a red-coloured complex. The intensity of this colour 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 coloured complex can be measured and used to determine its condensed tannin concentration. This method gives an approximation for tannin content, because interfering substances, e.g. ascorbate and chlorophyll, and underestimation of anthocyanins are known to affect the results (Sun et al., 1998).

2.3.2 Reagents

Prepare the following reagents:

- 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.
- A 7.2 N H_2SO_4 solution is created by mixing 21 mL of concentrated sulphuric acid with 79 mL of MeOH. This strong acidic solution aids in the extraction process.
- A 1% v/v H_2SO_4 in 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 coloured complex.

2.3.3 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% H_2SO_4 in MeOH solution to the sample for extraction.
- Vortex the mixture to ensure thorough mixing.
- Shake the sample for 90 min at room temperature using a table shaker to facilitate tannin extraction.
- Following shaking, centrifuge the sample for 10 min at 8,000 rpm and collect the supernatant in 2 mL Eppendorf tubes.

2.3.4 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 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 12.1.

Table 12.1. 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.

2.3.5 Determination and quantification

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% H_2SO_4 in MeOH
- 1.25 mL of the vanillin reagent
- 2.5 mL of 7.2 N H_2SO_4

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 H_2SO_4

This reaction mixture is incubated for 15 min at a controlled temperature of 35 °C. Following incubation, the absorbance of the reaction mixture is measured at a wavelength of 500 nm. The resulting absorbance value is then compared with the standard curve, allowing the determination of the condensed tannin concentration in the sample. To report the results accurately, it is 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 mg of condensed tannins (catechin equivalents CE) per 100 g of dry matter. This unit accounts for both the concentration of tannins and the weight of the sample's dry matter, providing a standardised and meaningful representation of the tannin content.

3. Other aspects

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 condensed tannin assay, in the 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/100 g dry matter).

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CHAPTER 13

Quantitative analysis of vicine and convicine in faba bean–based materials

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Summary

- Raw material: Faba bean
- Relevant applications: Solid, semi-solid, liquid fermented products containing faba bean flours, isolates, concentrates
- Fermentation-induced changes: Reduction of convicine and vicine content by lactic acid bacteria with specific enzymatic activities
- Analytical methods:
  - Reversed-phase high-performance liquid chromatography with UV detection (RP-HPLC-UV)

1. Introduction

Vicine and convicine are compounds mainly found in the faba bean, a legume plant from the Fabaceae family that grows in various climates. These two compounds are predominantly present in the cotyledons of faba beans, constituting about 1% of dry matter (Purves et al., 2018). Vicine and convicine are precursors of the aglycones divicine and isouramil (Crépon et al., 2010), which may trigger hemolysis after ingestion in people who suffer from favism, a genetic deficiency of the glucose-6-phosphate dehydrogenase (G6PD) enzyme (Arese et al., 1981). The severity of hemolysis can vary based on the specific mutation, resulting in milder symptoms for some individuals and more severe ones for others.

To enhance the use of faba bean, with its high nutritional quality, for food and feed, several studies investigated various processing methods and fermentation techniques to reduce the content of vicine and convicine in the faba bean. Cardador-Martínez et al. (2012) found that roasting and boiling can slightly reduce the content of these two undesired compounds. Hegazy and Marquardt (1983) investigated soaking in a glacial acetic acid solution at 40 °C for 72 h and found that the reduction can

be complete. Additionally, fermentation and enzymatic hydrolysis were studied by researchers including Coda et al. (2015), Rizzello et al. (2016) and Pulkkinen et al. (2019). They studied the application of selected LAB with 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 (Figure 13.1) (Pulkkinen et al., 2019). Recently, Björnsdotter et al. (2021) identified the key gene in the biosynthesis of vicine and convicine. Furthermore, they demonstrated that these pyrimidine glycosides are derived from purine metabolism through the riboflavin pathway.

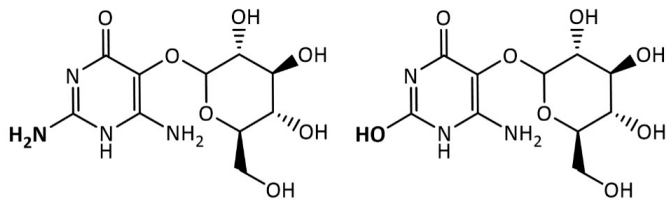

Figure 13.1. Molecular structure of vicine and convicine.

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. Specifically, regarding vicine and convicine, Coda et al. (2015) observed a decrease of more than 91% of vicine and convicine levels in faba bean flour samples fermented by a *L. plantarum* strain capable of synthesising ß-glucosidase. Rizzello et al. (2016) confirmed the efficacy of *L. plantarum* fermentation in this respect and investigated optimal fermentation conditions to enhance its application.

Pulkkinen et al. (2019) found that sourdough fermentation can lead to a reduction in these two compounds, whereby the hydrolysis efficiency varies depending on the strains and the fermentation conditions

employed. Controlled acidification plays a significant role in obtaining an acceptable sensory quality of the fermented food matrix.

2. Methods of analysis

2.1 General principle

Vicine and convicine are water-soluble compounds that can be extracted with water, separated by 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% perchloric acid. The method is described in detail by Pulkkinen et al. (2015). Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.2 Standard preparation

The purchased standards are vicine (≥98%; Cfm Oskar Tropitzsch GmbH, Germany) and uridine (≥99%, Sigma-Aldrich, Darmstadt, Germany). Uridine is used as an IS. 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 as vicine at 273 nm, convicine is quantified using vicine calibration curves (Pulkkinen et al., 2015). An 8 mg/mL uridine solution in water is prepared to be added as an IS to the samples. Stock solutions of standards are stored at –18 °C.

2.3 Sample preparation

Faba bean samples are milled to a 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, 2019).

2.4 Reagents and instruments

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

2.5 Extraction

Homogenised 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

HPLC separation is performed using RP-HPLC and isocratic elution using water containing 0.1% formic acid, followed by gradient elution with increasing proportion of acetonitrile containing 0.1% formic acid to wash the column. Elution is done at a 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: isocratic flow of 100% water containing 0.1% formic acid
- 8–15 min: linear gradient from 100% water containing 0.1% formic acid to 30%:70% water:acetonitrile containing 0.1% formic acid

- 15–22 min: isocratic flow of 30%:70% water:acetonitrile containing 0.1% formic acid
- 22–23 min: linear gradient from 30%:70% water:acetonitrile containing 0.1% formic acid to 100% water containing 0.1% formic acid
- 23–35 min: isocratic flow of 100% water containing 0.1% formic acid

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

3. Interpretation and analysis of results

The analytes and the IS elute in the order of vicine, convicine and uridine when 0.1% formic acid in water is used. 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), respectively, corresponding to retention factors of 2.10, 2.38 and 4.28, respectively.

With this method, vicine and convicine could be clearly separated from each other and from the IS, making analysis reliable (Figure 13.2).

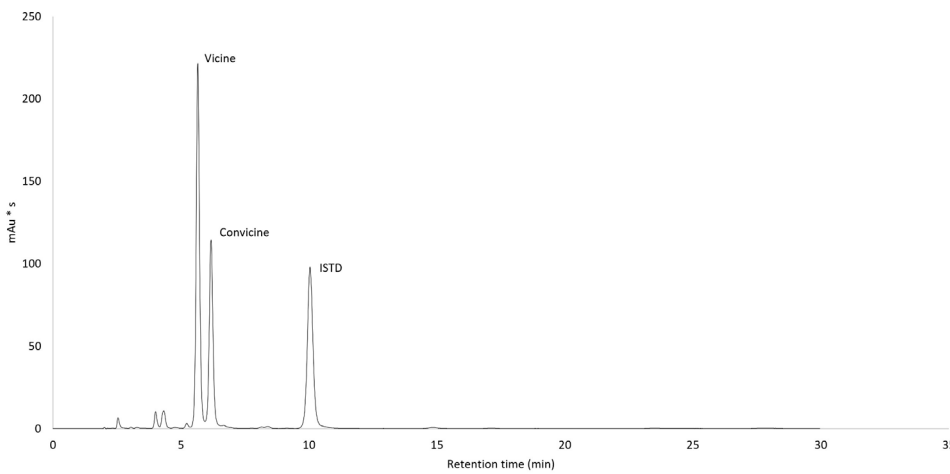

Figure 13.2. Example of a chromatogram obtained from the analysis of vicine and convicine via RP-HPLC.

4. Tips and 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 should be denatured, e.g. by placing test tubes in a boiling water bath for a few minutes prior to filtration.

Native faba beans contain notable concentrations of vicine and convicine, and therefore dilution of samples, e.g. from 15 mL to 25–50 mL, could be done to reduce the load on an analytical column.

With fermented samples, it may be good to run the samples first without the IS to ensure that fermentation would not produce compounds that would coelute with uridine.

To shorten the run time and to improve the separation of the analytes in chromatography, UHPLC may be used instead of HPLC.

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CHAPTER 14

Vitamin B12 analysis in fermented cereal- and pulse-based matrices

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Summary

- Raw materials: 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, other baked products) foods, semi-solid (dairy products, plant-based yoghurt) foods, and liquid (dairy products, plant-based milk) fermented food
- Fermentation-induced changes: *In situ* production of vitamin B12 in plant-based food through tailored fermentation induced by certain bacteria that are able to synthetize this compound
- Analytical methods:
  - Reversed-phase ultra-high-performance liquid chromatography separation with UV-detection (RP-UHPLC-PDA) (for determination and quantification)
  - Ultra-high-performance liquid chromatography separation with mass spectrometric detection (UHPLC-MS/MS) (for identification and confirming the structure)
  - Microbiological assay (MBA) (for estimation in fermented samples)

1. Introduction

Vitamin B12, also known as cobalamin, is an essential water-soluble micronutrient naturally present in foods 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 the proper functioning of two enzymes: methyl malonyl-CoA mutase and methionine synthase (Banerjee & Ragsdale, 2003). Since humans cannot acquire this vitamin from their enteric bacteria, as some animals can, it is essential for humans to obtain it through their diet (Stabler & Allen, 2004). The recommended intake for adults is 4 μg/day (EFSA, 2015; Blomhoff et al., 2023). Only certain bacteria and archaea can synthesise vitamin B12 (Martens et al., 2002). With the exception of some micro-algae, plant-origin foods do not contain

this vitamin; however, through the design of fermentation processes or fortification (Watanabe et al., 2013), plant-based products can also acquire a significant content of vitamin B12. Nowadays, subclinical vitamin B12 deficiency is prevalent worldwide, particularly among individuals with limited consumption of animal products, such as vegetarians and vegans (Herrmann et al., 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 a cost-effective method for fortifying food materials with micronutrients directly during the production process. A tailored fermentation process represents a powerful tool to enrich plant-based foods with vitamin B12, thereby preventing subclinical vitamin B12 deficiency in the future worldwide population. The choice of the microbes and fermentation processes need to be validated to ensure the production of the biologically active form of vitamin B12.

1.1 Chemical structure of vitamin B12

Vitamin B12 was initially identified in the, 1920s as a nutrient or an extrinsic factor that alleviated symptoms associated with pernicious anaemia by incorporating 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 using X-ray crystallography (Hodgkin et al., 1956). Their work revealed that the vitamin has a cyanide-bound, cobalt-containing, amidated tetrapyrrole structure, leading to its designation as cyanocobalamin. Vitamin B12 refers to a group of corrinoids characterised 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 (AdoCbl or coenzyme B12) and methylcobalamin (MeCbl). In contrast, the term vitamin B12 specifically denotes cyanocobalamin (CNCbl), which is the commercially produced form of vitamin B12 (Figure 14.1). The cyano group is a result of the extraction process used to isolate the compound from bacterial cultures. The vitamin B12 molecule can be 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 (DMBI); and an upper (beta) ligand derived from either an adenosyl group or a methyl group (Martens et al., 2002).

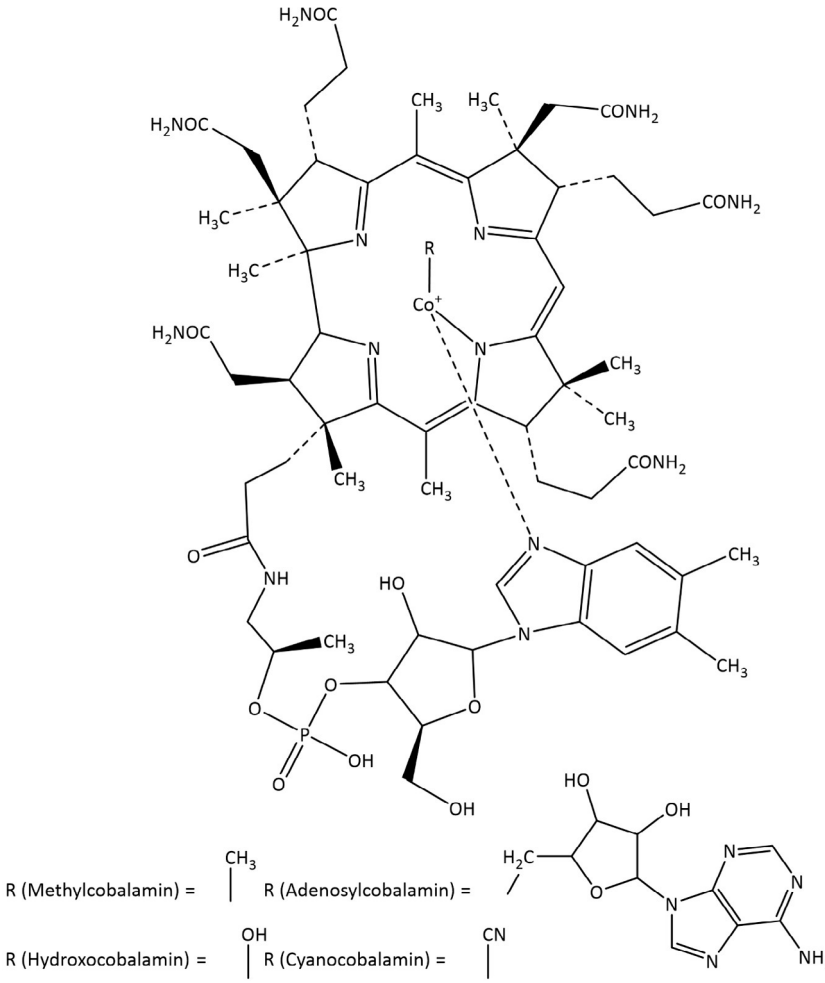

Figure 14.1. Structure of vitamin B12 as cyanocobalamin when R is CN. The primary biological forms of cobalamin have the cyano group replaced with an adenosyl, methyl or hydroxyl group, which are found in adenosylcobalamin, methylcobalamin and hydroxocobalamin, respectively. Modified from Smith et al., 2018.

In this vitamin, the cyano group represents the upper ligand, while DMBI is the lower ligand base. In biological systems, the upper cyano ligand can be substituted by an adenosyl group, resulting in AdoCbl, a methyl group leading to MeCbl, or by water, giving rise to hydroxocobalamin (OHCbl) (Smith et al., 2018). 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. AdoCbl is utilised 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). Some biological systems employ a different lower base to which the cobalt is bound; for instance, DMBI can be substituted with other bases, such as adenine, hydroxy or methoxy benzimidazole or phenolic compounds, including phenol or cresol (Hazra et al., 2013). Some LAB, for example *Levilactobacillus reuteri*, that were claimed to produce vitamin B12 turned out to produce pseudocobalamin with adenine as a lower ligand base instead of DMBI, as in vitamin B12 (Santos et al., 2007; Varmanen et al., 2016).

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

Chemical synthesis of vitamin B12 is technically challenging and expensive; therefore, commercial manufacturing of vitamin B12 is currently done via a biotechnological process, which involves using selected micro-organisms. Micro-organisms belonging to 20 genera are identified as B12-producing strains; however, due to the higher yields achieved, the industrial production via microbial fermentation mainly utilises strains belonging to species of *Propionibacterium freudenreichii*, *Bacillus megaterium* and *Pseudomonas denitrificans* (Hugenholtz & Smid, 2002). Following microbial fermentation, the 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 on the *in situ* production of vitamin B12 in cereal and other food matrices were conducted in the past decade, and as part of these, the use of *P. freudenreichii* strains was extensively investigated (Chamlagain et al., 2015, 2018, 2024; Edelmann et al., 2016; Wang et al., 2022; Xie et al., 2018, 2019, 2021). *P. freudenreichii* belongs to the propionic acid bacteria, which are mesophilic and aerotolerant Actinobacteria characterised by the ability to produce propionic acid as the main metabolic end product (Thierry et al., 2011). Although the yield achieved by using these strains in commercial vitamin B12 production is high, the *in situ* fortification of cereal food with vitamin B12 using these strains can be challenging.

1.3 Vitamin B12 analysis in fermented foods

Several studies have demonstrated the reliability of HPLC for vitamin B12 analysis in different food types. Furthermore, UPLC enhances the sensitivity and efficiency of vitamin detection (Chamlagain et al., 2015; Wang et al., 2021). Consequently, the AOAC has adopted a method that combines HPLC with immunoaffinity extraction as the recommended internationally recognised technique (Giménez & Martin, 2018). Additionally, MS combined with the HPLC allows the identification of the active form of vitamin B12, distinguishing it from inactive corrinoid compounds (Edelmann et al., 2019; Watanabe & Bito, 2018). MBA are also popular for vitamin B12 quantification. However, the test organism *Lactobacillus delbrueckii* can also use other analogues than active vitamin B12 for its growth. Thus, MBA may lead to an overestimation of vitamin B12 content in fermented samples containing corrinoid-like compounds (Chamlagain et al., 2015; Edelmann et al., 2019).

2. Methods of analysis

2.1 General principle

This chapter focuses on a UHPLC method of analysis, originally reported by Chamlagain et al. (2015, 2018), that is sensitive and can be adopted for analysis of plant-based food products when vitamin B12 is fortified *in situ*. The method includes an extraction step for converting natural active B12 vitamers to cyanocobalamin followed by a purification step using immunoaffinity chromatography. Vitamin B12 is separated and quantitated as cyanocobalamin with a UHPLC system. Further, the mass-spectrometric detection combined with a UHPLC separation can be used for identification and confirming structure of vitamin B12 and its analogues. If fermented samples are analysed for vitamin B12, MBA is not a recommended method. Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.2 Samples

The samples include fermented plant-based foods, both fortified and non-fortified. The analysis can be conducted on flour ingredients or freeze-dried samples. When analysing sourdough or other fermented samples, the moisture should be removed by freeze-drying prior to the analysis.

2.3 Chemicals, materials and reagents

- Sodium hydroxide, analytical grade
- Acetic acid, analytical grade
- Ethanol
- Acetonitrile, HPLC grade
- Trifluoroacetic acid
- Sodium cyanide
- Cyanocobalamin
- α-amylase (EC 232-588-1, A9857-5MU, *Aspergillus oryzae*)
- Certified reference material BCR 487 (lyophilised pig liver; Institute for Reference Materials and Measurements, Geel, Belgium; Sigma-Aldrich, Darmstadt, Germany)
- Milli-Q water
- Cyanocobalamin stock solution (200 µg/mL) prepared in 25% ethanol/Milli-Q water with the concentration measured by a spectrophotometer at 361 nm, following Indyk et al. (2002)
- Immunoaffinity column, Easi-extract® (RBiopharma, Glasgow, UK)
- Paper filters Ø 90 mm
- Syringe filters 0.45 µm
- Methanol
- Syringe filters 0.2 µm
- UPLC vials
- Waters Acquity UPLC system (Milford, MA, USA) equipped with a PDA (210–600 nm)
- Reversed-phase C18 column made of high-strength silica (HSS) T3 (Waters, Milford, MA, USA) (2.1 × 100 mm, 1.8 µm particles)
- Quadrupole time-of-flight (QTOF) mass spectrometer with an electrospray ionisation (Synapt G2-Si; Waters, Milford, MA, USA)
- Vitamin B12 assay medium, analytical grade (only in MBA)
- Tween 80 (only in MBA)

- 96 well microtiter plate (only in MBA)
- *L. delbrueckii* ATCC 7830 (only in MBA)
- Microplate reader (only in MBA)

2.4 Extraction

- Conduct all analytical processes under subdued light or protected from direct light.
- Weigh 1–2 g of cereal matrix or 0.5 g of freeze-dried sample (Wang et al., 2022) in extraction tubes in duplicate.
- Add 10–20 mL of buffer (8.3 mM sodium hydroxide and 20.7 mM acetic acid, pH 4.5) and 100 µL of 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 min.
- Cool the samples in an ice bath.
- If the 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 remaining pellet in 5 mL of extraction buffer, vortex, and centrifuge the mixture again (6,900 × *g*, 10 min).
- Filter the supernatants with paper filters and adjust the final volume to 20 or 25 mL with extraction buffer.

2.5 Immunoaffinity purification for UHPLC quantification

- Drain the buffer from the immunoaffinity column.
- Filter 5–20 mL (depending on vitamin B12 content) of the extract with 0.45 µm filters.
- Load the extract into column and let it eluate with the slow rate through the column, avoiding drying of the column.
- Wash the column with 10 mL of Milli-Q water and, finally, push air through the column with syringe to remove rest of water.
- Elute and collect vitamin B12 with 3 mL of methanol, and complete the elution with an additional 0.5 mL of methanol.
- Evaporate the eluate at 50 °C under a stream of nitrogen and reconstitute the residue with 300 µL of water, then syringe filter (0.2 µm) into a UPLC vial.

2.6 UHPLC-PDA

2.6.1 Instrumental settings

- Set the Waters UPLC system (Milford, MA, USA) equipped with a PDA (200–600 nm) and an Acquity HSS T3 C18 column (2.1 × 100 mm, 1.8 µm; Waters) for recording the chromatogram at 361 nm.
- Set the autosampler at 4 °C and the column temperature at 30 °C.
- Prepare the mobile phases consisting of 0.025% TFA in water (solvent A) and 0.025% TFA in acetonitrile (solvent B) and set the flow rate at 0.32 mL/min.
- Load the UPLC vials containing the sample into the sample holder.
- Set the injection volume of the sample to 2–15 µL and the autosampler in a partial-loop mode.
- Run the samples according to a gradient elution system shown in Table 14.1.
- Prepare an external calibration curve by injecting six cyanocobalamin standards (0.015–0.75 ng/µL) by using injection volumes of, e.g. 10–20 µL, in duplicate.

Table 14.1. A gradient system of UHPLC-PDA analysis for vitamin B12 (cyanocobalamin).

Time (min)	A (%)	B (%)	Temp. (°C)	Flow (mL/min)
0	95	5	30	0.32
0.5	95	5	30	0.32
5.0	60	40	30	0.32
6.0	60	40	30	0.32
10	95	5	30	0.32

2.6.2 Quantification

The identity of vitamin B12 is confirmed based on the retention time and UV spectrum of cyanocobalamin standard. Quantification of vitamin B12 (cyanocobalamin) is based on an external standard method using a multilevel calibration curve (e.g. 0.3–11 ng).

2.7 UHPLC-MS/MS analysis

- Prepare the mobile phase with 0.1% formic acid.
- Set the QTOF in a positive ion mode with a scanning *m/z* range of 50–1,500.
- Perform tandem mass spectrometry (MS/MS) for a specific m/z according to the target molecule, using helium as the collision gas, as per the instrumental settings detailed in Table 14.2.
- Identify the peaks and set the *m/z*, for example, CNCbl at 678.2 (doubly charged) and pseudo-cyanocobalamin at 672.2 (doubly charged). Calibrate the mass accuracy with a lock-spray mass correction standard (leucine-enkephalin; *m/z* 556.2771).

Table 14.2. Instrumental settings for UHPLC-MS/MS analysis.

Parameters	Set value
Capillary voltage	0.5 kV
Sampling cone voltage	40 V
Source off-set	80 V
Source temperature	150 °C
Desolvation temperature	600 °C
Desolvation gas flow	1,000 L/h
Nebuliser gas flow	6.5 bar
Cone gas flow	50 L/h
Trap collision energy ramp	15–90 eV
Trap gas flow	2 mL/min
Scan time	0.2 sec

2.8 Microbiological assay

The content of vitamin B12 in fermented samples can only be estimated using an MBA method. The MBA is performed using a 96-well microtiter plate, following Kelleher and Broin (1991). *L. delbrueckii* ATCC 7830 is used as the test organism, and cyanocobalamin is used as a calibrant. This method does not distinguish the active forms of vitamin B12 from the inactive forms. Therefore, the results need to be verified with the

UHPLC-PDA method and the vitamin forms need to be confirmed with the UHPLC-MS/MS method.

- After boiling, adjust the pH of the extract to 6.2 with NaOH before centrifugation and make up the volume to 25 mL with a pH 6.2 buffer.
- Dilute the sample extracts on the estimation of vitamin content at two levels with the extraction buffer (pH 6.2).
- Include a certified reference material, BCR 487 (pig liver) extract, as a reference sample in each set of samples, along with a blank sample.
- To create a calibration curve, inoculate 100 µL of cyanocobalamin standards of increasing concentration (0–8 pg/well).
- Inoculate sample extracts into the wells of the microtiter plate (4 wells for each concentration).
- Add 200 µL of freshly prepared sterile filtered vitamin B12 assay medium (pH 6.2) inoculated with the cryopreserved assay organism into each well.
- Incubate the plate under optimised conditions (35 °C for, 19 h).
- Measure turbidity with a microplate reader, at 595 nm.
- Generate a calibration curve of eight concentration levels against the turbidity values.
- Calculate the concentration of vitamin B12 in the samples.

3. Interpretation and analysis of results

The average retention time of cyanocobalamin is 3.5 ± 0.1 min. However, if pseudocobalamin is also present in a sample, pseudovitamin B12 (the cyano form of pseudocobalamin) eluates just before cyanocobalamin, at 3.4 ± 0.1 min (Figure 14.2A). Because a pseudovitamin B12 standard is not commercially available, the concentration of pseudovitamin B12 can be calculated using the same calibration curve obtained for quantifying the concentration of cyanocobalamin.

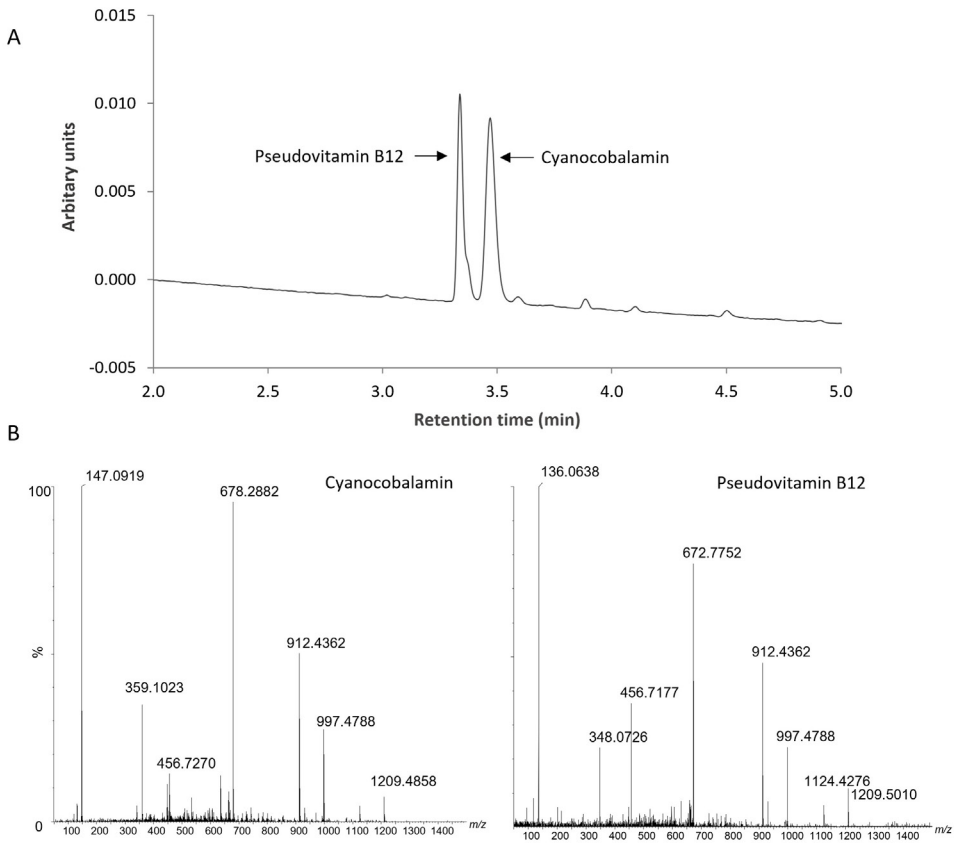

Figure 14.2. Example of a chromatogram showing (A) the cyanocobalamin and pseudovitamin B12 peaks obtained by running an immunoaffinity-purified extract of a fermented sample using UHPLC-PDA and (B) their corresponding mass spectra using UHPLC-MS/MS.

4. Other aspects

The volume of total extract (20 or 25 mL) and volume for purification can be controlled based on the estimated vitamin B12 content. Further, do not overload the immunoaffinity column with vitamin B12. The repeatability of the experiment can be checked by conducting two independent experiments on two different days.

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CHAPTER 15

Free fatty acid analysis in fermented cereal- and pulse-based matrices

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Summary

- Raw materials: Cereal and pulse ingredients (e.g. oat, faba bean, pea) in the form of flours or protein-rich fractions (e.g. protein concentrates, protein isolates)
- Relevant applications: Solid (meat, breads and other baked products), semi-solid (dairy products, plant-based yoghurt), and liquid (dairy products, plant-based milk) fermented foods
- Fermentation-induced changes: Release of free fatty acids into the food matrix through microbial lipase and lipoxygenase enzymatic activity
- Analytical methods:
  - Normal-phase high-performance liquid chromatography with evaporative light-scattering detection (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 cereal grains, FA are present in all parts, including in the membranes, spherosomes, starch and protein bodies (Chow, 2007). In pulse grains, lipids are located in the embryo axis and, in lower amounts, in the cotyledons and the seed coat. In cereal and pulse grains, as part of natural occurrences, lipid degradation plays a significant role in the release of free fatty acids (FFAs) that are more susceptible to chemical and enzymatic oxidation (Tiwari & Singh, 2012). Lipid oxidation occurs in three ways: autoxidation, enzymatic-catalysed oxidation and photo-oxidation. According to several studies included in the review by Shahidi and Oh (2020), Strecker degradation and Maillard reaction may involve lipids, resulting in the formation of multiple volatile compounds during food processing. Lipids significantly contribute to food flavour formation, especially in the presence of high amounts of unsaturated FA. In certain

foods, such as pulses (Tiwari & Singh, 2012) and meat (Shahidi, 2002), 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 flavour-active. In particular, aldehydes are highly reactive with other compounds, forming off-flavour molecules.

The enzymatic-catalysed oxidation of lipids is mainly due to the activity of lipid-modifying enzymes, such as lipase, lipoxygenase and peroxygenase (POX). Endogenous lipase activity leads to the release of FFAs (from their esters), which undergo oxidative reactions and the formation of hydroperoxides due to lipoxygenase (LOX) activity. POX leads to the formation of nonvolatile flavour molecules, known as epoxy and hydroxy FA, by degrading unsaturated FA in the 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-flavour release. Among the three lipid-modifying enzymes mentioned above, in oat, lipase and POX were the most active, while in faba bean, lipase and LOX were the most active. Oat-based raw kernels commonly undergo hydrothermal treatments, for instance kilning, to inactivate lipases and other enzymes. The hydrothermal treatment is necessary because of the high presence of polyunsaturated FA (shortened to PUFAs), e.g. linoleic and linolenic acids, which are easily oxidised (Lampi et al., 2015; Yang et al., 2023). Heat treatment is also considered effective for controlling LOX activity in legumes (Roland et al., 2017).

1.1 Chemical structure of free fatty acids

FA 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 structures of FA (Figure 15.1) (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.

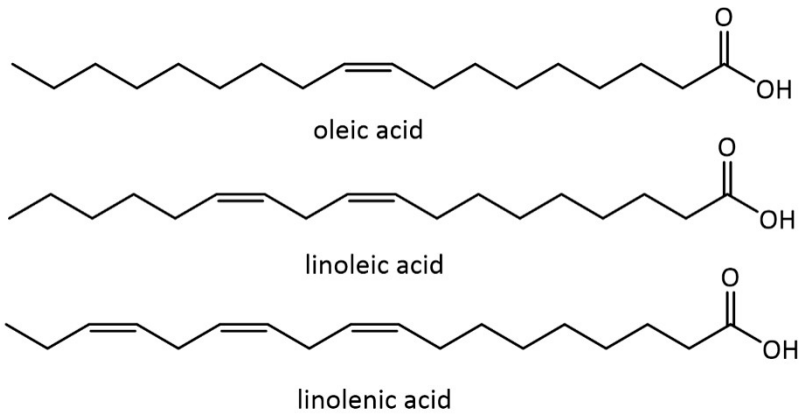

Figure 15.1. Structures of three free fatty acids with different unsaturation levels.

1.2 How can fermentation affect free fatty acids?

Fermentation plays a significant role in the development of the typical flavour in fermented food products. Microbial starters have the ability to synthetise lipases and phospholipases, which can release FFAs, which contribute to the formation of desirable flavour compounds. However, if the oxidation of lipids becomes excessive, the food product undergoes a decrease in nutritional and sensorial quality, resulting in spoilage. In contrast, some micro-organisms may be able to increase the antioxidant activity in the food matrix (Zhao et al., 2021). For instance, *Lactobacillus rhamnosus* showed a significant influence on lipid peroxidation inhibition in cereals (Đorđević et al., 2010) and led to the formation of antiradical compounds in soymilk (Marazza et al., 2012). Moreover, other studies found other micro-organisms with this ability, e.g. *Lactobacillus plantarum* in cowpea, *Cordyceps militaris* in chickpea, and *Streptomyces* sp., *Acetobacter* sp., *Lactobacillus* sp. and *Saccharomyces* sp. in soybean. In addition, decreasing pH has a marked effect on activities of lipid-modifying enzymes (Lampi et al., 2020). After the pH dropped below 7, no lipase activity was present in faba bean extracts.

2. Methods 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 is. Sourdoughs should be freeze-dried before analysis, whereas solid food models (e.g. meat alternatives) may be extracted as is or after freeze-drying, provided the lipid content is low.

2.2 General principle

The method for analysing (Figure 15.2) FFAs involves extracting and quantifying the FA 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 analysis coupled with an ELSD 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. After the sample cell has been sealed, the entire cell is placed in an extraction chamber. An inert gas, such as nitrogen, is used to pressurise 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 HPLC separates the FFAs based on their physical and chemical properties, such as MW 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 HPLC-ELSD analysis, the FFAs are eluted from the chromatographic column and

are carried into the ELSD detector. In the ELSD, the eluted FFAs are nebulised 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. Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

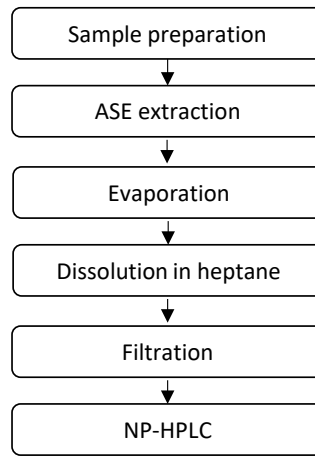

Figure 15.2. Schematic overview from sample preparation to NP-HPLC.

2.3 Reagents

- Ethanol
- Acetone
- Heptane
- 0.1% (v/v) acetic acid in heptane
- 3% (v/v) 2-propanol in heptane
- Oleic acid

2.4 Lipid extraction via ASE

Carry out the analysis in triplicate:

- Weigh the sample, ranging from 0.5–2 g of flour/freeze-dried sample, into an ASE cell (e.g. with ASE-200 instrument 11 mL extraction cell; Dionex, Sunnyvale, CA, USA) with a compatible cellulose filter at the bottom.
- Mix the sample with an equal volume of drying agent (e.g. Ottawa 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: 1,000 psi
    - Heating time: 5 min
    - Static cycle time: 10 min
    - Number of static cycles: 2
    - Flush volume: 60%
    - Purge time: 60 sec
    - Rinsing time: 1 min

2.5 Preparation for HPLC-ELSD analysis

- Carefully transfer eah extract from the ASE vials to a round-bottomed flask using ethanol. Ensure thorough rinsing to maximise analyte transfer and minimise loss.
- Evaporate the lipid solution using a rotary evaporator at a temperature below 37 °C.
- Redissolve the resulting lipid residue in approx. 2 mL of heptane.
- Transfer the re-dissolved lipid solution to a 10 mL volumetric flask with a Pasteur pipette. Rinse the round-bottomed flask with heptane

multiple times to ensure no residue remains and then fill the flask to the mark.
- Filter the extract, with e.g. a heptane-tolerant 0.45 µm syringe filter, into an HPLC vial.

2.6 NP-HPLC-ELSD

Separation is achieved as described by Yang et al. (2019) and Tuccillo et al. (2022), using a NP-HPLC method (Agilent 1200 HPLC system; Agilent Technologies, Santa Clara, CA, USA); ELSD detection (Waters 2420 ELSD; Waters, Milford, MA, USA); and with a column suitable for polar and slightly hydrophobic interactions (e.g. LiChrosorb, 5 µm, 3 × 100 mm, VDS; Optilab Chromatogaphie Technik GmbH, Berlin, Germany). Separation is done with eluent flow of 0.5 mL/min at 30 °C. Use the following gradient elution with solvents A (0.1% (v/v) of acetic acid in heptane) and B (0.1% (v/v) acetic acid, 3% (v/v) 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 polynomial (second-order) standard curve using oleic acid at 200-4,000 ng/injection. FFA content is given as mg/g.

3. Other aspects

Lipids can also be extracted with appropriate solvents, such as mixtures of chloroform and methanol or of heptane/hexane and isopropanol. Nowadays, UHPLC can be used instead of HPLC to separate lipid classes. There are other NP-HPLC-ELSD methods to analyse FFAs and other lipid classes, and one of them was published by Melis et al. (2021). It should be noted that ELSD responses vary with MW and unsaturation of lipids. Lipids used for calibration should be carefully selected.

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SECTION 5

Fermentation metabolites and intermediates

CHAPTER 16

Quantitative analysis of organic acids in fermented food matrices

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Summary

- Raw materials: 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 possess only 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 micro-organisms present can produce and consume a variety of organic acids (De Vuyst et al., 2021). Yeasts are also able to produce organic acids. Concerning LAB, a distinction must 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. Last, acetic acid bacteria can produce acetic acid and gluconic acid.

2. Method of analysis

Within this section, a method, based on De Bruyn et al. (2017), for the analysis of organic acid concentrations using UPLC coupled to tandem mass spectrometry (UPLC-MS/MS) is described. The method uses an Acquity system equipped with an HSS T3 column (length: 150 mm; inner diameter: 2.1 mm; particle size: 1.8 µm; pore size: 100Å; Waters, Milford, MA, USA) for compound separation. Detection is performed using a triple-quadrupole (TQ) tandem mass spectrometer with a ZSpray electrospray ionisation source operating in the negative ionisation mode (Waters).

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 (Figure 16.1) (Zhang et al., 2019).

Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

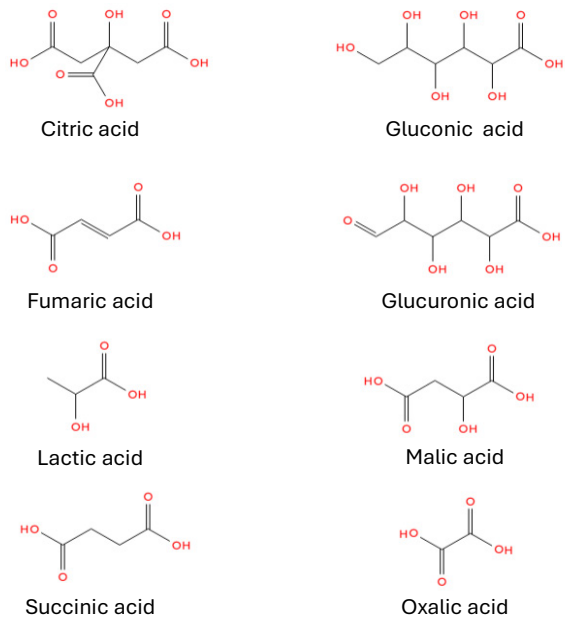

Figure 16.1. Chemical structures of the organic acids that can be quantified via UPLC-MS/MS.

2.1 Sample preprocessing

The organic acids to be measured can come from both liquid and solid matrices, and these matrices each require different handling of the sample.

For a liquid matrix, such as a liquid fermentation broth:
- Centrifuge the sample at 5,000 × *g* for 20 min.
- Transfer the supernatant to a new container for use in the further sample preparation.
- If the analysis is not to be performed immediately, freeze the supernatant at –20 °C.

For a solid matrix, such as a sourdough sample:
- Prepare a water extract by solid sample with ultrapure water (Milli-Q; Merck, Darmstadt, Germany) in a 1:5 ratio.
- Mix the diluted sample using a rotary mixer at 40 rpm for 15 min.
- Centrifuge the mixture at 5,000 × *g* for 20 min and transfer the supernatant to a new container for use in the further sample preparation.
- If the analysis is not to be performed immediately, freeze the supernatant at –20 °C.

2.2 Internal standard solution preparation

- Weigh 20 mg of salicylic acid into a 1 L bottle.
- Add 500 mL of ultrapure water (using a volumetric flask).
- Add 500 mL of methanol (using a volumetric flask).
- Mix well until all the salicylic acid is dissolved.

2.3 External standard preparation

- Weigh the appropriate amounts of pure compound for the compounds to be measured. Typically, a concentration of 2 g/L is aimed for in the stock solution except in the case of lactic acid, for which 20 g/L is used. These concentrations can be varied depending on the concentrations of the compounds present in the sample.
- Add ultrapure water in the appropriate amount to reach the above-described concentrations.

- Mix until all the compounds are fully dissolved.
- Prepare a dilution series of 12 dilutions using ultrapure water to serve as calibration points.
  - Dilution 1 = stock; Dilution 2 = 1:1 dilution of the stock; Dilution 3 = 1:1 dilution of dilution 2; …
- These dilutions will be further processed like the samples, as described below.

2.4 Sample preparation

All samples and external standards should be made in triplicate.

- Add 40 µL of sample to 760 µL of the IS solution.
- Vortex the sample for 2 min.
- Centrifuge at, 19,400 × *g* for 15 min at 10 °C.
- Filter the samples using a 0.2 µm polytetrafluoroethylene filter (Millex; Merck, Darmstadt, Germany) into capped chromatography vials (polypropylene screw neck vial N9 with an inner cone and screw closure N9 without slit; Macherey-Nagel, Düren, Germany).

2.5 UPLC-MS/MS run setup

- Run one series of external standards alternately with one series of samples to account for signal changes during the run.
- Parameters:
  - Flow rate: 0.3 mL/min
  - Injection volume: 10 µL
  - Eluents:
    - A: ultrapure water (Milli-Q; Merck) with 0.2% (v/v) formic acid (Merck)
    - B: mixture of methanol (Merck) and water (95:5) with 0.2% (v/v) formic acid (Merck)
  - Ionisation source should be used in the negative mode.
  - 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.

2.6 Quantification

- Manually check the automatic peak integrations.
- Use the IS to normalise the peak areas.
- Use the external standards to produce a standard curve for each compound.
- Determine the concentrations of each compound in each triplicate of the samples.
- Reduce the triplicate information to a single value by taking the average of the three concentrations per compound per sample.

3. References

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CHAPTER 17

Quantitative analysis of biogenic amines in fermented food matrices

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Summary

- Raw materials: 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 (Barbieri et al., 2019; Biji et al., 2016). These compounds are regularly found in food products that have a high protein content, such as fish, meat, dairy and other fermented food products (Barbieri et al., 2019; Biji et al., 2016; Decadt & De Vuyst, 2023). BA can be produced by a range of different micro-organisms, including enterobacteria, LAB and coagulase-negative staphylococci. The latter two microbial groups are often present in fermented food products, such as cheese and fermented sausages (Decadt et al., 2023; Van der Veken et al., 2020).

The presence of BAs in food products is generally unwanted, as some biogenic amines have a toxic nature (Benkerroum, 2016). Some BA, such as histamine, tyramine and β-phenylethylamine, exert a vasoactive effect on the human body, presenting intoxication issues when those BA are consumed in excessive amounts. Other BA, 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 to the quality of the food product (Van der Veken & Leroy, 2022).

2. Method of analysis

BA can be determined by UPLC-MS/MS (Van der Veken et al., 2020). This can be done using an Acquity UPLC system equipped with an HSS T3 column (length: 150 mm; inner diameter: 2.1 mm; particle size: 1.8 µm; pore size: 100Å) for compound separation (Waters, Milford, MA, USA). Detection can be performed using a TQ tandem mass spectrometer with a ZSpray electrospray ionisation 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 and tyramine (Figure 17.1) (Van der Veken et al., 2020). The following protocol is based on the method of Van der Veken et al. (2020).

Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

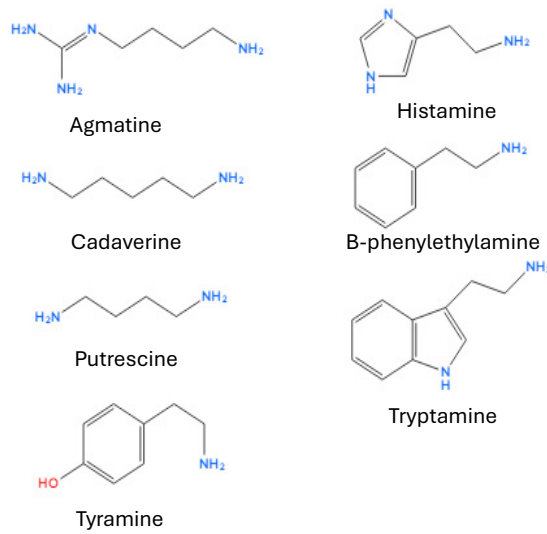

Figure 17.1: Chemical structures of the biogenic amines that can be quantified via UPLC-MS/MS.

2.1 Sample preprocessing

The BA to be measured can come from both liquid and solid matrices, and these matrices each require different handling of the sample.

For a liquid matrix, such as a liquid fermentation broth:

- Centrifuge the sample at 5,000 × *g* for 20 min at 4 °C.
- Transfer the supernatant to a new container for use in further sample preparation.
- If the analysis is not immediately performed, freeze the supernatant at –20 °C.

For a solid matrix, such as a sourdough sample:

- Prepare a water extract by adding together the solid sample and ultrapure water (Milli-Q; Merck, Darmstadt, Germany) in a 1:5 ratio.
- Mix the diluted sample using a rotary mixer at 40 rpm for 15 min.
- Centrifuge the mixture at 5,000 × *g* for 20 min at 4 °C and transfer the supernatant to a new container for use in the further sample preparation.
- If the analysis is not immediately performed, freeze the supernatant at –20 °C.

2.2 External standard preparation

- Weigh the appropriate amounts of pure compound for the compounds to be measured. Typical concentrations used for the stock solution are shown in Table 17.1. These concentrations can be varied depending on the concentrations of the compounds present in the sample.

Table 17.1. Typical concentrations of pure compounds in the external standard stock solution.

Histamine	4.6 mg/L
Tyramine	5 mg/L
Tryptamine	5 mg/L
Putrescine	5 mg/L
Cadaverine	6.5 mg/L
β-phenylethylamine	5 mg/L
Agmatine	4.4 mg/L

- Add ultrapure water in the appropriate amount to reach the above-described concentrations.
- Mix until all the compounds are fully dissolved.
- Prepare a dilution series of 12 dilutions using ultrapure water, to serve as calibration points.
  - Dilution 1 = stock; Dilution 2 = 1:1 dilution of the stock; Dilution 3 = 1:1 dilution of dilution 2; …
- These dilutions will be further processed like the samples, as described below.

2.3 Sample preparation

All samples and external standards should be made in triplicate.

- Add 250 µL of sample to 250 µL of acetonitrile containing 0.15% (v/v) of heptafluorobutyric acid (HFBA, ≥ 99%; Merck, Darmstadt, Germany) to precipitate all present proteins.
- Vortex the sample for 2 min.
- Centrifuge at 13,000 × *g* for 15 min at 4 °C.
- Filter the samples using a 0.2 µm polytetrafluoroethylene filter (Millex; Merck) into capped chromatography vials (polypropylene screw neck vial N9 with an inner cone and screw closure N9 without slit; Macherey-Nagel, Düren, Germany).

2.4 UPLC-MS/MS run setup

- Run alternately one series of external standards and one series of samples to allow taking into account signal changes during the run.
- Parameters:
  - Injection volume: 2 µL
  - Flow rate: 0.23 mL/ min
  - Eluents:
    - A: ultrapure water–acetonitrile mixture (95:5, v/v) with 0.1% (v/v) HFBA as an ion-pairing reagent
    - B: ultrapure water–acetonitrile mixture (5:95, v/v) with 0.1% (v/v) HFBA
  - The elution gradient is as follows: 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 SRM method was optimised via IntelliStart (Waters, Milford, MA, USA), 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.

2.5 Quantification

- Manually check the automatic peak integrations.
- Use the external standards to produce a standard curve for each compound.
- Determine the concentrations of each compound in each triplicate of the samples.
- Reduce the triplicate information to a single value by taking the average of the three concentrations per compound per sample.

3. References

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CHAPTER 18

Quantitative analysis of ethanol in fermented food matrices

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Summary

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

1. Introduction

Within a fermented food context, ethanol (Figure 18.1) is mostly produced by yeasts. through the glycolysis and subsequent pyruvate metabolism as a way to produce adenosine triphosphate (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 micro-organisms that produce ethanol in fermented food matrices is heterofermentative LAB, although they produce not just ethanol but, rather, a mixture of ethanol, lactic acid, acetic acid and/or formic acid as the end products of their carbohydrate metabolism.

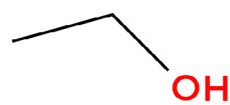

Figure 18.1: Chemical structure of ethanol.

2. Method of analysis

Ethanol concentrations can be determined by GC-FID (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).

Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.1 Sample preprocessing

The ethanol-containing samples can come from both liquid and solid matrices, and these matrices each require different handling of the sample.

For a liquid matrix, such as a liquid fermentation broth:

- Centrifuge the sample at 5,000 × *g* for 20 min.
- Transfer the supernatant to a new container for use in the further sample preparation.
- If the analysis is not immediately performed, freeze the supernatant at –20 °C.

For a solid matrix, such as a sourdough sample:

- Prepare a water extract by combining the solid sample with ultrapure water (Milli-Q; Merck, Darmstadt, Germany) in a 1:5 ratio.
- Mix the diluted sample using a rotary mixer at 40 rpm for 15 min.
- Centrifuge the mixture at 5,000 × *g* for 20 min and transfer the supernatant to a new container for use in the further sample preparation.
- If the analysis is not immediately performed, freeze the supernatant at –20 °C.

2.2 Internal standard solution preparation

- Prepare a bottle of acetonitrile (Merck, Darmstadt, Germany) and add 1% (v/v) of formic acid (Merck) to it.
- Add 0.2% (v/v) of 1-butanol (Merck) as an IS.
- Mix well to obtain a homogeneous mixture.

2.3 External standard preparation

- Prepare a solution of ultrapure water containing 20 g/L of ethanol.
- Mix well.
- Prepare a dilution series of 10 dilutions using ultrapure water to serve as calibration points.
  - Dilution 1 = stock; Dilution 2 = 1:1 dilution of the stock; Dilution 3 = 1:1 dilution of dilution 2; …
- These dilutions will be further processed like the samples, as described below.

2.4 Sample preparation

All samples and external standards should be made in triplicate.

- Add together the sample and the IS solution in a 1:4 ratio.
- Vortex the sample for 2 min.
- Centrifuge at, 19,400 × *g* for 15 min at 4 °C.
- Filter the samples using a 0.2 µm polytetrafluoroethylene filter (Millex; Merck, Darmstadt, Germany) into capped chromatography vials (glass screwcap vial N9 with a flat bottom and screwcaps N9 without slit; Macherey-Nagel, Düren, Germany).

2.5 GC-FID run setup

- Run alternately one series of external standards and one series of samples to allow taking signal changes during the run into account.
- Parameters:
  - Injection volume: 1 µL
  - Split flow with a 40:1 split ratio
  - Carrier gas: hydrogen gas (Nippon Gases, Madrid, Spain) at a constant flow rate of 1 mL/min

- Make-up gas: nitrogen gas (Nippon Gases)
- The injector and detector temperatures were set at 240 °C and 250 °C, respectively. The column temperature programme was 0 min, 40 °C; 10 min, 140 °C; 12 min, 230 °C; and 22 min, 230 °C.

2.6 Quantification

- Manually check the automatic peak integrations.
- Use the IS to normalise the peak areas.
- Use the external standards to produce a standard curve for each compound.
- Determine the concentrations of each compound in each triplicate of the samples.
- Reduce the triplicate information to a single value by taking the average of the three concentrations per compound per sample.

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CHAPTER 19

Quantitative analysis of sugar alcohols in fermented food matrices

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Summary

- Raw materials: 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)

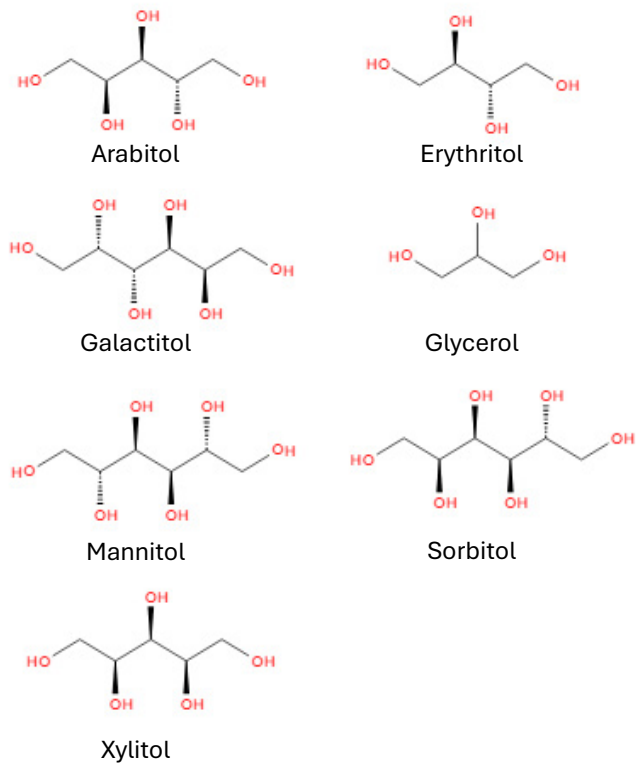
1. Introduction

Sugar alcohols, also called polyols (the P in FODMAPs, with undesired health-related consequences for some consumers), are carbohydrate derivatives in which the aldehyde or keto group has been substituted with a hydroxyl group (Bieleski, 1982; Grembecka, 2015). These polyols can be found naturally in small quantities in vegetables, fruits and mushrooms (Grembecka, 2015). Furthermore, sugar alcohols are produced by micro-organisms fulfilling different functions, such as regeneration of cofactors (glycerol in yeasts and mannitol and erythritol in LAB) and acting as osmoregulatory compounds (glycerol in yeast) (De Vuyst et al., 2017, 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 concentrations can be determined by 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 (Figure, 19.1). The following protocol is based on the method of Comasio et al. (2019).

Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.



Figure, 19.1 Chemical structures of the sugar alcohols that can be quantified via HPAEC-PAD.

2.1 Sample preprocessing

The sugar alcohols to be measured can come from both liquid and solid matrices, and these matrices each require different handling of the sample.

For a liquid matrix, such as a liquid fermentation broth:
- Centrifuge the sample at 5,000 × *g* for 20 min.
- Transfer the supernatant to a new container for use in the further sample preparation.
- If the analysis is not immediately performed, freeze the supernatant at –20 °C.

For a solid matrix, such as a sourdough sample:
- Prepare a water extract by solid sample with ultrapure water (Milli-Q; Merck, Darmstadt, Germany) in a 1:5 ratio.
- Mix the diluted sample using a rotary mixer at 40 rpm for 15 min.
- Centrifuge the mixture at 5,000 × *g* for 20 min and transfer the supernatant to a new container for use in the further sample preparation.
- If the analysis is not immediately performed, freeze the supernatant at –20 °C.

2.2 Internal standard solution preparation

- Weigh 50 mg of salicylic acid into a 1 L bottle.
- Add 500 mL of ultrapure water (using a volumetric flask).
- Add 500 mL of acetonitrile (using a volumetric flask).
- Mix well until all the salicylic acid is dissolved.

2.3 External standard preparation

- Weigh the appropriate amounts of pure compound for the compounds to be measured. Typically, a concentration of 1 g/L is aimed for in the stock solution. These concentrations can be varied depending on the concentrations of the compounds present in the sample.
- Add ultrapure water in the appropriate amount to reach the above-described concentrations.
- Muntil all the compounds are fully dissolved.

- Prepare a dilution series of 12 dilutions using ultrapure water to serve as calibration points.
  - Dilution 1 = stock; Dilution 2 = 1:1 dilution of the stock; Dilution 3 = 1:1 dilution of dilution 2; …
- These dilutions will be further processed like the samples, as described below.

2.4 Sample preparation

All samples and external standards should be made in triplicate.

- Add 100 µL of sample to 900 µL of the IS solution.
- Vortex the sample for 5 min.
- Centrifuge at, 19,400 × *g* for 15 min at 10 °C.
- Filter the samples using a 0.2 µm polytetrafluoroethylene filter (Millex, Merck, Darmstadt, Germany) into capped chromatography vials (polypropylene screwcap vial N9 with an inner cone and screwcap N9 with slit; Macherey-Nagel, Düren, Germany).

2.5 HPAEC-PAD run setup

- Run alternately one series of external standards and one series of samples to allow taking signal changes during the run into account.
- Parameters:
  - Injection volume: 10 µL
  - Column temperature: 30 °C
  - Eluents:
    - A: Ultrapure water
    - B: 500 mM NaOH solution
  - Flow rate: 0.4 mL/min
  - The following elution 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% B; 45.0 min, 0% A and 100% B; 45.5 min, 50% A and 50% B; and 55.0 min, 50% A and 50% B.

2.6 Quantification

- Manually check the automatic peak integrations.
- Use the IS to normalise the peak areas.
- Use the external standards to produce a standard curve for each compound.
- Determine the concentrations of each compound in each triplicate of the samples.
- Reduce the triplicate information to a single value by taking the average of the three concentrations per compound per sample.

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CHAPTER 20

Analysis of volatile compounds in (fermented) cereal- and pulse-based materials

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Summary

- Raw materials: 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:
  - Headspace solid-phase microextraction coupled with gas chromatographic separation and mass spectrophotometric detection (HS-SPME-GC-MS)

1. Introduction

1.1 What are volatile compounds?

Flavour is an important characteristic of food, and volatile compounds play a significant role in flavour (Wang et al., 2022). Plant raw materials contain a wide array of flavour-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 flavour of foods. Others, instead, are considered off-flavours. Aldehydes, alcohols, ketones, organic acids, pyrazines and sulphur compounds are the main groups responsible for the off-flavours of pulses (Rackis et al., 1979). Many of these compounds are generated during the oxidation of lipids, and they contribute to the development of grassy, beany and earthy flavours. Hexanal (Figure 20.1) is one instance; it is an odour-active monocarbonyl compound associated with the presence of aroma defects in food (Belitz et al., 2009).

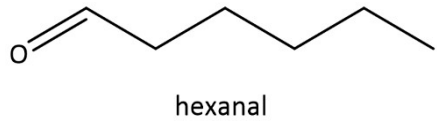

Figure 20.1. Chemical structure of hexanal.

The choice of raw materials can significantly impact the volatile profile of the food products. In comparisons of cereals and pulses as raw materials, for instance, variations in volatile compounds can be observed due to inherent differences in their biochemical composition (Karolkowski et al., 2021). 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 flavour of the final food products (e.g. bread, meat alternatives 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 flavour 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 flavours 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 micro-organismsI, such as bacteria, yeasts and moulds. They utilise carbohydrates, proteins and lipids as their sources of energy. They convert organic compounds into different degradation products, including volatile compounds (Pétel et al., 2017). The specific volatile compounds generated during fermentation are influenced by such factors as the type of micro-organisms 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 micro-organisms can produce volatile compounds that contribute to the unpleasant odours and off-flavours associated with food deterioration (Singh et al., 2004).

For instance, certain bacteria can produce volatile sulphur 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 odours (Bulushi et al., 2009). On the other hand, other micro-organisms 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-flavours (Verma et al., 2022). Remarkably, the presence of LAB has been found to induce significant modifications in the volatile profile of plant-protein ingredients, resulting in perceptible sensory enhancements (Arteaga et al., 2021; El Youssef et al., 2020; Leroy & De Vuyst, 2004; Wang et al., 2022).

2. Methods 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 (e.g. 2.0 g) into 20 mL amber solid-phase microextraction (SPME) vials. These vials are selected to ensure sample stability and minimise potential interactions with light. The sample amount should be weighed exactly, up to two decimals. 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 optimise efficiency, it is recommended to organise the sample order so that samples of the same replicate are analysed consecutively.

2.2 General principle

The volatile analysis method described here utilizes HS-SPME GC-MS following Tuccillo et al. (2022). This technique involves extracting volatile

compounds from the sample's headspace using a solid-phase microextraction fibre, followed by their separation and identification using GC-MS. This method leverages the unique property of volatile compounds to evaporate into the headspace, allowing their extraction using a solid-phase microextraction fibre. This fibre acts as a sorbent, capturing the volatile compounds in its coating. Subsequently, the volatile compounds are introduced into a 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 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 of a wide range of volatile compounds, from simple aliphatic hydrocarbons to complex aromatic compounds. Without using a standard for each compound and addition of an IS, the results from MS are only semiquantitative. However, comparison of relative peak areas of compounds can be applied in quantification.

Note that the method described below involves the use of specific columns and pieces of analytical equipment. These represent the conditions used by the authors. Of course, it is possible to perform a similar analysis with other systems, although this would require some optimisation, which is not included within the scope of this chapter.

2.3 Equipment and reagents

Gather the necessary equipment and reagents:

- HS-SPME injector (combiPAL; CTC Analytics. Zwingen, Switzerland) with a 1 cm divinylbenzene/carboxen/polydimethylsiloxane fibre (DVB/CAR/PDMS) with 50/30 µm film thickness (Sigma-Aldrich, Darmstadt, Germany)
- GC (HP 6890 series; Agilent Technologies Inc., Santa Clara, CA, USA), mass spectrometer (HP 6890 series, Agilent Technologies Inc.) equipped with a mid-polar/polar column (e.g. SPB-624, 30 m × 0.25 mm i.d., 1.4 µm film thickness; Supelco, Bellefonte, PA, USA)
- 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; Sigma-Aldrich)
- Mass spectra database for compound identification

2.4 HS-SPME-GC-MS

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 min
- Extraction time: 30 min with agitation (250 rpm)
- Incubation and extraction temperature: 50 °C
- Desorption temperature and time: 250 °C, 10 min
- 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 ionisation mode at 70 eV with ion source temperature at 230 °C and quadrupole temperature at 250 °C

2.5 HS-SPME-GC-MS of the alkane 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.

2.6 Identification and calculations

In the identification process, compounds are typically manually integrated to account for the potential impact of even small peaks on flavour perception. The mass spectra obtained from the analysis are compared with 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 utilised 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, tentative identifications can be made. Moreover, retention indexes (RI) can be employed as 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 with the RI values of the reference compounds to aid in compound identification. Quantification is primarily based on total ion responses from MS. Results are usually expressed as relative peak area (%) of all integrated peaks for each measurement. However, when an IS and standards for the analytes are available, quantitative results can be obtained.

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