Metabolomics and sensory studies to uncover asparagus flavour

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Metabolomics and sensory studies to uncover asparagus flavour

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Feel the colours of the flavours. Waste nothing. Explore.

Στο αστεράκι μου και στον τσακερδόνη μου.
(to my little star and my grandpa)
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Chapter 1

General Introduction
1.1. Asparagus crop: the harvest and the waste-products

*Asparagus officinalis* (asparagus) is just one and the most well-known of ca. 300 asparagus species in *Asparagaceae* family (Byng et al., 2016). Asparagus is a perennial crop which is widely appreciated for its high nutritional value and the distinctive flavour of its young shoots (spears). Asparagus fields are easily spotted in the south of the Netherlands thanks to the numerous long lines of furrows and soil ridges usually covered with a black or white plastic cover in spring and summer (Figure 1.1). These ridges are the so-called ‘asparagus beds’. The name ‘asparagus’ derives from the Greek word ‘asparagos’ which comes from the Proto-Indo-European word meaning ‘to spring up’ (source: vocabulary.com). This is exactly what happens with the spears which, under typical spring conditions, can grow more than 5-10 cm / day.

Figure 1.1 Asparagus fields in south of the Netherlands. Soil ridges (asparagus beds) are covered with an opaque plastic cover (left) to avoid exposure of the white spears to sunlight. An additional plastic cover is used in the beginning of the season to cover the asparagus beds and protect the spears from possible low temperatures (right). Pictures were taken in 2019 (left in May and right in March) in Helden, the Netherlands.

During the first months of spring, spears start to develop from the root crown of the plant and from then onwards harvesting can continue for approximately two months. Harvesting is often done manually (Figure 1.2) which is very laborious especially under full sunlight and when more than one field of approximately 5 hectares each are to be harvested. Only recently, asparagus robot harvesting machines which have been under development for many years, have been tested with positive results. This sets a new era for asparagus growers as more efficient, faster and standardized harvesting means less loss due to for instance wrong spear handling, and thus higher harvest yields.
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Figure 1. 2 Manual harvesting of white asparagus spears using a dedicated tool. Soil is removed around the emerging spear (left) and a harvesting tool enters the soil at a ca. 45° angle (middle). Spear is cut and carefully picked by hand (right). Pictures were taken in May 2019, in Helden, the Netherlands.

The delicate young asparagus spears are consumed in either green or white form. The key causal difference between the green and white forms is exposure to sunlight. For the cultivation of white spears, asparagus beds are 40 cm high and covered by opaque plastic covers which are used to exclude all light before harvesting (Figure 1. 1). For green asparagus, the beds are less high and spears grow in full sunlight until harvesting (Pegiou et al., 2020). Consumers characterize green asparagus as being more bitter and fresh than white spears which are considered as being slightly sweeter with a more subtle taste than green ones (Cuppert et al., 1997).

The growth and harvest season of asparagus lasts four months during spring and early summer although the harvest period for individual fields is usually not more than eight weeks. Consuming fresh asparagus is a culinary luxury which also explains the relatively high price, especially at the beginning of the season. Due to the perishability of the delicate spears and their tender tips, extra post-harvest care is needed particularly for white asparagus which is mainly cultivated in the Netherlands and is the form that is mainly focused upon in this thesis. Harvested white spears are washed and stored in clean cold water in darkness until the next day when they are manually classified for packaging and distribution. The market and commercial quality standards sets minimum requirements for classifying
asparagus spears to Class AA (superior quality or 'Extra Class'), A, or B (Table 1.1) (CXC 225-2001, 2013; United Nations Economic Commission for Europe, 2017). Interestingly there are no quality standards referring to the flavour of the crop, which one would say is more relevant for the consumer. Spears that are not graded into any of the classes are discarded as ‘waste’ (rejects). Another source of asparagus waste is the basal spear parts which are cut off to produce 22-cm spears and are used as a compost additive. The cut-offs together with all the ‘rejects’ sum up to a significant waste volume of ca. 30 % of the harvested white asparagus per season (J. Wang et al., 2013). This waste stream is currently either used for animal feed or compost. However, there may be potential to exploit this waste stream and have it further processed into a dried powder for use as a food ingredient for human consumption. This is the central underlying hypothesis of the work presented in this thesis.

### Table 1.1 Summary of the required characteristics for grading asparagus spears in Class AA, A, or B according to CODEX Standards for Asparagus (CODEX STAN 225-2001) published in 2013 and reviewed in 2019

<table>
<thead>
<tr>
<th>Class</th>
<th>Minimum diameter</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA / ‘Extra’</td>
<td>For white: 12 mm</td>
<td>Tips must be very compact.</td>
</tr>
<tr>
<td></td>
<td>For green: 3 mm</td>
<td>Base cut must be squared.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spears must be straight and well-formed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No woody parts allowed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For white: tips and shoots must be white.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For green: 95 % of length must be green.</td>
</tr>
<tr>
<td>A / I</td>
<td>For white: 10 mm</td>
<td>Tips must be compact.</td>
</tr>
<tr>
<td></td>
<td>For green: 3 mm</td>
<td>Base cut must be squared.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spears may be slightly curved.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For white: faint pink colour allowed; no woody parts allowed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For green: 80 % of length must be green; some woody parts allowed.</td>
</tr>
<tr>
<td>B / II</td>
<td>For white: 8 mm</td>
<td>Tips may be slightly open.</td>
</tr>
<tr>
<td></td>
<td>For green: 3 mm</td>
<td>Base cut may be slightly oblique.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spears may be curved.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Woody parts allowed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For white: tips may be coloured.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For green: 60 % of length should be green.</td>
</tr>
</tbody>
</table>

1The diameter of the shoots shall be measured 2.5 cm from the cut end.

1.2. Upcycling asparagus waste-products to powders

There is a great need to extract maximum value from our limited resources and hence certain main objectives within the food industry during the first half of the 21st century are to reduce
General Introduction

the use of critical natural resources, to extend the shelf-life of products and importantly to recover possible by-products from so called ‘waste’ (Jurgilevich et al., 2016). Food production must become as sustainable as possible. Waste must be minimized and any waste-products may become the starting point for the development of parallel pipelines for generating alternative products or ingredients of equal or higher value. Several research studies focus on specifically valorising vegetable waste-products in an efficient way for the production of natural food ingredients (Sabater et al., 2020; Sharma et al., 2021). A potential way to exploit vegetable waste streams is to dry them and generate powders for use as food ingredients. Currently, the methodologies applied aim for low cost and minimum energy use thus oven drying is usually applied (Santos et al., 2018). Although relatively inexpensive, oven-dried vegetable powders are characterized by little or no flavour and this is due to the loss of important aroma compounds (Nijhuis et al., 1998). To compensate for these ‘losses’, supplementary flavour components are often added to currently available commercial oven-dried vegetable powders. However, lately consumers are consciously choosing more for products containing ‘natural ingredients’ against those with ‘added flavour supplements’ (Bearth et al., 2014). Moreover, there is the EU Regulation (EC) No 1334/2008 which sets specific rules for labelling of food products regarding flavourings, and this eventually puts high pressure on the food industry to produce flavoursome natural ingredients (European Commission, 2008), thus without flavour supplements.

Alternative methods to oven (hot air) drying are vacuum drying, freeze drying and spray drying which are used in the food industry to preserve fruit and vegetable juices (Jafari et al., 2008; Karam et al., 2016; Shishir & Chen, 2017). Energy consumption during spray drying is lower compared to vacuum and freeze drying (Eijkelboom, 2020). Moreover spray drying is a rapid process which leads to encapsulation of flavour compounds and thus minimising potential heat-induced degradation of compounds present in the material (Jafari et al., 2008). This makes spray drying a promising candidate for drying asparagus waste-streams into powders which are hypothesised to have improved physical properties and flavour attributes compared to the available commercial hot air dried products (J. Siccama et al., 2019).

Spray drying requires a liquid starting material which is sprayed in droplets into a drying chamber. There, water from the surface of each droplet evaporates within a few seconds and
a semipermeable skin is formed. Because of this, flavour compounds are encapsulated and protected from the high temperatures of the chamber until further dried to powder particles (Coumans et al., 1994). The liquid starting material is first mixed with a carrier agent which assists in encapsulation and for this, maltodextrin is often used (Goubet et al., 1998; Jafari et al., 2008). As liquid starting material for spray drying asparagus, concentrated juice (concentrate) was used. Asparagus concentrate was produced using a split-stream processing strategy (Figure 1.3) where asparagus ‘waste’ material is pressed to separate the juice and fibre fractions (Figure 1.3A). The juice is then concentrated (Figure 1.3B) and the concentrate is spray-dried using maltodextrin as a carrier (Figure 1.3C). The hypothesized enhanced flavour of spray-dried compared to oven-dried asparagus is investigated in this thesis by performing both sensory evaluation and metabolomics analyses.

**Figure 1.3** Split-stream processing of asparagus ‘waste’. A schematic presentation of the novel approach ‘Split-stream processing’ for producing fibre and concentrated juice from asparagus by-products. Asparagus cut-offs are pressed to fibre and juice (A). Juice is concentrated (B) and the concentrate is spray-dried (C) using maltodextrin as carrier agent.

### 1.3. Sensory evaluation of food ingredients

A commonly-used analysis approach within food research is sensory evaluation of a product or ingredient. Professional sensory panels, team tastings, and consumer preference panels are examples of sensory evaluation procedures which are regularly used (Lawless & Heymann, 2010). Each of these can be chosen depending on the goal and objective of the analysis. For instance, a team tasting can be valuable to define initially the focus of those sensory attributes to be later scored by an expert panel (Rogeaux, 2015). Consumer
preference panels can be employed to score ‘liking’ and ‘preference’ after comparing two or more versions of a product or products (Murray et al., 2001). Evaluating the sensory profile and even further linking such information with chemical composition can assist in the understanding of the composition and matrix but also in the faster improvement of the product regarding its flavour. Human senses can be combined with advanced analytical instruments and high-tech detectors to provide a more advanced profiling and characterization of the food matrix under development. Gas chromatography-Olfactometry-Mass Spectrometry (GC-O-MS) is an example of combining analytical instrumental efficiency and our sense-of-smell as also performed in this thesis.

1.4. Plant Metabolomics

Plant metabolomics is used to study the composition of small molecules (< 1600 Da) produced by plant cells, tissues or organs. Although small, these molecules which can be stored in or excreted by plants, underpin biological processes that are central to plant development, growth and responses to biotic and abiotic environments. Plant metabolism is often divided into primary and secondary routes. Primary metabolism is highly conserved including photosynthesis, glycolysis, and amino acid biosynthesis. Products and intermediates of primary metabolic pathways and reactions, such as sugars, amino acids, organic acids and vitamins are those compounds essential for vital cell processes for the plant to grow, develop and survive. Primary metabolites are also involved in the biosynthesis of secondary metabolites, which is regulated by and related to the genetic background, developmental stage and environment of the plant. Secondary metabolites comprise a highly diverse array of compounds which play key roles in e.g. defence mechanisms of plants (e.g. glucosinolates, terpenes), pigmentation (e.g. carotenoids, flavonoids) and interaction with their environment (e.g. volatile aldehydes, alcohols). These functions of metabolites are translated to properties of the plants that are of interest for humans. Aroma and taste are such properties and are key crop quality traits. Plant properties based on the metabolite content are also e.g. antioxidant capacity, nutritional value and other health-promoting features (Falcone Ferreyra et al., 2012; Pagare et al., 2015; Pott et al., 2019) but are not within the scope of this thesis.

To understand the impact of genetic or environmental background of the crop, various differential materials should be studied. To study the composition of metabolites in depth,
sophisticated extraction techniques and cutting-edge analytical platforms are required. Processing and mining the large amount of data generated requires computer science expertise to extract relevant information. For the research presented in this thesis, I used mass spectrometry (MS) – based techniques due to their high sensitivity and potential to profile compounds in complex biological matrices in combination with providing information about their structure and thus enabling annotation of detected metabolites. Both liquid chromatography (LC) and gas chromatography (GC) were applied together with MS to elucidate the metabolite composition of asparagus.

1.5. Untargeted metabolomics workflow and data analysis
Untargeted metabolomics aims to explore the full profile of detected compounds (metabolome). However, there is no single platform that can fully cover the entire metabolome (Bjerrum, 2015). The general workflow I used to explore and investigate the metabolome of asparagus spears is here presented and described in Figure 1.4. As metabolites participate also in mechanisms that plants have evolved to respond to stress such as tissue damage which also occurs during harvesting, it is important to prevent compositional shifts occurring before the analysis. Therefore, freshly harvested spears were kept on ice (Figure 1.4A) before being ground in liquid nitrogen to minimise the activation of enzymes that would lead to the degradation and / or production of certain (new) metabolites - thus altering the in vivo metabolite fingerprint of the sample (Figure 1.4B). Here the focus is kept on secondary metabolites given their function and role in plants and thus their link to plant properties of interest. Non-volatile metabolites were extracted in acidified methanol with formic acid as described previously by De Vos et al. (2007). In the case of volatile organic compounds, these were routinely extracted from the headspace above the sample using solid phase microextraction (SPME) which is a method that does not require any solvent and the extracted profile resembles the aroma released by the analysed material. SPME conditions were adjusted from (Verhoeven et al., 2011). Subsequently, non-volatile metabolites were analysed by LC-MS and volatile metabolites by GC-MS (Figure 1.4C).

The generated raw data were processed to reduce background noise (baseline correction and peak picking) and to align mass signals across single runs of one sequence. Afterwards, mass signals were clustered and reconstructed into potential metabolites based on their
correlation within a specific retention time window and considering the chromatographic quality for each platform. Dedicated software has been designed to assist in the metabolomics data processing (Figure 1.4D), by using e.g. XCMS (Smith et al., 2006), MZmine (Pluskal et al., 2010) and in this thesis, I used MetAlign and MSClust, which have been developed and established (De Vos et al., 2007; Lommen, 2009; Y. M. Tikunov et al., 2012). The profiles of the putative metabolites can be further examined by applying dedicated statistical tools for multivariate or univariate analyses (Figure 1.4E).

**Figure 1.4** From asparagus spear to a data point. A schematic presentation of untargeted metabolomics workflow for analysis of asparagus materials from collection of starting material (A) to sample preparation (B), metabolite extraction (C) and profiling (D) to data analysis and visualisation (E).

To explore the overall chemical profile which often comprises a few hundred metabolites resulting in high-dimensional data, the first step in data analysis is usually to explore the overall structure of the dataset by investigating the main variation (differences and similarities) observed between samples. For this, multivariate statistical techniques for dimension reduction are employed. Chemometrics is the science employed to extract the valuable information when dealing with large and / or fused datasets. Chemometrics is the intersection of multivariate statistics, computer science and applied mathematics. The advent of chemometrics has highly facilitated our understanding of not only the composition of metabolites of plants but also their interaction and relationship with phenotypes and / or
environment of the plant itself or the food matrix, in case of flavour research (Hall et al., 2022; Jacobs et al., 2021; Pinto et al., 2012).

Commonly-used chemometric tools, either unsupervised or supervised, are principal components analysis (PCA), partial least square discriminant analysis or regression (PLSDA or PLSR) and hierarchical clustering (HCA). These dimension-reduction approaches assist in investigating the data in a stepwise manner, focusing on metabolites that may be discriminatory for a specific group of samples or show a specific pattern across samples. Inspecting the discriminatory and non-discriminatory compounds assists in better understanding of the background (e.g. genetic or environmental) of the samples. The key difference between supervised and unsupervised approaches is whether information about the samples (e.g. treatment, variety) is available or not to the model. It is advised that supervised approaches are followed only after a fully unbiased and untargeted investigation has been performed. The relevant or selected compounds can then be further examined by applying univariate statistical approaches such as analysis of variance (ANOVA) and Student’s t-test.

Compounds within the plant metabolome may interact and thus be correlated to each other or they might be involved in different biochemical pathways and thus not be directly correlated. Only using linear models limits our conclusions / interpretations to direct relationships within the metabolome. Langfelder and Horvath (2008) have developed a data mining approach (called Weighted Correlation Network Analysis - WCNA) which clusters compounds both based on direct as well as indirect relationships within the profile. WCNA has proven to be a useful, yet complicated data analysis approach for elucidating biochemical networks (Dekker et al., 2022; Ding et al., 2019; F. Y. Fan et al., 2021) and it has been also performed in this thesis for the purpose of elucidating the asparagus metabolome.

Data fusion techniques are lately often applied to enable us to make use of data from various classes of metabolite (e.g. volatiles and non-volatiles) or relating to other measurements, e.g. specific phenotypic traits, environmental conditions or sensory profiles (Hall et al., 2022). By bridging information obtained by various detectors / platforms, it may become easier to understand how the biological system under investigation functions than when focusing on datasets individually. However, once datasets obtained from different platforms are integrated, statistical analyses then tend to become more complicated and require
potentially more sophisticated techniques and / or combinations of tools that explore non-linear relations between variables (Cambiaghi et al., 2017). Machine learning approaches are then involved, e.g. Random Forest regression analysis (Breiman, 2001), which I also have used. As the interpretation of algorithms used in machine learning approaches is not always straight-forward, when I applied Random Forest, I also, performed the same analysis using PLS and compared the outcomes which gave me greater confidence to explain my findings.

1.6. Main objectives and thesis outline

In this thesis, I use untargeted metabolomics and various data analysis and mining tools to study numerous asparagus materials including raw and cooked spears as well as juice concentrate, dried powders and instant soup formulations, aiming:

1) To explore the chemical composition and the dynamics of the asparagus metabolome with regard to seasonal changes, harvest moments, cultivation methods and genetic background, as well as the compositional metabolite differences between raw and cooked spears.

2) To investigate the effect of spray drying asparagus waste-products on the formation and retention of flavour compounds.

3) To link the composition of secondary metabolites with (asparagus-specific) sensory attributes leading to the evaluation of known asparagus flavours and the discovery of new flavour candidates.

One of the main aims of the research described in this thesis was to understand better the metabolome changes that occur during the development and processing of asparagus spears. For this goal, obtaining the most complete overview of the asparagus metabolome was of high relevance. Chapter 2 gives a comprehensive review summarising recent studies on the composition of asparagus with a particular focus on flavour compounds but also secondary metabolites as a whole. The developmental stages of the asparagus plant from seed to harvested spear are presented, as well as the current knowledge of the main components of spear composition (minerals, sugars, fibres, vitamins). Studies on both green and white asparagus were taken into consideration although distinction between the two was not always made clear in previous studies. It was concluded that untargeted metabolomics studies would offer new knowledge on the asparagus metabolome. This formed the
foundation of the motives to compare the metabolite composition of green and white spears and to gain deeper insights into the mechanisms behind developmentally, genetically and environmentally-associated differences / changes.

In Chapter 3, both green and white spears of three varieties were analysed using untargeted metabolomics. This is one of the first studies to compare directly the metabolome of green and white asparagus and the first one to explore the composition of volatile organic compounds in both types. The material used for this study, was grown in the same field and harvested in two consecutive seasons for the purposes of repetition and validation. Volatile metabolites were profiled using SPME GC-MS and non-volatile metabolites were analysed using ultra-performance LC-MS. Multivariate and univariate statistics were performed to discover the discriminatory compounds between the two vegetable types and between varieties, as well as determining those compounds which do not vary significantly between the two spear types as these metabolites are likely to be relevant for the typical biochemistry of the crop. Finally, sections along the spears were profiled individually with respect to the spatial heterogeneity of volatile composition.

Chapter 4 presents a more in-depth study to look into the dynamics of the asparagus metabolome and aims to link these to development, genetic background and environment. Here I focus on white asparagus, as this is the type mainly cultivated in the Netherlands. By applying advanced statistics, the dynamics of the white asparagus metabolome throughout the harvest season could be illuminated. Those metabolites that have been reported as being flavour-relevant in asparagus were investigated in particular to assess the relationship between harvest moment or genetic background and the flavour of the harvested spears.

The next step was to inspect the metabolite composition shifts which may occur after cooking and test whether genetic background is relevant for the flavour as experienced by consumers. Chapter 5 details the changes in the metabolome of white spears of four selected varieties upon cooking. Here, I combined the results of metabolomics and team tasting using supervised multivariate statistics, and this highlighted potential relationships between volatile and non-volatile secondary metabolites with specific flavour attributes. The odour-active volatile metabolites in raw and cooked materials were detected by GC-O-MS. These results contribute to a better understanding of which potentially sensory-relevant asparagus
metabolites are already present in raw asparagus, and which may appear or disappear during cooking.

The second objective of the thesis was to evaluate the potential value of using asparagus waste materials as a source of flavour ingredients and assess the impact of spray drying on the metabolome of these asparagus waste-products. Chapter 6 presents the proposed strategy for processing asparagus waste, the so-called split-stream process (Figure 1.3). Maltodextrin was used as carrier agent and varying concentrations were tested. The optimal ratio of maltodextrin to asparagus solids was defined by evaluating the volatile profile using SPME GC-MS and the physical properties of the spray-dried powders as well as the starting material.

In Chapter 7, the split-stream processing method was applied to produce asparagus concentrate and spray-dried powder. The resulting asparagus fibre was oven-dried and milled into fine powder. These and a commercial asparagus powder were then used as ingredients for formulating instant soups. The commercial powder is made by oven-drying and then milling asparagus pieces but as it has moderate flavour it is usually supplemented with flavour additives. The in-house prepared formulations were compared to soups containing the commercial powder with and without the additional flavour mix by evaluating their flavour profile. Both sensory analysis by an expert panel and untargeted metabolomics were performed. Combining the data from both sources using multivariate chemometric and machine learning approaches led to the confirmation of known as well as proposing new compounds impacting asparagus flavour. GC-O-MS was performed to confirm the odour-activity of the proposed aroma compounds.

In Chapter 8, I integrate and discuss all the findings of the thesis. I discuss the general biochemistry and flavour composition of asparagus based on the examined dynamics of the metabolome primarily of white spears and suggest an updated list of key sensory-relevant compounds. I also discuss the potential of metabolomics when combined with sensory analyses to examine (new) food ingredients. I, furthermore, touch upon the application of the split-stream processing and spray drying of other vegetable waste materials, the societal impact of the scope of my research with respect to food waste and sustainability, and I round off with future perspectives and concluding remarks.
Chapter 2

Green and white asparagus (Asparagus officinalis) – a source of developmental, chemical and urinary intrigue

Eirini Pegiou, Roland Mumm, Parag Acharya, Ric C.H. de Vos, Robert D. Hall

This chapter is an updated version of the review published in Metabolites (2020) DOI:10.3390/metabo10010017
Abstract:

Asparagus (*Asparagus officinalis*) is one of the world’s top 20 vegetable crops. Both green and white shoots (spears) are produced; the latter being harvested before becoming exposed to light. The crop is grown in nearly all areas of the world, with the largest production regions being China, Western Europe, North America and Peru. Successful production demands high farmer input and specific environmental conditions and cultivation practices. Asparagus materials have also been used for centuries as herbal medicine. Despite this widespread cultivation and consumption, we still know relatively little about the biochemistry of this crop and how this relates to the nutritional, flavour, and neutra-pharmaceutical properties of the materials used. To date, no-one has directly compared the contrasting compositions of the green and white crops. In this short review, we have summarised most of the literature to illustrate the chemical richness of the crop and how this might relate to key quality parameters. Asparagus has excellent nutritional properties and its flavour/fragrance is attributed to a set of volatile components including pyrazines and sulphur-containing compounds. More detailed research, however, is needed and we propose that (untargeted) metabolomics should have a more prominent role to play in these investigations

Keywords: asparagus; secondary metabolites; asparagus aroma; flavour; phytonutrients; plant metabolomics
2.1. The asparagus crop

Asparagus (A. officinalis) is predominantly a food crop, solely eaten in the form of its very young thickened shoots, called spears. However, it and its related species also have been, and are still used as a source of medicinal bioactives. The crop has been cultivated and harvested for thousands of years by many ancient civilizations, including the Egyptians, ancient Greeks and Romans and so the crop has a rich and varied past. Its appearance as an offering, illustrated on an old Egyptian frieze, is a first indication of its use, although it is not clear whether the plant had a food or medicinal application at that time. There are many references to its use in ancient Greek and Roman times both as a food and as a source of herbal medicine. It is known to have been grown in French monasteries in the mid-15th century but apparently only arrived a century later in Germany and England. Introduction to North America occurred much later, in the mid-19th century. Today, it is widely consumed right across the world. Furthermore, its potential healing powers are still recognised in Traditional Chinese Medicine practices (Winter, 2005). Taxonomically, when the monocot family Liliaceae was divided, Asparagus became a member of the new family Asparagaceae (Byng et al., 2016). A. officinalis is only one of many Asparagus spp. and is an herbaceous perennial native to (Northern) Europe and is found in Asia as far east as Mongolia. However, through farming / escapes the species is now widespread as a (persistent) weed in a much broader range of countries including those in N. America, Scandinavia and Australia (Figure 2.1).

Figure 2.1 Distribution of cultivation of Asparagus officinalis around the world. The origin is believed to be eastern Mediterranean, however it grows also in central Europe, the Caucasus and western Asia (green). It was brought centuries ago to North America, Northern Europe and parts of South America, North Africa and Australia (purple). ©Copyright 2017 World Checklist of Selected Plant Families. http://creativecommons.org/licenses/by/3.0.
Successful production of the asparagus crop requires strict cultivation conditions, such as a specific soil composition, good drainage and the correct temperature range. Seeds are germinated and the plants are grown for one year before being transplanted to the final production location with the correct agronomic features (Figure 2.2 A-C). Plants must then be allowed to grow there for at least a second year in order to develop a strong root crown for the coming years production (Figure 2.2 B-C). From the third year on, spears can be harvested for consumption (Figure 2.2 D-F). The commercial production period can last for up to 10-12 years after which the plants should be discarded and the field used for the cultivation of a crop other than asparagus. Harvesting of the emerging spears can only be done for a specific period as the plant must then be allowed to produce a leafy crown to build up photosynthetic reserves to survive through the following winter and produce the next year’s spears. In Europe, the season is usually from late-April until mid-June but earlier harvesting (already in February/March) is becoming possible through either greenhouse production or even by warming the soil below the roots using a modified central heating system. Harvesting too late or using incorrect storage conditions can result in inedible woody spears (Herppich et al., 2008; Huyskens-Keil et al., 2005; Song et al., 2015).

*Figure 2.2 Asparagus officinalis* (white) from seed to harvested product. (A): asparagus seeds, primed in water in order to help germination when sown. (B): asparagus root crown, one-year old, ready to be planted in the field. (C): new asparagus field with two-year old plants. (D): asparagus field in harvesting season with plastic covers to raise soil temperature. The black plastic covers the plants during the whole harvesting period to eliminate light exposure to the spears. (E): white asparagus shoots emerging from 40cm deep in the soil. (F): harvested white asparagus spears. All pictures taken in Helden, The Netherlands (spring 2019).
The asparagus crop recognises two main forms – green (and green-purple) and white. Some countries (The Netherlands, Belgium, Peru) are more familiar with / favour the white varieties, while others (e.g. the UK) usually only see the green form in supermarkets. The green form traditionally has a much bigger global market to the extent that some countries only see this form and consumers may not even be aware of the existence of the white variant. This has led to some scientific papers not even mentioning which form was used in the research (so one has to assume that it is green). Agronomically, the key difference is whether the shoots are harvested above ground (green) or underground before they reach the surface/light (white) (Figure 2.2). Botanically, both types are a single species and while, in some cases, the same variety can be used to produce both variants, breeders have chosen to breed for varieties which are better suited to one of the two production methods.

Asparagus is a rather challenging crop requiring intense manual labour efforts during the short harvesting period lasting 7-8 weeks in the spring. However, once harvesting stops, the plants still require extensive care while the produced foliage provides the required sugars through photosynthesis for storage in the roots until die-back in the early autumn.

While asparagus was considered for many years as a luxury / gourmet food item, the crop has become more broadly available and more widely eaten in recent years. For example, production in Europe almost doubled to more than 310,000 tonnes/year between 1968 and 2020 (Asparagus Production in the World, 2020). However, the time and cost needed to establish, manage and manually harvest asparagus fields, as well as the crop’s strong seasonality limited to just the first few weeks of growth, leads to the high price of the crop. This is likely to keep asparagus in the most expensive vegetable category at least until production methods require less input. Automated harvesting machines for white asparagus have been under development for years and in 2021 for the first time used in the Netherlands (source: freshplaza.com, 2021). The selective automated harvesting of spears may lead to a major change in the industry. Nevertheless, in 2021 the crop was already in the top 20 most eaten vegetables in both the USA and Europe (Top 20 Fruits and Vegetables Sold in the U.S., 2021). China leads global production with more than 7.3 million tons in 2020 (ca. 73 % of global production; mostly green). In N. America, in Mexico and the USA respectively, 300,575 and 33,733 tons were produced in 2020 (mostly green) and in S. America, Peru leads the way with 370,532 tons (mostly white). In Europe, Germany (both white and green) is by far the
biggest grower and producer having reached 117,560 tons in 2020 – bit less than twice its nearest rival, Spain where ca. 65,000 tons were produced (Shahbandeh, 2020). In 2017, market predictions indicated that the global asparagus market will continue to grow by ca. 3% per year to reach a global market volume of ca. 10 million metric tonnes by 2027 with a total market value of 30 billion USD (An Incisive, In-Depth Analysis on the Asparagus Market, 2017). Interestingly, already in 2020, global production had reached 10 million tons, indicating the fast growth of the Asparagus industry (Shahbandeh, 2020).

Considering the importance of the crop, its growing global market across all continents, its value as a nutritious flavoursome food and its potential healing properties (see further below), there is a clear growing interest in gaining deeper knowledge of asparagus and its composition. Asparagus is considered high in basic nutrients, including vitamins, minerals and amino acids (Al-snafi, 2015; López et al., 1996; Takacs-Hajos et al., 2013) and is a fibre-rich food (Amaro-López et al., 1998; Fuentes-Alventosa et al., 2009; Hamdi et al., 2018) (Table 2.1). However, despite its long history, there is relatively little knowledge of the crop and especially of its chemistry and its impact on human physiology after ingestion. Flavour and fragrance are key food quality traits which for asparagus, have only infrequently been the subject of detailed study. In this short review we present an overview of our current knowledge of the crop and describe our advancing need for new information. While we shall occasionally touch on the non-food aspects, the focus shall mainly be placed on the vegetable market for which most data are available. We shall also address how modern scientific approaches such as metabolomics may help us gain deeper knowledge and allow us to design new strategies for developing a crop with improved quality and which meets future consumer needs.

2.2. Asparagus biochemistry

The genus Asparagus consists of almost 300 species (Negi et al., 2010). In the past, roots of some species were used by herbalists for their various properties, e.g. Asparagus curilus for diabetes and dysentery (Gaur, 1999), Asparagus filicinus for rheumatism (Rana & Pandey, 2007) and Asparagus racemosus for epilepsy, night blindness and hypercholesteremia (Kirtikar & Basu, 1984; Visavadiya & Narasimhacharya, 2009) while Asparagus officinalis aqueous root extracts have been associated with the regulation of main reproductive hormones and oogenesis in mammals (Karimi Jashni et al., 2016).
Green and white asparagus (*Asparagus officinalis*) – a source of developmental, chemical and urinary intrigue

Table 2.1 Nutritional overview of raw and cooked asparagus (100 g) and for reference, the Recommended Daily Intake (RDI) of the nutrients based on a 2000 kcal diet. Indicated are also the values of the listed nutrients present in spinach considered as the No. 1 of the 14 healthiest vegetables in the world (Link, 2022).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Value per 100 g raw asparagus</th>
<th>Value per 100 g cooked asparagus</th>
<th>Value per 100 g raw spinach (1st in the top-14 healthiest vegetables)</th>
<th>RDI based on a 2000 kcal diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Nutrition facts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calories</td>
<td>20 kcal</td>
<td>20 kcal</td>
<td>23 kcal</td>
<td>2000 kcal</td>
</tr>
<tr>
<td>Dietary fibres</td>
<td>2.1 g</td>
<td>2 g</td>
<td>2.2 g</td>
<td>25 g</td>
</tr>
<tr>
<td>Sugars</td>
<td>1.9 g</td>
<td>1.3 g</td>
<td>0.4 g</td>
<td>90 g</td>
</tr>
<tr>
<td>Proteins</td>
<td>2.2 g</td>
<td>2.4 g</td>
<td>2.9 g</td>
<td>40-50 g</td>
</tr>
<tr>
<td>Fat content</td>
<td>0.12 g</td>
<td>0.22 g</td>
<td>0.4 g</td>
<td>65 g</td>
</tr>
<tr>
<td>Vitamins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B1, thiamine</td>
<td>0.143 mg</td>
<td>0.162 mg</td>
<td>0.078 mg</td>
<td>1.4 mg</td>
</tr>
<tr>
<td>Vitamin B2, riboflavin</td>
<td>0.141 mg</td>
<td>0.139 mg</td>
<td>0.189 mg</td>
<td>1.6 mg</td>
</tr>
<tr>
<td>Vitamin B3, niacin</td>
<td>0.978 mg</td>
<td>1.1 mg</td>
<td>0.724 mg</td>
<td>15 mg</td>
</tr>
<tr>
<td>Vitamin B9, folate</td>
<td>52 μg</td>
<td>149 μg</td>
<td>194 μg</td>
<td>400 μg</td>
</tr>
<tr>
<td>Vitamin C, ascorbic acid</td>
<td>5.6 mg</td>
<td>7.7 mg</td>
<td>28.1 mg</td>
<td>75 mg</td>
</tr>
<tr>
<td>Vitamin E, alpha-tocopherol</td>
<td>1.13 mg</td>
<td>1.5 mg</td>
<td>2.03 mg</td>
<td>10 mg</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>41.6 μg</td>
<td>50.6 μg</td>
<td>482.9 μg</td>
<td>80 μg</td>
</tr>
<tr>
<td>Minerals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium, Ca</td>
<td>24 mg</td>
<td>23 mg</td>
<td>99 mg</td>
<td>1000 mg</td>
</tr>
<tr>
<td>Copper, Cu</td>
<td>0.19 mg</td>
<td>0.19 mg</td>
<td>0.13 mg</td>
<td>0.9 mg</td>
</tr>
<tr>
<td>Iron, Fe</td>
<td>2.14 mg</td>
<td>0.91 mg</td>
<td>2.71 mg</td>
<td>15 mg</td>
</tr>
<tr>
<td>Magnesium, Mg</td>
<td>14 mg</td>
<td>14 mg</td>
<td>79 mg</td>
<td>350 mg</td>
</tr>
<tr>
<td>Manganese, Mn</td>
<td>0.158 mg</td>
<td>0.158 mg</td>
<td>0.897 mg</td>
<td>5 mg</td>
</tr>
<tr>
<td>Potassium, K</td>
<td>202 mg</td>
<td>224 mg</td>
<td>558 mg</td>
<td>3500 mg</td>
</tr>
<tr>
<td>Selenium, Se</td>
<td>2.3 μg</td>
<td>10.8 μg</td>
<td>1 μg</td>
<td>35 μg</td>
</tr>
<tr>
<td>Sodium, Na</td>
<td>2 mg</td>
<td>14 mg</td>
<td>79 mg</td>
<td>1500 mg</td>
</tr>
<tr>
<td>Zinc, Zn</td>
<td>0.54 mg</td>
<td>0.54 mg</td>
<td>0.53 mg</td>
<td>15 mg</td>
</tr>
</tbody>
</table>

These various properties attributed to asparagus would seem to underline a complex and interesting biochemistry. Studies on the phytonutrients of asparagus have showed a rich mixture, including saponins, flavonoids and other phenolics (Guo et al., 2020; Negi et al., 2010; H. Zhang et al., 2018). In this section, an overview of our knowledge of the biochemical composition of *Asparagus officinalis* (asparagus) is given in relation to the main groups of compounds discovered.

2.2.1. Steroidal saponins

Saponins are high molecular-weight glycosides consisting a conjugate of an oligosaccharide to a triterpene or a steroid aglycone. They naturally occur in plants and have often been
associated with medicinal properties (K. & Marston, 1995). The aglycone of a steroidal saponin is usually a spirostanol or a furostanol (Sahu et al., 2009). Steroidal saponins play an important role in the biological and pharmacological activities of different Asparagus species (Negi et al., 2010; Shao et al., 1999; Shimoyamada et al., 1996, 2011; Sullivan et al., 2017). The main saponins present in most of the green and white commercial varieties (Asparagus officinalis) are asparanin A (Liu et al., 2009), protodioscin (Lee et al., 2010; Shao et al., 1999; Vázquez-Castilla, Jaramillo-Carmona, et al., 2013; M. Wang et al., 2003), sarsasapogenin (X. F. Huang et al., 2008; X. Huang & Kong, 2006) and yamogenin (X. Huang & Kong, 2006; Z. Sun et al., 2010). Interestingly the saponin profiles of other Asparagus spp., such as A. maritimus and A. prostrates which are from southern Europe, were different than the one from A. officinalis, where indeed protodioscin was the main saponin (Jaramillo-Carmona et al., 2017). Investigating the properties of all detected saponins in different species and varieties of asparagus might suggest alternative uses of the non-food Asparagus spp. Studies on asparagus saponins have revealed in vivo their hypolipidemic effects due to their contribution in decreasing LDL and total cholesterol levels, improving the health state of rats that were fed a high-cholesterol diet (García et al., 2012; Vázquez-Castilla, Jaramillo-Carmona, et al., 2013). Saponins extracted from asparagus shoots have also been associated with having antitumor (X. F. Huang et al., 2008; Jaramillo et al., 2016; Ji et al., 2012; Liu et al., 2009; Shao et al., 1996; J. Wang et al., 2013) and antifungal (Shimoyamada et al., 1996, 2011) effects in vitro. Apart from such important biological activities, these compounds are also invaluable for the characteristic bitter taste of asparagus (Dawid & Hofmann, 2012a, 2012b, 2014; Kawano et al., 1975, 1977) as described further below.

2.2.2. Vitamins

Asparagus is a valuable nutritional source of several vitamins. In Table 2.1, the levels of important vitamins that are present in 100 g of asparagus (raw and cooked) are listed. In the top-14 healthiest vegetables worldwide, asparagus ranks 10th (Link, 2022), and in Table 2.1, it is shown that the levels of B vitamins do not deviate much from the No. 1 vegetable in the top-14. B vitamins, especially vitamin B6, vitamin B12 and vitamin B9, contribute to maintaining a healthy level of homocysteine in blood. Elevated levels of homocysteine increase the risk of atherosclerosis and other cardiac disorders (Tucker et al., 2004). Furthermore, folate, next to maintaining healthy levels of homocysteine in the body, through
Green and white asparagus (Asparagus officinalis) – a source of developmental, chemical and urinary intrigue

conversion to methionine, plays a crucial role in cell division and the formation of DNA (Carmel et al., 2005). The high folate content of asparagus shoots emphasizes the added nutritional value of the vegetable and the advantage of its consumption especially, for example, during pregnancy (Fischer et al., 2017; Pitkin, 2007). Traces of vitamin B12 are also found in asparagus and this vitamin is essential for general cell physiology and is a co-factor in the effective biosynthesis of DNA, amino acids and fatty acids (Watanabe et al., 2014). Asparagus also contains smaller amounts of vitamin K and vitamin E (Table 2. 1), which also contribute to a healthy diet.

2.2.3. Minerals

Many essential minerals are present in asparagus. These include selenium, iron, calcium, copper, zinc, magnesium, potassium and phosphorus (Table 2. 1). The content of these minerals was studied as a function of the position within the spears for two asparagus varieties and significant spatial variation was observed (Amaro-López et al., 1998). Minerals were found to be most concentrated in the upper sections of the spears, close to the tip. This indicates a gradient of chemistry in asparagus which is discussed further in later parts of this review. In Table 2. 1, the recommended daily intake value of all nutrients is given, showing how much an excellent source of minerals asparagus is. Levels presented entail that a reasonable asparagus consumption (a full portion of asparagus corresponds to 180 g) makes a valuable contribution to the dietary intake of these essential minerals.

2.2.4. Flavonoids and other phenols

Flavonoids and other phenolic compounds have been the focal point of many analyses of asparagus materials, motivated by their potential antioxidant and anticarcinogenic properties (Ali Ghasemzadeh, 2011; Guo et al., 2020; X Zhang et al., 2019). Evaluation of these properties was performed through the analyses of the total phenols (R. Fan et al., 2015; Fanasca et al., 2009; Jiménez-Sánchez et al., 2016) or specific phenolic subclasses such as flavonoids, comparing different varieties and / or cultivation systems (Fuentes-Alventosa et al., 2008; Ku et al., 2018). Fuentes-Alventosa et al. (2008) analysed 32 commercial hybrids of green asparagus and 65 genotypes of the Spanish asparagus variety Triguero, which is a wild green-purple asparagus consumed in Southern Spain. Clustering analysis revealed that the flavonoid profiles of some Triguero genotypes are significantly different from the American commercial hybrids that were analysed. This is of potential importance as Vázquez-Castilla
et al. (2013) were able to show that the flavonoid content of Triguero spears was one of the characteristics linked to improving plasma lipid and liver antioxidant status in hypercholesterolemic rats. As an example of one important flavonoid in asparagus, the quantification of rutin (quercetin-3-O-rutinoside), revealed that this flavonoid is present in the upper sections of green asparagus spears (1.51 – 7.29 mg/g dry weight) (Lee et al., 2010) explaining a possible correlation of rutin content and the green asparagus tissues that are exposed longer to sunlight. Next to this, traces of rutin have also been detected in white asparagus (below 0.5 mg/g dry weight), but still following a concentration gradient with highest levels in the upper parts (Lee et al., 2010). Earlier data showed similar results when comparing the content of rutin in different parts of (assuming green) asparagus (M. Wang et al., 2003). Rutin was detected in the upper sections at levels 0.03 – 0.06 % fresh weight, but also in the lower parts at levels below 0.01 % fresh weight. Both studies (Lee et al., 2010; M. Wang et al., 2003) imply a concentration gradient as also seen for minerals (Amaro-López et al., 1998) as well as for saponins in white spears (Dawid & Hofmann, 2014).

Rutin, together with 93 more compounds (of which 32 phenolic compounds), was also detected in green spears in an untargeted analysis by Jiménez-Sánchez et al. (2016), who performed Reversed-Phase High-Performance-Liquid-Chromatography Electro-Spray-Ionization Quadrupole-Time-of-Flight Tandem Mass-Spectrometry (RP-HPLC-ESI-QTOF/MS²) and annotated 74 compounds. This list included hydroxycinnamic acids, which play a role in the cell wall biochemistry of asparagus shoots (Rodríguez-Arcos et al., 2004). These studies highlight the various roles that phenolics might have in the complex biochemistry of asparagus.

2.2.5. Volatile sulphur compounds and their precursors

Sulphur-containing compounds (S-compounds) have a prominent role in plants and derived products, relating to plant protection aspects (Bloem et al., 2005), to human health-promoting activities (Nakabayashi & Saito, 2017; Petropoulos et al., 2017) and to flavour and fragrance attributes (Mcgorrin, 2011). S-compounds form a rather interesting compound class in asparagus that have attracted the attention of several studies that we discuss and refer to in this review. Asparagusic acid (1,2-dithiolane-4-carboxylic acid) is an S-compound which has been reported as being unique in asparagus (Mitchell & Waring, 2014; Tressl, Bahri, et al., 1977; Yanagawa et al., 1972) and has been of great interest concerning both
pharmacological (Jang et al., 2004; Mitchell & Waring, 2014) and flavour properties (Dawid & Hofmann, 2012a; Tressl, Bahri, et al., 1977; Tressl, Holzer, et al., 1977) of asparagus. A number of S-compounds were found to contribute to the typical asparagus flavour. These include dimethyl sulphide (DMS), methanethiol and methional (Tressl, Bahri, et al., 1977; Ulrich et al., 2001). From these S-volatiles, DMS is considered the main key odorant in cooked asparagus (Ulrich et al., 2001) and the fact that it is formed upon cooking / processing means that its precursor, S-methylmethionine (Scherb et al., 2009) is an equally crucial compound in the vegetable. Recently, another novel S-compound, asparaptine has been discovered and detected in both green and white asparagus spears, and shown to have an inhibitory activity against the Angiotensin-Converting Enzyme (ACE), in vitro (Nakabayashi et al., 2015), yet there has been no indication whether asparaptine has any sensory-relevant properties. Bergamasco et al. (2022) have recently reported a comprehensive profiling of S-metabolites in Asparagus officinalis.

In a completely different context, some S-compounds have also been highlighted as being major contributors to the characteristic urine odour following asparagus consumption (Mitchell, 2001). However, there is still a lot of confusion as to whether these are the main odorants contributing to the smell, or if there are other compounds derived from several biosynthetic pathways (Akers & Venkatasubramanian, 1997; Mitchell, 2001; Mitchell & Waring, 2014). This particular topic is described in more detail at the end of this article. Considering the importance of volatile compounds to various consumer-related quality attributes, exploring the biochemical pathways of the S-volatiles in asparagus in more detail is needed to help shed more light on the origins of these important compounds, also in the context of food quality and health (Nakabayashi & Saito, 2017).

2.3. Cultivation, harvesting, and storage influences on asparagus quality

Asparagus cultivation is a rather challenging process that requires commitment and dedication as well as patience and flexibility to adapt to the varying weather conditions which can highly influence the productivity and final crop quality of the asparagus field. Testing the soil composition is crucial to determine the nutrient needs both for the new and the established fields. Based on soil type, different concentrations of phosphorus, potassium and trace elements are recommended for the healthy growth of asparagus roots, shoots and ferns. Careful nutrient applications during the harvest years ensures for continued healthy

```
spears and high productivity. After 10-12 years of harvesting, an asparagus field is cleaned and prepared for the cultivation of another crop. It is advised not to continue harvesting spears for much longer than a decade from the same field, because as well as lower harvest yields and thinner spears (Feller et al., 2010; Heißner et al., 2006), there is an increasing chance that the spears are infected by pathogens. The main fungal pathogens infecting asparagus are *Fusarium spp.* which cause the roots to rot (Blok & Bollen, 1995; Borrego-Benjumea et al., 2014; Fiume & Fiume, 2003). Breeders are concerned and engaged in the development of resistant varieties, as it is not possible to directly control *Fusarium spp.* and the best approach is to prevent the initial infection (Papizadeh et al., 2018; Y. Zhang et al., 2022).

Concerning the productivity of asparagus, another factor is the sex of the plants. There are productive differences between male and female asparagus plants that make the male plants sprout earlier and produce both larger and more spears per rootstock weight than the female plants do (Bracale et al., 1991; Uragami et al., 2016). Consequently, asparagus growers prefer male plants, and due to the indistinguishable morphological differences between the two sexes at early stages of growth (Bracale et al., 1991), attempts to differentiate males from females based on seedlings (Shiobara et al., 2011) or flower (Sheng & Tang, 2019) phenotype and molecular markers (Gao et al., 2007; Gebler et al., 2007; Ji et al., 2012; Loeptien, 1979) have kept breeders busy for long time. These studies have allowed researchers to construct genetic maps that help in the identification of sex chromosomes in *A. officinalis* (S. F. Li et al., 2014; Moreno et al., 2018) and to exploit asparagus as a model plant for studying the origin of sex chromosomes (Harkess et al., 2017; S. F. Li et al., 2021).

Focusing back to the method of cultivation, evaluation of different cultivation systems of asparagus by multivariate statistics showed separation between conventionally and organically grown spears based on analysed bioactive compounds (Ku et al., 2018). Furthermore, also in this study, comparing different parts of the crop (cladodes and spears), showed that different tissues have significantly different compositions of the bioactive polyphenolics. Another study, aiming to correlate taste differences among different spear sections of asparagus, showed that the content of phenolic and organic acids was dependent on the position within the spear (increasing from base to top) (Slatnar et al., 2018). Consequently, these results, in combination with similar conclusions concerning minerals
Green and white asparagus (Asparagus officinalis) – a source of developmental, chemical and urinary intrigue

(Amaro-López et al., 1998) and saponins (Dawid & Hofmann, 2014) concentrations, imply a differential profile of metabolites within the asparagus shoot which is developmentally regulated. However, it is not clear how this might be further affected by environmental conditions present during growth and storage. In addition, while genetic factors together with the environment evidently influence the final quality of the crop, the (phyto)chemical composition and thus potential health of the plants can also be affected by microbes in the environment in both a positive and negative way. For example, in the case of A. officinalis, the profile of natural headspace volatiles was shown to vary between healthy and black cutworm-induced stems (Morrison et al., 2016). Consequently, studies focusing specifically on genotype variation should also bear in mind the influence of both abiotic and biotic stresses in these analyses.

Cultivation practice and the moment of harvesting (e.g. soil, temperature, environmental conditions at harvest) may influence productivity (Heißner et al., 2006; Kmitiene et al., 2009) and the final quality of crop. These quality differences have been correlated with levels of specific compound classes, such as the phenolics and flavonoids, in connection to their antioxidant capacity (Ku et al., 2018; Papoulias et al., 2009). Based on the USDA Agricultural Marketing standards, the basic attributes for asparagus grading are the length, diameter and colour uniformity. However, considering the ever-increasing demands of the modern consumer, we would propose that more attention be given to additional consumer-relevant characteristics such as flavour as described previously (Cuppett et al., 1997; Hoberg et al., 1999, 2003, 2008).

Harvesting has a direct impact on the metabolism of plant materials, as it can lead to the activation of plant defence and stress mechanisms and eventually senescence and decay, leading to the production of several protective secondary (volatile and non-volatile) metabolites and proteins (Matsui, 2006). The impact of cultivation conditions, harvesting and storage of asparagus on its antioxidant capacity have been thoroughly examined (Jaramillo-Carmona et al., 2008; Jaramillo et al., 2007; Ku et al., 2018; Papoulias et al., 2009; Rodríguez-Arcos et al., 2004; Takacs-Hajos et al., 2013). In white asparagus, so far the focus has been on the total phenolic content (Papoulias et al., 2009) and specifically, ferulic acid derivatives (Jaramillo et al., 2007; Rodríguez-Arcos et al., 2004). It was shown that an increased level of ferulic acids is associated with post-harvest spear hardening (Rodríguez-
Arcos et al., 2004), and that post-harvest storage temperature plays an important role in cell wall hardening (Herppich et al., 2008; Huyskens-Keil et al., 2005; Jaramillo-Carmona et al., 2008; Jaramillo et al., 2007; You et al., 2021). Other studies have shown that storage temperatures above 10 °C can have significant impact on both the appearance and the texture of the spears (A. S. Siomos et al., 2000). Such indications imply the high perishability of asparagus meaning a relatively short shelf life, that short farm-to-shop times are essential and strictly controlled conditions are required for maintaining the quality of the crop after harvest. The whole topic of the influence of environmental, genetic and post-harvest storage factors on asparagus quality has been recently well reviewed by A. Siomos (2018) who again stressed the need for a better understanding of the influence of environmental factors specifically on sensory-active phytochemicals.

Cold storage of asparagus spears does not completely deactivate all biochemical mechanisms that might play a role in final crop quality, including texture (Huyskens-Keil et al., 2005; You et al., 2021). The accumulation of ferulic acid, a hydroxycinnamic acid, has been verified to increase in the cell walls of asparagus under different storage conditions and contributes to the hardening of the spears (Jaramillo et al., 2007; Rodríguez-Arcos et al., 2004). In this hardening process the content of the polysaccharide heteroxylan in the spear cell walls also increases, which has made asparagus a plant model also for studying the heteroxylan biosynthesis (Song et al., 2015). Concerning the colour of the spears, an increase in anthocyanins in white asparagus was reported upon post-harvest light-exposure leading to a slight purple colorization (A. S. Siomos et al., 2000). This also entails a reduction in quality / market value. Therefore, a conscious understanding of the biochemical background of asparagus is needed in order to define the important determinants influencing the quality of the vegetable and, therefore the optimal post-harvest handling strategy.

2.4. Asparagus and health

A significant daily fruit and vegetable consumption is considered essential for a healthy diet as promoted by the ‘5 per day’ campaign running in the USA, UK and other European countries and on the basis of WHO advice (Five A Day Campaign, 2003). Apart from nutritionists and health professionals, a growing audience is tending to pay ever-growing attention to the potential health benefits and properties of especially the vegetables and fruits that are consumed, as well as how they are prepared. Being rich in vitamins and
Green and white asparagus (Asparagus officinalis) – a source of developmental, chemical and urinary intrigue

Antioxidants (see above) and with a high fibre and relatively low-calorie content (Table 2.1), consuming A. officinalis spears makes an excellent contribution to a healthy diet. Furthermore, its mineral content (Table 2.1) makes it of value to specific patient types such as those with hypertension (Staruschenko, 2018) considering the potassium content of asparagus. However, this and other Asparagus spp. have also been linked to more medicinally based applications in the form of herbal medicines and here we also briefly provide an overview of these non-food applications.

Traditionally, Asparagus spp. have been especially used in China and Korea, as a source of herbal medicines. For example, thanks to its diuretic properties, the vegetable has found application for the treatment of urinary problems (Al-snafi, 2015; Negi et al., 2010; Thapa et al., 2020; Winter, 2005). In India, asparagus root extracts have been used to strengthen the female reproductive system, promote fertility and increase breast milk production (Winter, 2005). In both ancient Eastern and Greek medicine, asparagus extracts have been used as a tonic for the prevention and cure of several ailments including those for the kidney, bladder, rheumatic, liver disease, asthma and cancer (Al-snafi, 2015; Bandaïphet & Kennedy, 2004; Debuigne & Couplan, 2009; Winter, 2005). Nevertheless, despite these long-term traditional medicinal uses, such applications have never been FDA-approved and hence, pharmacological use beyond those as ‘traditional medicines’ has not gained wide acceptance due to insufficient convincing clinical evidence. However, new studies using more advanced approaches to find the potential bioactive components using proper combinations of cell-based assays and clinical trials may contribute to the discovery of novel medicines from Asparagus spp. in the global quest for new pharmaceuticals.

Being a rich source of phytochemicals with antioxidant attributes has highlighted asparagus shoots as being a very valuable dietary component (Fanasca et al., 2009; Gębczyński, 2007; Negi et al., 2010). Asparagus spear extracts have been correlated to suppressive effect on elevated blood glucose in type-2 diabetic rats (Hafizur et al., 2012). Fan et al. (2015) compared different extraction solvents and methods focusing on the antioxidant compounds in asparagus waste materials using HPLC-MS². They detected ferulic acid, rutin, kaempferol, quercetin and isorhamnetin in the analysed samples, without mentioning whether they were detected as aglycones or glucosides. All of these compounds had been also identified in the context of the high antioxidant status of the crop (Jiménez-Sánchez et al., 2016; M. Wang et
al., 2003) and they have been promoted earlier for their potentially health-boosting characteristics (Martin et al., 2011). As mentioned above, another main class of the chemical constituents in asparagus is the steroidal saponins, which were of great interest in a number of earlier studies (Shao et al., 1996, 1999; Shimoyamada et al., 1996). Next to the reported antitumor properties of asparagus saponins, it was suggested that there are also antifungal properties of the saponin which was first reported as yamoscin (Shimoyamada et al., 1996). The structure of this saponin is 3-O-[α-L-rhamnopyranosyl (1-2)α-L-rhamnopyranosyl (1→4)-β-D-glucopyranosyl](25s)spirost-5-ene-3-β-ol which is likely also the saponin named yamogenin II (Z. Sun et al., 2010). The reported saponin was isolated from the lower parts of white asparagus spears which are generally discarded as waste (Shimoyamada et al., 1996). This together with the analysis of Fan et al. (2015) represent possibly interesting bioresource application opportunities for the crop. More recent studies have taken advantage to expand on these data and enrich our knowledge of the potential anticancer properties of asparagus steroidal compounds (X. F. Huang et al., 2008; Liu et al., 2009; J. Wang et al., 2013; F. Zhang et al., 2020).

There are also health-related aspects regarding the group of S-compounds for which asparagus has become well known (see above). Organic compounds such as isothiocyanates, allicin, and sulphides that are present in garlic, onion (Allium spp.) and broccoli (Brassica oleracea) are already being applied to help decrease LDL (bad cholesterol) and blood pressure, and in preventing cancer (Le Bon & Siess, 2000; Parcell S., 2002). Asparagus spp. have indeed already been associated with hypocholesterolaemia effects (García et al., 2012; Vázquez-Castilla, De la Puerta, et al., 2013; Visavadiya & Narasimhacharya, 2009). As discussed above, in asparagus the central S-compound is asparagusic acid. Derivatives of asparagusic acid, together with some other S-compounds from asparagus were observed to inhibit cyclooxygenase 2 (COX-2) activity, which is an inducible enzyme associated with inflammatory diseases and carcinogenesis (Jang et al., 2004). A targeted metabolomics approach for S-compounds, using LC – Fourier Transform Ion Cyclotron Resonance (FTICR) MS, detected a new S-compound in asparagus spears, which they called asparaptine (C_{10}H_{19}N_{4}O_{3}S_{2}) and contains the same 1,2-dithiolane ring present in asparagusic acid. Asparaptine has been proposed to have inhibitory activity against the ACE, which plays a role in hypertension regulation in humans (Nakabayashi et al., 2015; Sanae & Yasuo, 2013). These
findings might imply that asparaptine shall gain a crucial role for the pharmacological image of asparagus (Miyoshi et al., 2018; Nakabayashi et al., 2021).

Despite there still being no reported human trials to verify the many potential health benefits of asparagus consumption, or the compounds listed above, interest in asparagus phytochemicals is growing. References to traditional medicinal applications and consideration of the aforementioned studies on potential health benefits of different compound groups present, place asparagus again in the spotlight as a potential source of novel bioactives for which the pharmaceutical industry is urgently searching.

2.5. Asparagus flavour

Asparagus flavour can be divisive; many love the delicate bitter complexity, while others are revolted from what can seem a strange vegetal tang. The typical asparagus flavour plays a central role in its promotion. The so-called ‘white gold’ (white asparagus) is usually considered to have a milder and more delicate taste than its green counterpart. Being considered a delicacy of the vegetable world, when in season (March till mid-June in Europe) asparagus provides the main ingredient in several dishes, both in haute cuisine as well as in traditional food dishes. Asparagus is often reported with aroma descriptions similar to grassy white wines such as unoaked Sauvignon Blanc (Aroma of the Week: Wine Aroma Kit: Asparagus, 2016). Other equivalent wine descriptors that have been linked to asparagus include fresh, savoury and bitter senses (Seal, 2017). These experiences are accredited to specific volatile compound classes and in particular to aldehydes, pyrazines and S-compounds. The intensity balance of the compounds can affect how appealing, or not, the overall flavour is to an individual consumer. While as yet there are few publications on asparagus flavour, a start has been made. A summarized list, based on the literature, of the main key odorants in cooked asparagus is presented in Table 2.2. The associated perceived aroma attributes have also been added where these are known, together with the odour thresholds of these compounds in water. Based on these figures, clear differences in potential flavour impact are observed. However, odour thresholds are dependent on the type of food matrix (Perry & Hayes, 2016; Tournier et al., 2007) and this must be taken into consideration in any evaluation.

Flavour can be studied both in a descriptive and an analytical manner. The descriptive way corresponds to the results of human sensory panels, where trained panellists assess the
flavour, in the form of aroma and/or taste and texture of a food product (J.A. et al., 2002; Tournier et al., 2007). The analytical route refers to the techniques used to obtain the metabolite profile of the food product of interest and the two might be joined to some extend by exploiting Gas Chromatography Olfactometry (GC-O) approaches. Which metabolites are observed depends not only on which are actually present in the sample but also which specific extraction, separation and detection methods will be used for the analysis. Metabolomics tools are nowadays increasingly being applied for both qualitative and quantitative analysis of food materials (Creydt & Fischer, 2020a, 2020b; Diez-Simon et al., 2019). Non-volatile compounds that may contribute to the taste, such as semi-polar secondary metabolites, are usually analysed using NMR (Nuclear Magnetic Resonance) or LC-MS methods, while volatile compounds that may contribute to the aroma are analysed using GC-MS techniques. However, the application of comprehensive (untargeted) metabolomics approaches have hardly yet been exploited for asparagus. Analytical chemistry studies on the asparagus flavour date from four decades ago (Hoberg et al., 1999; R. Sun et al., 2002; Tressl, Bahri, et al., 1977; Ulrich et al., 2001; Ulrich & Hoberg, 2002). Combinations of analytical techniques with sensory panels have also been used with the aim to unravel the aroma and taste of asparagus in terms of individual chemicals and to correlate these to cultivars and environmental factors (Dawid & Hofmann, 2014; Hoberg et al., 2003; Tressl, Bahri, et al., 1977; Ulrich et al., 2001; Ulrich & Hoberg, 2002). However, most of the studies performed have tended to focus on a limited pre-defined list of compounds, which have been proposed to be the key odorants. This list, reported here in Table 2. 2, contains C6 alcohols and aldehydes and other volatile organic compounds derived from the fatty acid degradation pathway, as well as some S-compounds and pyrazines which may be formed during cooking / processing, due to heat treatment. As a result, such an approach may be biased in driving conclusions repeatedly to existing known compounds and runs the risk of misinformation. This might better be tackled with more advanced approaches using initially an untargeted more holistic analytical methodology.

The final flavour of asparagus, as of any vegetable, is a rather complex combination of aroma and taste. The levels of bitterness seem to be a crucial characteristic for acceptance by consumers (Hoberg et al., 1999, 2008; Vilgis & Vierich, 2017). Bitter-tasting saponins (mainly furostanol saponins (Kawano et al., 1975, 1977)) likely play an important role here
and interestingly, different saponins are responsible for the bitter taste in fresh and in cooked asparagus (Dawid & Hofmann, 2012a, 2012b, 2014). Hoberg and Ulrich (2008) proposed what might be the right balance between sweetness and bitterness, as based on European consumer preference.

Figure 2. 3 represents a re-worked summary of the data from that study where it was recommended that breeders and producers should aim for increased sweetness and reduced bitterness in white asparagus cultivars for an improved consumer sensory experience (Hoberg et al., 2008). However, flavour perception and assessment are highly dependable on societal habit and local culture. Therefore, any such conclusions should take into consideration the season as well as the geographical location / social custom of the reported analysis. For the clearest picture, sensory evaluations should be combined with analytical assessments, in order to allow us to build up the most detailed picture and to correlate specific (groups of) compounds with specific sensory experiences.

All in all, asparagus flavour is a complex delicacy for those who admire it. Based on the literature and the aroma attributes of specific volatile compounds, we constructed the sensory wheel shown in Figure 2. 4. This can be seen as the Asparagus Sensory Wheel, summarizing all the aromas, the right balance of which creates the characteristic cooked asparagus aroma (the ‘typical odour’ as indicated in Figure 2. 3).
### Table 2.2 Summarized list of the key odorants in cooked asparagus flavour, based on the literature. The concentration in asparagus, odour threshold in water (*Odor & Flavor Detection Thresholds in Water Database*, 2019) and the characteristic aroma attribute of each volatile are also indicated if known (MW: molecular weight in g/mol, nq: not quantified, nd: not determined).

<table>
<thead>
<tr>
<th>Volatile Compound, Molecular formula (MW)</th>
<th>Concentration in asparagus (ppb)</th>
<th>Odour Threshold in water (ppb)</th>
<th>Aroma attribute</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>dimethyl sulphide, C₂H₆S (62.14)</td>
<td>3300</td>
<td>0.12</td>
<td>Sulphurous, onion-like, asparagus</td>
<td>(R. Sun et al., 2002; Tressl, Bahri, et al., 1977; Ulrich et al., 2001; Vilgis &amp; Vierich, 2017)</td>
</tr>
<tr>
<td>2,3-butanedione (diacetyl), C₄H₆O₂ (86.09)</td>
<td>nq</td>
<td>8.6</td>
<td>Sweet, buttery, caramel</td>
<td>(Tressl, Bahri, et al., 1977; Ulrich et al., 2001)</td>
</tr>
<tr>
<td>3-methylthio-propionanal (methional), C₄H₈O (104.17)</td>
<td>nq</td>
<td>0.2</td>
<td>Sulphurous, cheesy, cooked egg, baked potato</td>
<td>(Ulrich et al., 2001; Vilgis &amp; Vierich, 2017)</td>
</tr>
<tr>
<td>2,3 -pentanedione, C₅H₁₀O (100.12)</td>
<td>nq</td>
<td>20</td>
<td>Buttery, caramel, roasted, nutty</td>
<td>(Tressl, Bahri, et al., 1977; Ulrich et al., 2001; Vilgis &amp; Vierich, 2017)</td>
</tr>
<tr>
<td>trans-2-hexenal, C₆H₁₀O (98.14)</td>
<td>13</td>
<td>17</td>
<td>Green, fresh, fruity,</td>
<td>(R. Sun et al., 2002; Tressl, Bahri, et al., 1977; Ulrich et al., 2001; Vilgis &amp; Vierich, 2017)</td>
</tr>
<tr>
<td>Hexanal, C₆H₁₂O (100.16)</td>
<td>100-260</td>
<td>4.5</td>
<td>Green, fresh, grass, woody</td>
<td>(R. Sun et al., 2002; Tressl, Bahri, et al., 1977; Ulrich et al., 2001; Vilgis &amp; Vierich, 2017)</td>
</tr>
<tr>
<td>2-methoxy-3-isopropyl pyrazine, C₈H₁₂N₂O (152.19)</td>
<td>nq</td>
<td>0.002-10</td>
<td>Earthy</td>
<td>(Tressl, Bahri, et al., 1977; Ulrich et al., 2001)</td>
</tr>
<tr>
<td>2,3-octanedione, C₁₀H₁₄O (142.2)</td>
<td>nq</td>
<td>nd</td>
<td>Cooked, buttery, dill-like, broccoli-like</td>
<td>(Tressl, Bahri, et al., 1977; Ulrich et al., 2001; Vilgis &amp; Vierich, 2017)</td>
</tr>
<tr>
<td>1-octen-3-ol, C₁₀H₁₄O (128.21)</td>
<td>42-300</td>
<td>1</td>
<td>Earthy, mushroom-like</td>
<td>(Hoberg et al., 2003; R Sun et al., 2002; Tressl, Bahri, et al., 1977; Ulrich et al., 2001; Vilgis &amp; Vierich, 2017)</td>
</tr>
<tr>
<td>2-methoxy-3-isobutyl pyrazine, C₁₀H₁₄N₂O (162.22)</td>
<td>nq</td>
<td>0.002-0.016</td>
<td>Spicy, earthy, green, sprout-like</td>
<td>(Ulrich et al., 2001; Vilgis &amp; Vierich, 2017)</td>
</tr>
<tr>
<td>2-pentylfuran, C₁₀H₁₄O (138.21)</td>
<td>2-165</td>
<td>6</td>
<td>Buttery, earthy</td>
<td>(Tressl, Bahri, et al., 1977; Ulrich et al., 2001)</td>
</tr>
</tbody>
</table>
**Figure 2.3** Re-worked summary of the data from (Hoberg et al., 2008). The sensory profile of asparagus with low (pink) and high (green) acceptability as was calculated based on the average of the sensory profiles of 12 different cultivars in Europe in 2004. The typical odour corresponds to the right balance of compounds and aroma attributes presented in Table 2.2 and Figure 2.4. The musty odour is basically due to unbalanced ratios of C₈ ketones and alcohols, based on the findings of (Harris et al., 1986).

### 2.6. Asparagus and the smelly urine story – a catalogue of misconceptions

One of the more noteworthy features of eating asparagus is the often observed and indeed, discussed consequence - in the form of the distinctly odorous urine of the consumer. Human physiology seems to go into overtime as the rapidity of this occurrence is remarkable in that this can be noticeable within just a few minutes (Ramamoorthy et al., 2018). Our growing understanding of this process makes for an interesting story of physiological, sensory and genetical intrigue. Table 2.3 gives a brief potted history of key seminal moments in this smelly urine story.
Figure 2.4 The Asparagus Sensory Wheel - constructed based on key odorants and sensory attributes from the literature reported in this review. The key odorants from Table 2.2 are included next to the main aroma attribute of their contribution for the typical asparagus odour. The key to a pleasant aroma profile is the right balance of all the aroma attributes and therefore, concentrations of the key odorants.

The phenomenon of odorous urine was already observed long ago, already being noted by Lemery in 1702 and later also referred to by many prominent authors and polymaths such as Arbuthnot (1731), Benjamin Franklin (1770) and Proust (1913). Only later did it become clear that not everyone actually produces smelly urine and furthermore, not everyone can smell it. These first reports from around the mid-18th century have interestingly been proposed to be linked to a change in agronomic practice at that time related to the start of using inorganic and organic S-containing fertilizers to boost plant yield (Waring et al., 1987). ‘Recognition of smelly urine appears coincidental with the start of the use of S containing fertilizers’ (S. C. Mitchell, 2001) and this relevance of sulphur quickly became clear once the first chemical investigations were carried out.
Looking into which components were potentially causal to asparagus-associated urine odour, in 1891 Nencki first reported a potential link to the presence of one S-volatile, methanethiol. Later results were subsequently questioned as it was considered that the extraction / detection methods used might induce the appearance of artefacts rather than reveal the true in vivo compounds (Waring et al., 1987). Later however, growing numbers of S-compounds were reported (Garhart & Pierce, 1977; White, 1975). Waring et al. (1987), using GC-MS, detected six S-compounds, including the originally reported methanethiol, as well as dimethyl sulphide - a highly volatile, low odour threshold compound. Furthermore, both these compounds were also confirmed using trained panellists and standard compound solutions as ‘having a smell reminiscent of asparagus urine odour’. In 2001, Leitner reported in total 12 volatile S-compounds detected in asparagus urine, several of which also have low odour thresholds. Summarising the literature to date, Pelchat et al. (2011) provided the most extensive table of sulphur-containing odorants (n = 29) so far found in urine after asparagus consumption. These results strongly suggest that while S-compounds are potentially causal, it is also likely that the urine odour is the result of a complex mixture rather than a single component. The origin of these S-compounds is still a cause of speculation. Many of these odorants are not found in fresh asparagus (Waring et al., 1987) and also not after cooking (where indeed, their highly volatile nature would likely lead to their loss) (Tressl, Bahri, et al., 1977). This infers that they arise as the result of human metabolism working on chemical precursors, perhaps through the digestion of compounds such as S-methylmethionine and asparagusic acid (Tressl, Bahri, et al., 1977; Waring et al., 1987). Interestingly, the latter is considered to be unique to asparagus and is proposed as the ‘most probable culprit’ as the source of the specific asparagus urine odour (Stephen C. Mitchell & Waring, 2014).

A theoretical set of chemical conversions from asparagusic acid to the observed odorants has been proposed as being feasible but no evidence has yet been provided (Mitchell, 2001; Tressl, Bahri, et al., 1977). To elucidate this further we might consider a combination of an untargeted GC-based metabolomics approach coupled to two detection methods in parallel. A Mass Spectrometer would give in depth chemical information on those compounds present and a second detector – the human nose, in the form of a GC-O analysis using a sniffing port, would help us link individual compounds to bioactivities (odour).
Table 2.3 Seminal moments in the history of smelly asparagus urine.

<table>
<thead>
<tr>
<th>Year</th>
<th>Observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1702</td>
<td>Asparagus... causes a powerful/filthy and disagreeable smell in the urine as everybody knows.</td>
<td>(Lemery, 1702)</td>
</tr>
<tr>
<td>1731</td>
<td>‘...Of the Stems of Plants, some contain a sine Aperient Salt, and are Diaretick and Saponaceous, as Asparagus which affects the Urine with a Fetid Smell (especially if cut when they are white)...’ (- and later included in the definition of Asparagus in Samuel Johnstons First Dictionary of the English Language Vol 1 Edition 1 (1755))</td>
<td>(Arbuthnot, 1731)</td>
</tr>
<tr>
<td>1770</td>
<td>‘A few Stems of Asparagus eaten, shall give our Urine a disagreeable Odour’</td>
<td>(Franklin, 1770)</td>
</tr>
<tr>
<td>1913</td>
<td>but what fascinated me would be the asparagus, &gt;&gt;&gt; all night long after a dinner at which I had partaken of them, they played &gt;&gt;&gt; at transforming my humble chamber into a bower of aromatic perfume.</td>
<td>(Proust, 1913)</td>
</tr>
<tr>
<td>Scientific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1891</td>
<td>Urine smell proposed to be related to S-compound first identified as methanethiol</td>
<td>(Nencki, 1891)</td>
</tr>
<tr>
<td>1956</td>
<td>Polymorphism reported within 115 individuals – two genes associated with ability to produce/excrete the S-compound (methanethiol) in urine after eating Asparagus; excretor gene is dominant</td>
<td>(Allison &amp; McWhirter, 1956, 1957)</td>
</tr>
<tr>
<td>1975</td>
<td>Adding two S-compounds to urine resulted in the characteristic odour ‘likely through formation of methanethiol’</td>
<td>(White, 1975)</td>
</tr>
<tr>
<td>1980</td>
<td>328 Israelis divided into smellers/non-smellers but concluded that production was ‘universal’ and suggest also a genetically determined odour hypersensitivity in 10% of volunteers.</td>
<td>(Lison et al., 1980)</td>
</tr>
<tr>
<td>1987</td>
<td>800 volunteers eat asparagus – just under half 43% are excreters. Family studies also confirmed genetic polymorphism / Autosomal dominant gene and phenotype not age or sex related</td>
<td>(Mitchell et al., 1987)</td>
</tr>
<tr>
<td>2001</td>
<td>12 S-compounds identified (many with low odour thresholds) using SPME GCMS and excretion dynamics followed 10 min – 16h</td>
<td>(Leitner, 2001)</td>
</tr>
<tr>
<td>2010</td>
<td>First web-based GWAS survey of asparagus eaters. 63% smelled; But failed to recognise that there may be non-producers/smellers. Appears to stem from a single switched base-pair mutation in a cluster of 50 genes coding for olfactory receptors</td>
<td>(Eriksson et al., 2010)</td>
</tr>
<tr>
<td>2011</td>
<td>GWAs but solved issue limiting conclusions from Eriksson related to discriminating non-producers / smellers etc. Basis of inability to produce is still unknown but inability to smell is linked to an SNP in a 50 gene cluster on Chromosome 1.</td>
<td>(Pelchat et al., 2011)</td>
</tr>
<tr>
<td>2016</td>
<td>Confirmed results of Eriksson but again made the mistake of not discriminating non-producers from non-smellers.</td>
<td>(Markt et al., 2016)</td>
</tr>
</tbody>
</table>

In this way, we would not only get an unbiased overview of the chemical mixture, but we could reveal any potential links between individual components and their sensory relevance.

More than half a century ago it was recognized and reported for the first time that the phenomenon of humans producing smelly urine is actually not universal (Allison &
McWhirter, 1956). Allison and McWhirter (1957) reported a polymorphism within 115 individuals for the ability to produce and excrete odorous methanethiol after asparagus consumption, with the excreter genotype being dominant. Mitchell et al. (1987) reported that just 43% out of 800 volunteers were ‘excreters’ after asparagus consumption. In 1980, from a study of 328 Israelis of different ethnic backgrounds it was concluded that some people could not smell asparagus urine and that ca. 10% were the opposite, being ‘hypersensitive’ and still able to detect the odour after significant dilution (Lison et al., 1980). This phenomenon of anosmia (smell blindness) is a recognized genetically determined trait (Lison et al., 1980) as is hyperosmia or an over-sensitivity for certain odour compounds. Although there may be some ethnical relationships (S. C. Mitchell, 2001) it is evident that the global population comprises both excreters and non-excreters as well as smellers and non-smellers and indeed, in all possible combinations. Eriksson et al. (2010) performed the first 23andMe web-based analysis using voluntary information obtained from 10,000 subjects on the ability to smell ‘asparagus urine’. Despite failing to recognize the complication that many volunteers in the cohort will have been in the ‘smeller but non-excreter’ category and hence will give false negative information, the analysis did allow the identification of a genetic link to a region of Chromosome 1. A single switched base pair mutation was detected and interestingly, within a cluster of 50 olfactory receptor genes. Later, but taking into account the smeller / excreter complexity issues, Pelchat et al. (2011) were able to confirm the Eriksson finding and also concluded that the abilities to excrete / smell appear not to be genetically linked. A later study by Markt et al. (2016) remarkably also failed to recognize the complexity of this odour phenomenon but again reported 50-60% of individuals were anosmic and polymorphic for an SNP in the same Chromosome 1 region where the OR2 olfactory receptors are located as was previously identified.

To conclude this historical investigation which is still ongoing, the central current dogma is that firstly, almost certainly not all asparagus varieties produce the same amounts of substrate, and secondly, while certain individuals can secrete the smelly compounds but not smell them, others can smell the compounds despite not making them themselves and there are those that do both or neither. Thirdly, there also seems to be quantitative variation in both abilities.
2.7. The potential of asparagus metabolomics

Asparagus is a crop which has long been at the centre of attention due to its unique cultivation requirements, its typical flavour and its health-promoting potential. For *A. officinalis* there are many reports of not only its characteristic unique flavour (Dawid & Hofmann, 2012a, 2014; Hoberg et al., 1999, 2003; R. Sun et al., 2002; Tressl, Bahri, et al., 1977; Ulrich et al., 2001; Ulrich & Hoberg, 2002) but also its medicinal properties (Al-snafi, 2015; Bandaiphet & Kennedy, 2004; Debuigne & Couplan, 2009; Fan et al., 2015; Fanasca et al., 2009; Guo et al., 2020; Sara Jaramillo et al., 2016; Nakabayashi et al., 2015; Negi et al., 2010; Shimoyamada et al., 1996, 2011; J. Wang et al., 2013; Winter, 2005; F. Zhang et al., 2020). All of these features, as well as visible differences between e.g. varieties, cultivation strategy, etc. are directly related to the biochemical composition of the spears. However, our knowledge is still rather limited despite the importance of the crop on a global scale. Untargeted metabolomics approaches could very effectively be exploited to this purpose as these have great potential to advance our chemical knowledge and help us unravel the complex biochemical pathways in asparagus, covering both primary and secondary (volatile and non-volatile) metabolites. Plant metabolomics can contribute by exposing the complex physiology and biochemistry of plants as reported before (Hall, 2006; Hall et al., 2022). Furthermore, MS-based imaging (MSI) techniques such as laser ablated electrospray ionisation (Laser Ablation Electrospray Ionization LAESI; (Bartels & Svatoš, 2015; Etalo et al., 2018) and Matrix-assisted laser desorption/ionization – imaging MS MALDI-IMS (Nakabayashi et al., 2019), can provide additional useful information on the localization of metabolites *in planta*. Untargeted metabolomics approaches have already successfully been applied to investigate the metabolome of for instance eggplant fruits (Hanifah et al., 2018), tomato fruit (De Vos et al., 2011), potato tubers (Dobson et al., 2010) and coffee leaves (Souard et al., 2018), providing useful information for breeders and manufacturers. A combination of robust metabolomics technologies for analysing primary and both volatile and non-volatile secondary metabolites in asparagus will contribute in expanding and improving our knowledge about the crop.
For asparagus, most analytical chemical analyses carried out to date have generally been focused on specific (sub)groups of metabolites and have failed to reveal the true chemical complexity of the materials. This is also evident, by examining the currently used metabolite databases (October 2022): searching the key word ‘asparagus’ in the current largest food metabolite database FooDB (www.foodb.ca) retrieves 126 compounds. In the KNAPSACK-DataBase (www.knapsackfamily.com) 201 compounds are registered and the Dictionary of Natural Products (dnp.chemnetbase.com) includes 194 and of course these overlap to a great extent. Clearly, many more metabolites must be present but have yet to be identified and registered and metabolomics can play an important role here. Recently, *A. officinalis* spears (green) were used in a proof-of-concept study, applying a newly-developed tissue sectioning protocol to visualize and locate specific metabolites within plant tissues using MALDI-IMS (Miyoshi et al., 2018; Nakabayashi et al., 2019). Here, MALDI-IMS allowed the localization of asparaptine (recently detected as a new compound (Nakabayashi et al., 2015)) and revealed another metabolite ‘gradients’ in the spear adding to those from previous studies covering minerals and phenolics. Nakabayashi *et al.* (2019) also focused on flavonoids and correlation analysis revealed the flavonoid rutin to co-localize with a number of other metabolites across the different tissues analysed, suggesting a common biochemical regulation. The same study also showed a lower accumulation of rutin in those spear tissues not exposed to sunlight. This potentially contradicts previous results (Lee et al., 2010) where rutin was found to be more predominant in the upper spear sections than in lower ones. MS-based high-resolution imaging methodology (like MALDI-IMS) offers an additional spatial dimension for metabolite analysis and, together with complementary, comprehensive metabolomics approaches may help reveal the detailed metabolome of asparagus in both spatial and temporal terms. To our knowledge, an untargeted (unbiased) metabolomics approach, aiming to detect and compare as many metabolites present in asparagus as possible had not yet been applied earlier than 2019. Furthermore, few biochemical pathways related to quality traits in asparagus have been fully unravelled.

**2.8. General conclusions**

Asparagus is a major global crop of growing importance. Several *Asparagus spp.* have been used for their medicinal properties (Gaur, 1999; Kirtikar & Basu, 1984; Rana & Pandey, 2007; Visavadiya & Narasimhacharya, 2009) but nowadays, asparagus is mainly used and valued
as a vegetable. Appreciation from consumers depends on certain quality parameters mainly focused on appearance. Included here are colour uniformity, the length and thickness of the spear and the tendency of the head to split making the spear unsellable. However, important consumer-relevant quality parameters such as aroma and taste have largely been ignored by breeders. This is mainly because we still have only a meagre understanding of which chemical factors determine these phenotypic attributes and of their underlying biochemical pathways. Furthermore, how these quality parameters are genetically and environmentally influenced also remains largely a black box. Some studies have led to the proposal that there are certain key odorants (specific volatile compounds) that are considered mainly responsible for the typical asparagus flavour. However, the exact importance and contribution of each of these components still requires clarification. Exploiting unbiased, comprehensive metabolomics approaches might take us some way further in defining the complex biochemistry of this crop and identifying all the key compounds which underlie sensory quality and other crop properties.

Supplementary Tables are available online at http://www.mdpi.com/2218-1989/10/1/17/s1.
In the meantime we were harvesting, and later cooking, processing and tasting the spears.
Chapter 3

Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis)

Eirini Pegiou, Qingrui Zhu, Paraskevas Pegios, Ric C. H. de Vos, Roland Mumm, Robert D. Hall

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Abstract: Green and white asparagus are quite different crops but can be harvested from the same plant. They have distinct morphological differences due to their mode of cultivation and they are characterised by having contrasting appearance and flavour. Significant chemical differences are therefore expected. Spears from three varieties of both green and white forms, harvested in two consecutive seasons were analysed using headspace GC-MS and LC-MS with an untargeted metabolomic workflow. Mainly C₅ and C₈ alcohols and aldehydes, and phenolic compounds were more abundant in green spears, whereas benzenoids, monoterpenes, unsaturated aldehydes and steroidal saponins were more abundant in white ones. Previously-reported key asparagus volatiles and non-volatiles were detected at similar or not significantly different levels in the two asparagus types. Spatial metabolomics revealed also that many volatiles with known positive aroma attributes were significantly more abundant in the upper parts of the spears and showed a decreasing trend towards the base. These findings provide valuable insights into the metabolome of raw asparagus, the contrasts between green and white spears as well as the different chemical distributions along the stem.

Keywords: *Asparagus officinalis*; asparagus metabolome; GC-MS; LC-MS; crop metabolomics; spatial metabolomics
3.1. Introduction

Asparagus (Asparagus officinalis) is a perennial crop, where the spears (shoots), can be eaten as a nutritious vegetable. It has two main types: green and white, which can be derived from the same variety depending on the cultivation method. Green asparagus is harvested above-ground, while white asparagus grows fully immersed in soil wherein it is also harvested (Pegiou et al., 2020). The key difference in cultivation relates to growth with or without sunlight. Green asparagus is, by far, the most widely grown whereas white asparagus is more country-specific. In Europe for example, green asparagus is more commonly grown and consumed in e.g. UK and Spain while white asparagus is more popular in e.g. Germany and the Netherlands. In some countries, consumers speak about ‘asparagus’ without distinguishing the type as they are unaware of the white alternative (Pegiou et al., 2020).

For cultivating asparagus, the crowns should be planted such that they are ca. 20 cm deep. For green asparagus, once the shoots start to grow, they emerge above the soil, are exposed to sunlight, and elongate further. For white asparagus, ridges are made using an extra ca. 40 cm of topsoil and the shoots are harvested while still beneath the soil as they must remain in the dark (Pegiou et al., 2020). Exposure to light not only induces the synthesis of pigments such as chlorophyll and anthocyanins, but also, strongly determines spear morphology (Figure 3.1A). Green asparagus spears bear more prominent cladodes (leaf-like organs) (Figure 3.1B). Furthermore, white spears are generally significantly thicker, as in darkness, cells from the primary thickening meristem proliferate both periclinally and anticlinally (Maung et al., 2019; Schweingruber & Börner, 2018).

Figure 3.1 Clear differences in appearance are visible between white and green asparagus spears: (A) Three freshly harvested white and green spears; (B) Zoom in to reveal the cladodes and general tip morphology.
The composition of secondary metabolites in plants is tissue-specific and is influenced by developmental stage and genetic/environmental perturbation (Pott et al., 2019). Many different pathways are involved in secondary metabolite biosynthesis, including the phenylpropanoid, methyl-erythritol-4-phosphate (MEP) and mevalonate (MVA) pathways, which lead to the formation of many different volatile and non-volatile (poly)phenolic and terpenoid compounds (Pott et al., 2019). Volatile organic compounds (VOCs) formulate the aroma profile and, in addition to the aforementioned pathways, many other aroma compounds are formed by the conversion/degradation of fatty acids and amino acids into straight and branched medium-chain aldehydes, alcohols and esters (Gigot et al., 2010; Gonda et al., 2010; Pott et al., 2019), as well as heterocyclic compounds such as methoxypyrazines (Mortzfeld et al., 2020).

Aroma is one of the two key determinants of asparagus flavour (Hoberg et al., 2008; Tressl, Bahri, et al., 1977; Tressl, Holzer, et al., 1977; Ulrich et al., 2001). Thanks to the studies of Hoberg, Ulrich et al. on the aroma profile of cooked white asparagus (Hoberg et al., 1999, 2008; Ulrich et al., 2001; Ulrich & Hoberg, 2002), we can now refer to specific VOCs (e.g. dimethyl sulphide, 2-methoxy-3-isopropyl pyrazine, methional) as being the ‘key odorants’ also currently used as industrial references for flavour quality. Remarkably little research has been done on the metabolite profile of the raw vegetable. Such investigations are needed to provide the fundamental chemical basis to understanding the physiology and chemistry of the crop and to explore precursor pathways involved in the formation of flavour, also after food preparation. It is hypothesized that the flavour chemistry of green and white asparagus should have many commonalities, as they both have a recognizable asparagus taste, while contrasting compounds will be the basis of the nuances in green / white flavour.

Several studies on green or white asparagus have focused on the chemical composition of the crop, including e.g. minerals (Amaro-López et al., 1998), flavonoids (R. Fan et al., 2015; Jiménez-Sánchez et al., 2016) and saponins (X. Huang & Kong, 2006; Onlom et al., 2017). However, most studies only involve green asparagus although sometimes the type is not actually mentioned. A few studies have compared the chemical composition of both types, covering mineral composition (Makus, 1994), sensory characteristics (Cuppert et al., 1997), antioxidant capacity (Maeda et al., 2005), and the quantification of specific chemicals such as the flavonoid rutin and the saponin protodioscin (Lee et al., 2010). These last two studies
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis)

also compared different regions within the spears. Nevertheless, conclusions were limited as this work was performed using materials from different cultivation locations or used shop-bought materials of unknown varietal origin or cultivation history.

During / after harvest, much asparagus material is discarded (Pegiou et al., 2020). There is now growing interest to exploit this material to extract flavour compounds for the food industry, aiming for a more sustainable food production system. Most of this waste comprises stem bases (J. W. Siccama, Pegiou, Eijkelboom, et al., 2021). The study described here was performed with two main goals: to exploit untargeted metabolomics to investigate the chemical differences between green and white spears and to relate chemical differences to the contrasts in spear development and growth regime. We also performed spatial metabolomics to localize sensory-relevant metabolites within the spear in the context of exploiting waste materials in a circular bioeconomy strategy. The strength of our conclusions has benefitted greatly from the fact that we have been able to access all materials, both green and white, of three known, validated varieties all of which have been grown at a single commercial field location and all have been harvested, at the same specific times.

3.2. Results

3.2.1. Untargeted metabolomics of green and white asparagus spears

Three asparagus varieties in both green and white form were harvested and analysed per platform in a single randomised sample sequence, for two successive seasons (2019 and 2020). Both GC-MS and LC-MS raw data were processed in an untargeted manner based on the dedicated MetAlign - MSClust workflow. A number of technical replicates of a mixed sample were also analysed in each series to assess technical reproducibility (Quality Controls: QCs). The average variation in metabolite abundance within the QCs (RSD) was < 20 %, which is within acceptable limits for untargeted metabolomics studies.

Clear quantitative and qualitative differences were revealed with a first inspection of the raw GC-MS and LC-MS data plotted as total ion current (TIC) and base peak chromatograms, respectively (Figure 3. 2).
In total, 104 VOCs were detected in the headspace of green and white asparagus samples from the two harvest years, of which 88 were identified (including 61 common metabolites from 2019 and 2020). Of these 88 VOCs, 33 were unambiguously identified and 55 were tentatively annotated. The asparagus volatile profile mainly consisted of alcohols, aldehydes, esters, ketones and short-chain fatty acids with 2 to 13 C atoms (C₂ – C₁₃). Furthermore, 9 monoterpenoids and 10 benzene derivatives were tentatively identified. Other identified VOCs included 5 sulphur-containing compounds (S-compounds), namely methanethiol, dimethyl sulphide, dimethyl disulphide, dimethyl trisulphide and methional, together with 4 furans and 2 methoxypyrazines (Tables S1 and S2). The abundances of > 60 % of the detected VOCs in both years were significantly different between the two asparagus types (Table S3). The VOCs detected in both years as being discriminatory for the two types are summarized in Table 3.1A.

A total of 442 semi-polar compounds were detected in the asparagus spears. Based on the reconstructed (in-source) mass spectra provided by the MSClust tool, 76 compounds could be assigned with a monoisotopic mass and 56 could be putatively identified. Mainly flavonoids, other phenolic compounds, triterpenoid glycosides (saponins) and S-compounds were among the identified non-volatile secondary metabolites. At the beginning of the
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis)

chromatograms, additional polar compounds could be detected, and within these, arginine, aspartic acid, citric acid, sucrose, raffinose and uridine diphosphate glucose were tentatively identified (Tables S4 and S5). However, due to the high possibility of ion suppression effects in this crowded part of the chromatogram, these polar primary metabolites were not further examined in this study.

Not all non-volatile components were detected both in green and white asparagus. Both in 2019 and 2020, > 50 % of the detected compounds were discriminatory for the two asparagus types (Table S6). For those overlapping in both seasons, the ones that could be assigned with a monoisotopic mass and identified are summarized in Table 3.1B.

3.2.2. Comparison of the metabolome of green and white asparagus in 2019

Following the processing of the GC-MS and LC-MS data, a Principal Components Analysis (PCA) was performed separately per platform for the green and white asparagus data. In both cases, the QC samples are closely grouped and located in the middle of the plots, showing good technical reproducibility of both analytical platforms (Figure 3.3). Clearly, both the volatile and the non-volatile profiles of the two types are distinct, as in both datasets the green and white asparagus spears are separated along the first principal component (PC1), which explained 57.3 % and 50.6 % of the variation between the samples, respectively (Figure 3.3).

For both platforms, most (>50 %) compounds were detected at significantly different levels (adjusted \( p \) value \( \leq 0.05 \)) between the two types, confirming the PCA findings (Tables S3 and S6). In the case of VOCs, some alcohols and aldehydes, as well as a few short chain fatty acids and S-compounds were more abundant in the green type, while most benzene derivatives, monoterpenoids and some aldehydes were more abundant in the white type (Figure S3.1A and Table S3). Concerning the non-volatiles, the clearest distinction between the two asparagus types was in the composition of phenolics (more abundant in green) and saponins (more abundant in white) (Figure S3.1B and Table S6).
Table 3.1 Overview of characteristic (A) volatiles and (B) non-volatiles detected in either significantly different abundances between raw green and white asparagus (adjusted p value ≤ 0.05) or in only one asparagus type based on the two seasons. The CAS numbers (when applicable), experimental retention indices (RIs) and calculated monoisotopic mass (M) are given. The level of identification (LOI) provided is based on the Metabolomics Standards Initiative guidelines (Sumner et al., 2007). For a complete list of all detected compounds in both seasons (2019 and 2020) see Tables S1 – S6.

(A) VOLATILES

<table>
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<th>RI</th>
<th>LOI</th>
<th>Metabolite name</th>
<th>CAS</th>
<th>RI</th>
<th>LOI</th>
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(B) NON-VOLATILES

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<th>LOI</th>
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<th>CAS</th>
<th>M</th>
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</tbody>
</table>

Significantly more abundant in green

Significantly more abundant in white

Significantly more abundant in green

Significantly more abundant in white
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis)

Figure 3.3 PCA score plots based on (A) 84 volatiles and (B) 442 non-volatiles detected in the green and white asparagus spears harvested in 2019: The first two PCs are presented and the explained variance is shown in parenthesis on the axes. Colours indicate the asparagus type, symbol shapes indicate asparagus variety.

In both PCA plots, PC2 depicts an additional ca. 10% of the overall variation (Figure 3.3). Although the white asparagus varieties are all closely grouped for both platforms, the green varieties are separated across PC2 (Figure 3.3). Specifically, with regard to the volatile profiles the green varieties are all separate (Figure 3.3A), and for the non-volatile profiles, especially the green Avalim had a distinct profile (Figure 3.3B). The abundances of 11 VOCs and ca. 16% of the detected non-volatiles were found to significantly vary between the three green varieties (Tables S1 and S4).

In 2019, for practical reasons not all green varieties could be harvested at exactly the same time; Gijnlim and Grolim were harvested in calendar week 21 and the green Avalim was harvested in calendar week 23. Given this harvest-day variation between the green varieties, their metabolite profiles (volatiles and non-volatiles) were compared to determine if there was a potential harvest-day effect. The levels of the same compounds varying between the green varieties were also found to vary between the green asparagus harvested on the two different days in 2019 indeed suggesting a harvest-day effect. These compounds were ethanol, acetic acid, ethyl acetate, (E,E)-2,4-heptadienal, 2-methoxy-3-isopropylpyrazine, acetaldehyde, chlorogenic acid, and other detected flavonoid glucosides (Tables S1 and S4). A more in-depth analysis was therefore performed in the follow-up validation study in 2020.
3.2.3. Comparison of the metabolome of green and white asparagus in 2020

To validate and follow-up on the results from the first year’s analyses, in 2020, the same green and white varieties were harvested from the same field as in 2019. All varieties were harvested twice, with three weeks in between, to investigate potential genotype and harvest time-point differences. The sample preparation, the analyses, the examination and processing of the raw data were all carried out in the same way as in 2019.

PCA was performed separately on the complete 2020 datasets, both for the GC-MS and the LC-MS data. The QCs again indicate good reproducibility of the analyses (average RSD based on compounds present in QCs <20 %) and the green and white asparagus samples are again separated along PC1 (Figure 3. 4). Further statistical analysis showed the discriminatory metabolites for the two asparagus types of the 2020 season which corresponded to 64 % and 75 % of the detected VOCs and non-volatiles respectively (Tables S3 and S6). In agreement with the 2019 data, aldehydes and alcohols were highly abundant in the green varieties while white spears were characterized with high levels of a few other aldehydes. The differences in the levels of all benzenoids and monoterpenoids between green and white asparagus were less distinct based on the differential PC1 values (Figure S3.2A). With regards to the non-volatiles, the metabolites separating the green and white spears comprised polyphenols and saponins, respectively (Figure S3.2B), confirming the findings of 2019. Additionally, PCA was performed on the combined 2019 and 2020 GC-MS data on the 61 identified volatiles detected in both seasons verifying the distinct differences in the volatile composition of green and white spears (Figure S3.3).

The separation of the green varieties based on their volatile and non-volatile profiles along PC2 that was observed in 2019 data, was less evident in the 2020 data (Figure 3. 4). Further analyses comparing the profiles of the three varieties were performed, separately for the green and white asparagus and only 6 volatiles and 0 non-volatiles were found at significantly different levels (adjusted \( p \) value ≤ 0.05) between the three varieties in either the green or white type (Figures S3.4 – S3.5, Tables S2 and S5).
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis)

Figure 3.4 PCA score plots based on (A) 80 volatiles and (B) 255 non-volatiles detected in the green and white asparagus spears harvested in 2020: The first two PCs are given and the explained variance is shown in parenthesis on the plots axes. The ellipses indicate asparagus type, colour indicates the calendar harvest week, symbol shape indicates asparagus variety.

Within each asparagus type ca. 25% of the total variation in the profiles of volatiles and non-volatiles was caused by the harvest time-point, which also appeared to be type dependent (Figures S3.4, S3.5). The compounds significantly changing in calendar week 23 compared to week 20 are 2-methylfuran, toluene, 2-methoxy-3-isopropylpyrazine, 2-methoxy-3-isobutylpyrazine and methanethiol for the white spears and chlorogenic acid, one asparagusic acid ester isomer, heptanal, 3-methylthiopropanal (methional), nonanal and octanal for the green spears (Tables S2 and S5).

3.2.4. Non-discriminatory compounds in green and white asparagus

Metabolites that were detected at similar (FC < 2) or not significantly different levels (p value > 0.05) between green and white asparagus are also of interest. Such metabolites can be expected to be involved in general asparagus chemistry and contribute to the typical asparagus flavour characteristics, common to both types.

For both years, the metabolites with less than a two-fold difference between the two types were 2-heptanone, 2-methyl-3-buten-2-ol, 2-methylfuran, (E)-2-hexenal, methanethiol, (E)-2-octenal, 1-octen-3-one, hexanal, heptanal, (E)-2-heptenal, 2-pentylfuran, pentanal, dimethyl sulphide, dimethyl disulphide, 2-methoxy-3-isopropylpyrazine, asparagusic acid and its ester isomers (Tables S3 and S6). Moreover, the two asparagus types did not significantly differ with regards to 3-methyl-1-butanol, (E)-2-hexen-1-ol, 1-octen-3-ol,
dimethyl trisulphide, 2-methoxy-3-isobutylpyrazine and asparaptine (Tables S1 – S2 and S4 – S5).

3.2.5. Spatial metabolomics of the asparagus spear
Throughout the asparagus season, large waste-streams are generated which do not just consist of complete spears and indeed mainly comprises stem bases. It was hypothesized that there is a difference regarding the volatile composition along the spear. To test this hypothesis, a GC-MS analysis was performed to profile the secondary VOCs of 5 (for green) to 6 (for white) different spear sections taken from top to base (Figure S3. 6B).

The majority of the detected VOCs showed compositional heterogeneity along the asparagus spear for both the green and white types. A number of VOCs (20 in white, 11 in green spears) were more abundant in the upper parts with a decreasing trend towards the base, as exemplified by e.g. 1-penten-3-ol, 1-pentanol and 1-octen-3-ol. The opposite pattern was also found, as in the case of 4-methyl-3-pentanone. Interestingly, a few compounds, such as 2-methoxy-3-isopropyl pyrazine and 2-pentylfuran, showed contrasting gradients between the two asparagus types (Figure 3. 5).

**Figure 3. 5** Relative abundance of selected identified volatiles in shoot sections of the asparagus varieties in their green (5 sections) and white (6 section) forms. Colours are based on the asparagus type. The numbers on the x axis correspond to the relevant spear sections as indicated on the right of this figure; each of the upper 3 sections represents 2 cm of spear, while the following 3 sections are each 4 cm in length. Green spears were shorter than the white as demonstrated in Figure 3.1, thus there were fewer green spear sections available for analysis.
3.3. Discussion

This comprehensive (untargeted) metabolomics research has explored the complexity of green and white asparagus chemistry, how this is environmentally, developmentally, spatially and genetically influenced and attempts have also been made to link this to differences in asparagus flavour and sensory relevance.

3.3.1. Asparagus metabolome and differences of green and white spears

This study is the first to compare green and white asparagus volatile profiles in a holistic way revealing greater detail of their biochemical composition. Some of the identified aldehydes have been detected earlier in asparagus, such as pentanal, hexanal, (E)-2-hexenal, heptanal, and octanal (Chen et al., 2015; Hoberg et al., 2003; R. Sun et al., 2002). Other aldehydes detected in this study are reported in raw asparagus for the first time. Some of these, such as phenylacetaldehyde (benzeneacetaldehyde) and 3-methylbutanal are known vegetable VOCs, and their aroma attributes are green, fresh, floral, and fruity (source: www.foodb.ca). Among the unambiguously identified alcohols and ketones, 1-octen-3-ol, 1-penten-3-ol, 1-pentanol, and 2-butanone have been reported in (cooked) asparagus (Hoberg et al., 1999; R. Sun et al., 2002; Tressl, Holzer, et al., 1977; Ulrich et al., 2001; Ulrich & Hoberg, 2002). Furthermore, most of the monoterpenes and benzenoids, which are here reported for the first time in asparagus, are also already-known vegetable / fruit VOCs. For instance, styrene and limonene have been detected in blackcurrant, cauliflower, apple, cabbage, celery, carrot, etc., and their aroma attributes contain several fruity, fresh, and floral notes (source: www.foodb.ca).

Interestingly, in this study, a number of VOCs that have previously been reported in other asparagus materials (e.g. cooked white spears) was not detected. A few examples are 2,3-butanedione, 2,3-pentanedione, or 2,5-dimethylpyrazine (Pegiou et al., 2020). These are volatile compounds usually formed via heat-induced reactions (H. Zhang et al., 2020) and so this might explain their absence in the raw spears. With regard to the non-volatiles, a large number of the identified compounds has been previously reported in asparagus. These include compounds such as rutin (Lee et al., 2010), 3-O-feruloylquinic acid (Rodríguez-Arcos et al., 2004), asparaptine (Nakabayashi et al., 2015), protodioscin (Jaramillo-Carmona et al., 2017), as well as asparagusic acid (Tressl, Bahri, et al., 1977) and its ester isomers (Dawid & Hofmann, 2012a). However, it should be emphasized that here, when using a non-targeted
LC-MS approach without (semi)automated MS/MS spectral analysis, the majority of the metabolites detected could not readily be identified, indicating that there is a wealth of chemical information still waiting to be discovered.

One highlight of the study presented here is the list of discriminatory secondary metabolites for green and white asparagus, which was validated after analyses in two consecutive seasons (Table 3.1). In particular, some of the compounds that characterized the white spears were 1,2-dimethoxybenzene, \((E,E)\)-2,4-decadienal, shatavarin and protodioscin, while green spears were distinguished by significantly higher levels of 1-penten-3-ol, 3-pentanone, octanal, rutin and most of the identified phenolics (Tables 3.1, S3 and S6, Figure S3.3).

These differences might primarily arise due to their growing environment and the contrasting developmental processes which the two types follow. Green asparagus grows above the ground, while white asparagus stays below ground and often under impermeable plastic foil to avoid any exposure to sunlight. The distinct chemical differences between the two asparagus types (Figures 3.3 and 3.4, Table 3.1) imply that biochemical routes are differentially active in green and white shoots. These contrasts may be directly connected to the light / dark conditions of growth as we are comparing photosynthetic and non-photosynthetic systems where many differences might be anticipated. However, it is perhaps surprising how many common metabolites were found within the two systems although significant quantitative differences could be observed which may or may not be linked to the photosynthetic state. Worthy of mentioning here is that the metabolomics methods applied are not suitable to analyse the specific photosynthetic (apolar) thylakoid-bound compounds in green plant tissues like chlorophylls and carotenoids.

Different environments lead to the development of different plant traits (van Geem et al., 2013), and this can also be translated into variation in their metabolomes. Next to light, green asparagus is also more readily exposed to any changes in weather conditions and especially, temperature fluctuations (Herppich et al., 2008). Weather changes can result in the production and emission of \(C_5 - C_8\) aldehydes and alcohols (ul Hassan et al., 2015), which here were found to be more abundant in the green spears (Tables 3.1A and S3). The biosynthesis of plant polyphenols is linked to light and especially, UV exposure, where induced accumulation of phenolic compounds has been linked to plant protection (Neugart
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis) & Bumke-Vogt, 2021. In this study, such compounds were detected only (e.g. 3-O-feruloylquinic acid), or were at considerably higher levels (e.g. rutin), in green asparagus (Tables 3.1B and S6). On the other hand, white asparagus shoots grow entirely underground which can have a certain buffering / protective capacity to the growing shoots thus limiting weather-related abiotic stress fluctuations. In this study, a few benzenoids, monoterpenoids, unsaturated aldehydes and saponins were found to be more abundant in white asparagus (Tables 3.1 and S3). Subterranean organs in plants have evolved specific protective (chemical) mechanisms to withstand attack by pathogens and microorganisms (Döll et al., 2021). Some of these protectants might be carried over from the roots to the young shoots when still below ground. For example, higher saponin levels are typical of asparagus roots (Hayes et al., 2008; X. Huang & Kong, 2006) as well as white shoots compared to green ones (Table 3.1). In this regard, Dawid & Hofmann (2014) and Lee et al. (2010) found higher concentrations of saponins in the stem sections closer to the crown / roots of white asparagus, compared to the young asparagus tips.

This differentiation of the profiles in flavonoids (more abundant in green) and saponins (more abundant in white spears) might have been anticipated since previous studies have reported generally high flavonoid contents in photosynthetic tissues (Tohge et al., 2017), and high saponin levels in plant parts growing below-ground (Hayes et al., 2008; X. Huang & Kong, 2006). Using a complementary approach, Yi et al. (2019) performed de novo transcriptome sequencing in both green and white types of a commercial variety (Atlas) and found significant differences between the two asparagus types in that 21 % of the genes involved in the biosynthesis of the flavonoid rutin were upregulated in green and 4 % of the genes involved in the biosynthesis of the saponin protodioscin were upregulated in white asparagus (Yi et al., 2019). These results followed and confirmed other studies, which focused on quantifying these two compounds in green and white asparagus, as well as other flavonoids and saponins (Dawid & Hofmann, 2012a, 2014; Fuentes-Alventosa et al., 2008; Lee et al., 2010; M. Wang et al., 2003), suggesting that indeed metabolic pathways are differently active in green and white asparagus shoots.

The distinct differences in the metabolome of green and white asparagus also likely contribute to the distinct green and white flavour profiles, respectively. Cuppett et al. (1997) performed a sensory analysis on raw materials, showing that the main flavour variations
were: green, grassy and bitter notes for the green asparagus, and sweet corn, potato and buttery notes for the white type. This flavour variation can at least partially be explained by the specific VOCs that were shown in this study to be predominantly abundant in each asparagus type (Tables 3.1 and S1 – S3).

### 3.3.2. Non-discriminatory asparagus compounds in green and white spears

Green and white asparagus spears are different but they also have many commonalities and compounds that did not significantly differ between the two types (e.g. 1-octen-3-ol, dimethyl trisulphide, 2-methoxy-3-isobutylpyrazine and asparaptine) (Tables S1 – S2 and S4 – S5) can be proposed as ‘typical’ asparagus compounds, which may contribute to the general asparagus flavour characteristics, common to both types. In addition, the known cooked white asparagus odorants dimethyl sulphide, methional (3-methylthiopropanal), 2-methoxy-3-isopropyl pyrazine (Tressl, Bahri, et al., 1977; Ulrich et al., 2001), were also detected here in raw asparagus in both green and white types. These commonalities may emphasize the potential general importance of these sensory-relevant compounds to the overall asparagus sensory experience, as they are present in both types. Concerning the metabolites with FC<2 (e.g. 2-methylfuran, (E)-2-hexenal, methanethiol and (E)-2-octenal), despite how small the differences between the two types, these may still have impact in flavour perception (Chambers IV & Koppel, 2013). Future investigation and focus on such compounds is of relevance for confirming the nature of any flavour deviations.

### 3.3.3. Variety and harvest time-point effects on the asparagus metabolome

Concerning genetic background, relatively minor differences were observed especially for the white asparagus type (Figures 3.1 – 3.2, Tables S2 and S5). This might imply that fewer chemical differences mean similar taste and aroma profiles but this still needs to be tested. For the green type of the varieties, there did appear to be more varietal discrimination, especially in the 2019 season, which might also be assigned to the impact of the different harvest days. Initially, as harvest day differed by just a few weeks, this effect was not anticipated but to investigate, in 2020 the validation study was specifically designed to include two harvest time-points with three weeks in between. The set-up of this 2020 study was such that the only source of variation was the cultivation method (green or white) or the genetic background, at each harvest time-point. Again, no significant variation between the three varieties was found, in either green or white forms, thus confirming the findings.
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis) from 2019. This apparent lack of genotypic influence, as compared to spear type and harvest period, is perhaps surprising but may be related to all three varieties having been generated by the same breeding company. It is perhaps also worthy of mentioning here that while Grolim is usually only used to produce white asparagus, while the other two cultivars (Avalim and Gijnlim) are considered suitable as both green and white crops, this did not appear to be reflected in the biochemical profiles.

In the 2020 data, despite just a three week difference between harvests, clear harvest-day effects were observed in the metabolomes, which interestingly appeared to be type dependent (Tables S2 and S4). Importantly, these included the VOCs dimethyl disulphide, methional and 2-methoxy-3-isopropyl pyrazine. All three are known asparagus odorants (Ulrich et al., 2001). Such temporal variation in the asparagus metabolome has also been observed by Creydt et al. (2018), who suggested that even a one week period can have a high impact. These observations suggest that the asparagus aroma profile is dynamic and likely also (harvest) time dependent, but this would need further investigation using larger sample sets, in combination with taste trials.

For the non-volatiles, only a few compounds (27 % of total detected) appeared to be dependent on harvest day, but these did not include the putatively identified polyphenols and saponins, which were most abundant in green and white asparagus, respectively (Tables 3.1 and S4). Soteriou et al. (2021) have recently shown that there was a significant increase in total phenolics and a significant decrease in total sugars towards the end of the asparagus harvest season. This can be due to the fact that they sampled at longer time intervals across a 10-week season while we used only two time-points separated by 3 weeks. Future research should focus on fully defining the dynamics of variation on the asparagus metabolite profiles throughout a complete harvest season.

### 3.3.4. Metabolite distribution along the asparagus spear

Aiming to understand better the potential sensory quality of raw asparagus and whether there is a variation of metabolite composition along the shoot, different regions of the asparagus spear were analysed using GC-MS. Several VOCs were observed to have a compositional gradient from top to bottom (e.g. 1-penten-3-ol) or from bottom to top (e.g. 4-methyl-2-pentanone) of the spear (Figure 3.5). This observed heterogeneity in the distribution of the volatiles along the green and white spears matches with previous studies.
on asparagus which have focused on other types of analytes. For example, Amaro-López et al. (1998) and Makus (1994) studied the distribution of important minerals in the green and white asparagus shoots, and Creydt & Fischer (2020) have recently investigated the distribution of primary metabolites in white asparagus spears. Moreover, Dawid & Hofmann (2014) have demonstrated a quantitative gradient of asparagus saponins along the white asparagus shoot, while Lee et al. (2010) and Wang et al. (2003) have also shown a gradient of protodioscin along asparagus spears. Together, all these complementary observations clearly indicate considerable heterogeneity in metabolite presence within asparagus spears. Given the rapid growth of the asparagus shoots during the season, various developmental stages can be found along the spear, from the large upper meristem comprising mainly mitotic cells to the lower regions where only cell expansion is likely. Therefore, a more active metabolome might be expected in the young asparagus tips compared to the lower parts and considering their greater vulnerability to herbivore and pathogen attack, a more extensive protective chemical arsenal may have evolved. In this study, the metabolite composition in the tips was indeed generally chemically richer (Figure 3.5) than that of the basal regions and complements preliminary findings by Nakabayashi et al. (2021), who described the localization of asparaptine in asparagus shoot apical meristems. This observed heterogeneity in the chemical composition has particular relevance in the context of circularity and the exploitation of waste streams (J. W. Siccama, Pegiou, Eijkelboom, et al., 2021). During the asparagus harvest season, ca. one-third of the harvested asparagus material is discarded as waste; both as imperfect whole spears as well as the trimmed-off stem bases removed to produce equally-long spears for the supermarket (Pegiou et al., 2020). This huge volume of biomass is seen to be a potentially valuable source of asparagus aroma and flavour chemicals (J. W. Siccama, Pegiou, Zhang, et al., 2021). Considering the chemical variation observed here, it should therefore be born in mind that different waste streams may produce different quality end-products. For example, as the majority of the VOCs with fruity, fresh, bitter and floral aromas, were found to be more abundant in the upper parts of the asparagus spears, waste materials containing more of this tissue and fewer stem bases will likely yield a richer aroma profile which is also more typical of the whole spear. However, this needs further investigation and verification as not all
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis)

Compounds contribute equally to sensory impact hence such an analysis needs to involve both profiling of the key bioactive metabolites and proper aroma and flavour evaluation.

3.4. Materials and Methods

3.4.1. Asparagus plant materials

All materials used in this study originated from a single field location where the green and white crops were grown side by side. The green and white spears of three asparagus varieties were included, Avalim, Gijnlim and Grolim. All three varieties are 100% hybrid F1 plants. It is relevant to note that while both Avalim and Gijnlim are considered suitable for both green and white asparagus production, Grolim is less suited as a green crop because it can have a tendency to give more open heads under sub-optimal temperatures. The asparagus plants were fully established as a commercial crop and had been cultivated by a local grower (Bennekom, the Netherlands) since 2017. White asparagus was grown in drills under translucent plastic foil, while green asparagus plants were grown in the standard manner in an open field. The shoots were harvested in the 2019 and 2020 seasons.

In both seasons, harvesting of spears was done on a single day of a specific week and always around 10 a.m. In 2019, all white asparagus samples were collected on 2nd May (calendar week 18); green spears of Gijnlim and Grolim were harvested on 22nd May (calendar week 21), and green Avalim on 5th June (calendar week 23). In 2020, all varieties were harvested on 13th May (calendar week 20) and on 5th June (calendar week 23).

Nine asparagus spears were collected per variety and per time-point and were directly transferred on ice to the lab. These were carefully rinsed under cold water and separated into three pooled samples of three spears per variety. Each pooled sample was prepared as described in Figure S3.6. All pooled samples were subsequently ground in liquid nitrogen using a grinding mill (IKA®, Germany), and the powders were stored at -80 °C until further analysis.

3.4.2. Chemicals and analytical standards

Calcium chloride (CaCl₂) and Ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (the Netherlands). Ultrapure water (Milli-Q™ Reference Ultrapure Water Purification System) was used for the preparation of the EDTA solution (stock solution of 90 mM). A mix of n-alkanes (C₆ – C₂₁) was prepared. All alkanes were purchased from Sigma-Aldrich (the Netherlands). The analytical standards used for metabolite identification had a
purity between 96-99 %. All standards were all purchased from Sigma-Aldrich (the Netherlands) except for methanethiol, 1,3-diethylbenzene, styrene and 1,4-diethylbenzene which were purchased at Greyhound Chromatography (UK). All standards were dissolved in methanol (Biosolve BV, The Netherlands). Methanol, formic acid and acetonitrile (Sigma-Aldrich, the Netherlands) were used for the extraction and analysis of the non-volatile semipolar compounds.

3.4.3. Sample preparation for volatile analysis

For each ground sample, 500 (±3) mg fresh weight was transferred to a glass 10-ml GC screw-cap vial, with an ND18 magnetic screw cap fitted with a 8 mm silicone/PFTE septum (BGB Analytik, Harderwijk, The Netherlands). Samples were kept in liquid nitrogen until 0.73 g CaCl$_2$ dihydrate and 0.5 ml 90 mM EDTA-NaOH (pH 7.5) were added to give final concentrations of 5 M and 50 mM, respectively, to deactivate endogenous enzymes as previously described by Tikunov et al. (2005). Subsequently, samples were thoroughly vortexed until homogeneous. For the preparation of the QC samples, 500 mg of all ground green and white spears were mixed.

3.4.4. Extraction of volatiles and analysis with HS-SPME GC-MS

The trapping of volatiles by SPME and the GC-MS settings were essentially as described by Diez-Simon et al. (2020) with certain modifications. The asparagus extracts were incubated at 50 °C for 15 min with agitation (300 rpm), and subsequently, volatiles in the headspace were extracted by inserting the SPME-fiber PDMS/DVB/CAR 50/30 μm diameter, 1cm length, (Supelco, PA, USA) for 15 min at 50 °C without agitation using an MPS-2 autosampler (Gerstel, Germany). Analytes were transferred to an Agilent GC7890A gas chromatograph coupled to a 5975C quadrupole mass spectrometer by thermal desorption in a Gerstel CIS4 at 250 °C for 2 minutes in split-less mode under a constant helium flow of 1ml/min. The column used was a Zebron ZB-5MSplus with dimensions 30 m x 0.25 mm i.d. x 1.00 μm film thickness (Phenomenex, the Netherlands). The GC oven temperature started at 45 °C for 2 min, then increased at a rate of 8 °C/min to 250 °C, and finally at a rate of 15 °C/min to 280 °C and maintained for 3 min. The column effluent was ionised by electron impact at 70 eV with a scan range of m/z 33–330. The MS interface temperature was set to 280 °C. A mix of n-alkanes (C$_6$ – C$_{21}$) was analysed with the same method to calculate the retention indices (RIs).
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3.4.5. Extraction of semi-polar non-volatiles and analysis with LC-MS

The extraction and analysis of non-volatile semi-polar components were performed based on the method described previously by De Vos et al. (2007). An aliquot of 300 (±3) mg fresh weight from each ground sample was taken to extract the semi-polar compounds in 99 % methanol containing 0.133 % formic acid (FA) in a 3:1 ratio (ml methanol-FA : mg sample) (final concentration 75 % methanol/0.1 %FA), followed by sonication and centrifugation for 15 min each. Chromatographic separation was done using an Acquity UPLC module (Waters) using a reversed Luna C18 column with dimensions 2.0 × 150 mm and 3 µm (Phenomenex, the Netherlands), at 40 °C, using a linear gradient from 5 to 75 % acetonitrile acidified with 0.1 % FA at a flow rate of 0.19 ml/min in 45 min. The injection volume was 5 μl. Compounds eluting were detected using a photodiode array detector (Waters 2996) and subsequently with a high-resolution Orbitrap FTMS mass spectrometer (Thermo Fisher Scientific, the Netherlands) in negative electrospray ionization (ESI) mode (m/z 95–1350), using the mass calibration and other settings as recently described by van Treuren et al. (2018). In the second year, tandem MS (MS/MS) spectra were also generated on QC samples for manual identification of specific compounds.

3.4.6. Processing of mass spectrometry data

Raw data were processed following an untargeted metabolomic workflow. Both GC-MS and LC-MS raw data were processed using the MetAlign software (Lommen, 2009) for baseline correction, peak picking (S/N > 3) and alignment of the mass signals. Mass signals that were present in less than three samples were discarded, considering that three biological replicates were analysed per variety. Subsequently, mass features were reconstructed to potential compounds using the MSClust workflow (Y. M. Tikunov et al., 2012). Volatile metabolites were identified based on matching of the reconstructed mass spectra and calculated RIs with authentic reference standards and using the NIST17 Mass Spectral library and in-house databases. Non-volatile metabolites were putatively identified based on their molecular ion mass match with the online databases KnaPSAck (http://www.knapsackfamily.com/), Dictionary of Natural Products (http://dnp.chemnetbase.com/) and mzCloud (https://www.mzcloud.org/) within a mass deviation of 5 ppm. In addition, MS/MS fragmentation patterns of selected compounds were manually compared to those in literature. Having analysed in negative ESI mode, the main
detected ions were \([\text{M-H}]\), its formic acid adduct \([\text{M+FA-H}]\), and for a few compounds also \([2\text{M-H}]\). The in-source and MS/MS fragments for most compounds comprised a loss of hexose (neutral loss of 162.05282), deoxyhexose (neutral loss of 146.05791), pentose (neutral loss of 150.052823), a methyl group (neutral loss of 14), \(\text{CO}_2\) (neutral loss of 44), and the aglycone of the related metabolite. All these indicative masses were used, when possible, to verify the annotations of the identified compounds. The levels of identification given follow the Metabolomics Standards Initiative suggestions (Sumner et al., 2007); level 1 identified compounds have been verified with authentic reference standards, level 2 identified compounds are those with a high match of mass spectrum (and RI for VOCs); level 3 are compounds with low match of mass spectrum (or RI for VOCs); level 4 are compounds that the match of mass spectrum (and RI for VOCs) is too low to provide a putative annotation.

3.4.7. Statistical analysis and visualisation tools
Prior to unsupervised multivariate statistical analysis, processed GC-MS and LC-MS data were log-transformed and autoscaled (van den Berg et al., 2006). The LC-MS dataset from 2020 was further corrected for batch effects based on injection order followed by an additional step of peak filtering, using the R-code package BatchCorrMetabolomics 0.1.14 as described by Wehrens et al. (2016). The processed GC-MS and LC-MS data were subjected to principal components analysis (PCA) separately per year. Furthermore, Student’s t-tests were performed for comparing the profiles between green and white types or the two time-point measurements, and analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) post hoc tests were performed for the comparisons between the three varieties per type. The packages used for these tests were sklear 0.24.1 (Pedregosa et al., 2011) and statsmodels 0.12.2 in Python version 3.9.2 (2021-02-19). The obtained \(p\) values were adjusted for False Discovery Rate (FDR) using the algorithm of Benjamini & Hochberg (1995). FDR adjusted \(p\) values equal or lower than 0.05 were considered significant. Visualization of data was performed using RStudio with R version 4.0.3 (2020-10-10), Python version 3.9.2 (2021-02-19) and Microsoft Excel for Microsoft 365 (version 2104, 2021).
3.5. Conclusions

In conclusion, the contrasting metabolite profiles of green and white asparagus have been observed and illustrated. The main differences were reproducible across two consecutive harvest years and can be linked to the cultivation method and the direct environment of the growing shoots. Certain previously-reported key asparagus volatiles and non-volatiles were detected at similar levels in the two types and many new metabolites (both identified and unknowns) have also been described. Such metabolites are proposed to contribute to the characteristic asparagus profile and may potentially influence sensory impact. Concerning the asparagus flavour profile, a considerable number of aroma compounds were found to be highly abundant in the upper parts of the shoots, suggesting these may have a richer flavour compared to the basal stem parts. These findings provide valuable insights into the metabolome of raw asparagus which can help to better understand crop chemistry. Finally, these results can contribute to reconsidering the applicability of discarded asparagus waste after harvesting as a valuable source of flavour compounds.
3.6. Supplementary Figures

**Figure S3.1** PCA of the GC-MS and LC-MS data of the green and white asparagus spears harvested in 2019. The first two PCs are presented and the explained variance is shown in parenthesis on the axes: (A) Loading plot of the volatile profiles. Colours indicate the compound class of the identified volatiles. Unidentified volatiles are coloured grey; (B) Loading plot of the non-volatile profiles. All non-volatiles are colored black except for identified saponins and polyphenols which indicated by the red and blue circles respectively.
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis)

**Figure S3.** 2 PCA of the GC-MS and LC-MS data of the green and white asparagus spears harvested in 2020. The first two PCs are given and the explained variance is shown in parenthesis on the plots axes. Colours indicates compound class of the identified metabolites. Unidentified compounds are coloured grey: (A) Loading plot of the volatile profiles; (B) Loading plot of the non-volatile profiles.
Figure S3. 3 PCA of the 61 identified volatiles detected in the green and white asparagus spears harvested and analysed in 2019 and 2020. Data were log-transformed and auto-scaled before fusing them: (A) Score plot of the first two PCs where the explained variance is shown in parenthesis on the plots axes. Colours indicate the asparagus type, symbol shapes indicate asparagus variety; (B) Loadings of the first PC sorted in descending order.
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis)

**Figure S3.** 4 PCA of the volatile profiles of the white and green asparagus spears, separately, harvested in 2020; The first three PCs are given and the explained variance is shown in parentheses on the axes. In the score plots, colour indicates the calendar week of harvest, symbol shape indicates asparagus variety: (A) Score and loadings plot of the second and third PCs of the analysis of the white varieties; (B) Score and loadings plot of first two PCs of the analysis of the green varieties; in the loading plots, colour indicates the compound class of the tentatively identified volatiles and unidentified volatiles are colored grey.
Figure S3. 5 PCA of the non-volatile profiles of the white and green asparagus spears; The first three PCs are given and the explained variance is shown in parentheses on the axes. In the score plots, colour indicates the calendar week of harvest in 2020, symbol shapes indicate asparagus variety. In the loading plot, colour indicates the compound class of the tentatively identified volatiles. Unidentified volatiles are colored grey: (A) Score and loadings plot of the first two PCs from PCA of the non-volatiles of white spears; (B) Score and loadings plot of the first and third PCs from PCA of the non-volatiles of white spears; (C) Score and loadings plot of the first two PCs from PCA of the non-volatiles of green spears.
Metabolomics reveals heterogeneity in the chemical composition of green and white spears of asparagus (A. officinalis).

**Figure S3.** Schematic representation of the composition of a pooled sample produced for the analysis of the asparagus varieties. For each pooled sample of three spears, the shoots were cut into 2 cm sections: (A) One piece per section (X) was taken across three spears to generate a pooled biological replicate sample representative of a whole spear; (B) For the spatial metabolomics pilot, the equivalent pieces from three spears were collected to make pooled samples per section. The numbers given in (B) correspond to the annotations in Figure 3.5; For green spears, 5 sections are shown as green shoots were shorter than white asparagus (Figure 3.1).

Supplementary Tables are available online at https://www.mdpi.com/2218-1989/11/10/708.
Chapter 4

Unravelling the seasonal dynamics of the metabolome of white asparagus spears using untargeted metabolomics

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Abstract
Introduction The white asparagus season lasts four months while the harvest period per field is eight weeks. Different varieties are better suited for harvesting early or late in the season. Little is known of the dynamics of secondary metabolites of white asparagus during the production season.

Objective Characterization of the metabolome of white asparagus spears covering volatile and non-volatile composition in relation to quality aspects.

Methods Eight varieties, harvested repeatedly during two consecutive seasons were analysed following an untargeted metabolomics workflow using SPME GC-MS and LC-MS. Linear regression, cluster and network analyses were used to explore the profile dynamics, unravel patterns and study the influence of genotype and environment.

Results The metabolite profiles were influenced by the harvest moment and genetic background. Metabolites that significantly changed over time were distributed across seven clusters based on their temporal patterns. Two clusters including monoterpenes, benzenoids and saponins showed the most prominent seasonal changes. The changes depicted by the other five clusters were mainly ≤ 2-fold relative to the harvest start. Known asparagus aroma compounds were found to be relatively stable across the season / varieties. Heat-enhanced cultivation appeared to yield spears early in season with a similar metabolome to those harvested later.

Conclusion The dynamics of the white asparagus metabolome is influenced by a complex relationship between the onset of spear development, the moment of harvest and the genetic background. The typical perceived asparagus flavour profile is unlikely to be significantly affected by these dynamics.

Keywords: White asparagus; Asparagus officinalis; untargeted metabolomics; seasonal dynamics; linear modelling; network analysis
4.1. Introduction

Asparagus (*Asparagus officinalis*) is a perennial crop, the shoots (spears) of which are mainly harvested during the spring and early summer months. Different varieties have been bred to be better suited for harvest at the beginning or end of the crop season and, for logistical reasons, there are often only one or two varieties cultivated in each field. The usual harvest period for each field is eight weeks, during which spears are harvested every other day. Thereafter, the plants are allowed to produce leafy shoots as normal in order to support root crown development and nutrient reserve accumulation for the following year. Based on these different parameters, growers plan their harvest regime to be able to provide products continuously over a ca. 4-month period (Pegiou et al., 2020).

The metabolite composition of plants is highly linked to the developmental stage, growth rate and genotype as well as to response mechanisms to abiotic and biotic stress. Changes in the metabolite profiles of crops underpin these processes and may also have significant impact on crop quality (e.g. texture, colour, flavour) (Hall, 2006; Jaramillo et al., 2007; van Treuren et al., 2018). During the asparagus season - for example in North Europe between March and June (Pegiou et al., 2020) - temperature fluctuations and weather changes regularly occur. Asparagus growers have developed cultivation method modifications not just to protect their crops from the uncontrollably changing weather conditions which could be detrimental to the spear yield and quality (Heißner et al., 2006), but also to enable earlier spear development than usual. Although not severe, the typically varying climatological conditions during the asparagus season might lead to metabolic changes of relevance to specific crop quality traits. Plastic mini-tunnels are regularly used but more impactful modifications such as using a warm water soil heating system (heated fields) or the use of greenhouses can be applied (Pegiou et al., 2020).

The quality of asparagus spears is strongly connected to the multitude of secondary metabolites formed during development (Pegiou et al. 2020). One important aspect of asparagus quality is its flavour and the metabolites that significantly contribute to the asparagus flavour profile comprise chemically-diverse molecules. These include the sulphur-containing compounds dimethyl sulphide, methional and asparagusic acid as well as 2-methoxy-3-isopropyl pyrazine, medium-chained carbonyls such as hexanal and
1-octen-3-ol, and bitter-related compounds such as saponins and flavonoids (Dawid & Hofmann, 2012a; Hoberg et al., 2008; Kawano et al., 1975; Ulrich et al., 2001).

Changes during the asparagus metabolome throughout the harvest period have so far been poorly studied. Using green asparagus, Soteriou et al. (2021) showed a decrease in total sugars and an increase in total phenolics during the harvest season. Earlier, using white spears, Creydt et al. (2018) studied the profile of secondary non-volatile compounds and the variation in materials harvested in various European countries and from different years. They suggested that there is a strong influence of both the local climate and specifically the temperature, and soil composition on asparagus metabolites. In a comparative metabolomics study using three asparagus varieties in both green and white forms, grown at a single field location and harvested at two time-points within one season, Pegiou et al. (2021) showed that even a 3-week harvest interval can have significant influence on the composition of secondary metabolites although the genetic background effect was minor. Altogether, these initial studies indicate that the metabolome of white asparagus is indeed influenced by environmental factors (e.g. harvest time-point, meteorological conditions, geographical location). However, limited knowledge is available regarding the genetic background effect, temporal changes of the secondary metabolome of asparagus spears and their interactions which may occur during the complete harvest period and which are of potential sensory relevance.

The central aim of the study presented here was to explore thoroughly the metabolite composition of white spears during the harvest season. It was hypothesized that the majority of secondary metabolites would show temporal changes and we aimed to investigate the extent of these temporal patterns. Complementary metabolomics platforms were used to analyse white spears of eight varieties grown and harvested at different times across the total asparagus season (early, middle and late). Spears were harvested in two consecutive seasons (2019 and 2020), from selected fields in the Netherlands where also contrasting cultivation methods were applied. We focused on the analysis of volatile and semi-polar non-volatile metabolites as both groups comprise compounds which are highly important for flavour and quality (e.g. Pegiou et al. 2020, 2021). In particular we explored the impact of environmental (harvest time-point, cultivation method) and genetic (variety)
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factors on the dynamics of the biochemical composition of the spears using the extensive 2019 data. Material from 2020 was analysed to validate observations made in 2019.

4.2. Material and Methods

4.2.1. Asparagus material

White spears of eight varieties were kindly provided by the commercial grower Teboza BV (Helden, The Netherlands). All varieties were grown following standard commercial cultivation practices, as described below. All fields had been enriched with natural fertilizers to ensure optimal nutrient concentrations and pH adjusted for the healthy growth of the crop. Spears were harvested from fields located in the South of the Netherlands in Limburg and North Brabant, all within a 25 km radius. For every sample, the variety, exact cultivation and harvest history could be unambiguously assigned. Post-harvest treatment of all spears was standardized. The varieties were chosen based on their growing phenology and included so-called early varieties (i.e. Avalim, Gijnlim, Fortems, Cumulus), harvested mainly in the beginning to middle of the season and late varieties (i.e. Backlim, Grolim, Primems, Tallems) harvested middle – end of the season. The four cultivation methods applied were 1) the standard open field method, 2) using mini-tunnels, 3) heated fields and 4) a greenhouse. The last two methods were applied specifically when the aim was to harvest spears artificially early in the season. In all cases, asparagus beds were covered with an opaque plastic foil to avoid exposure of the spears to sunlight following standard production practice.

Each variety was sampled at 3-8 time points from one specific field and the harvesting scheme, in both years, was arranged based on the planning for each variety, field and the grower’s advice (Table S1). On each sampling day, asparagus spears were freshly harvested and within 2-3 hours, transported on ice to the lab. Then the spears were carefully rinsed with cool water and further treated as described previously (Pegiou et al., 2021). Three biological replicates were generated, each consisting of three spears per variety and per harvest day (2019: 123 biological samples; 2020: 57 biological samples). Subsequently, the spears were cut into pieces, ground in liquid nitrogen, and powders were stored at -80 °C until further analysis (Pegiou et al., 2021).
4.2.2. Untargeted metabolomics

Solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) was applied to collect the volatile metabolite data as described previously (Pegiou et al., 2021). For this, 0.5 g frozen asparagus powder was mixed with 0.73 g CaCl$_2$ dihydrate (Sigma-Aldrich, The Netherlands) and 0.5 ml 0.09 M EDTA-NaOH (Sigma-Aldrich, The Netherlands) (pH 7.5) in a pre-cooled 10-ml ND18 headspace glass vial (BGB®, Germany). Vials were closed with magnetic screw caps (8 mm hole) with Silicone/PTFE septa (BGB®, Germany). A mix of all sample powders was prepared and 0.5 g aliquots were used as quality control (QC) samples which were analysed together with the biological samples. A series of n-alkanes (C$_6$ – C$_{21}$) (Sigma-Aldrich, the Netherlands), was analysed with the same SPME GC-MS method to calculate retention indices (RIs).

Ultra-performance liquid chromatography MS (LC-MS) was performed for the profiling of the semi-polar non-volatile compounds. The semi-polar compounds from each sample were extracted by mixing 0.3 g frozen powder with 0.9 ml 32.04 M methanol containing 0.035 M formic acid (Sigma-Aldrich, The Netherlands) followed by sonication and centrifugation, as described previously by De Vos et al. (2007). The LC-MS calibration and analysis settings were as previously (Pegiou et al., 2021). A mix of all biological samples was prepared and 0.3 g aliquots were used as QC samples and analysed together with the biological samples. All samples were analysed using both analytical platforms. GC-MS analyses were performed in 4–6 batches of 32 injections each. For LC-MS, samples were analysed in a single sequence of 2 batches. Per sequence or batch, 4–6 QCs were analysed. Raw data were processed following the established untargeted metabolomics workflow using the software packages MetAlign and MSClust (De Vos et al., 2007; Lommen, 2009; Y. M. Tikunov et al., 2012). After baseline correction, peak picking and alignment of the mass signals, mass features that were present in more than two biological replicates for each sample were retained. Subsequently, mass signals were reconstructed into potential compounds based on their correlation across retention time and the intensity pattern across samples. The relative abundances of the reconstructed compounds in the processed GC-MS and LC-MS data were log2-transformed and a correction for between-batch differences and within-batch signal drift was carried out, based on the QCs (Wehrens et al., 2016).
Volatile metabolites were identified by matching the obtained reconstructed mass spectra and calculated RIs with those of authentic reference standards and using the NIST17 Mass Spectral library and in-house databases. Non-volatile compounds were putatively identified by matching their molecular ion mass and other in-source fragments with those in online databases KnaPSAck (http://www.knapsackfamily.com/), and mzCloud (https://www.mzcloud.org/) and in previous reports on asparagus materials (Dawid & Hofmann, 2012a, 2014; Nakabayashi et al., 2015; Pegiou et al., 2020, 2021). Levels of identification (LOI) were assigned following the Metabolomics Standards Initiative guidelines (Sumner et al., 2007).

4.2.3. Statistical analysis and visualization tools

Statistical analysis and combining analyses outputs for visualization of data was performed using RStudio with R version 4.0.3 (2022.02.3+492), Microsoft Excel and PowerPoint for Microsoft 365 (version 2104, 2021).

The metabolite profiles were investigated by hierarchical clustering (HCA) of the compounds using a correlation-based distance and average linkage. The clustering of the temporal patterns was visualized in a heatmap displaying for each variety, harvest week, and cultivation type the average abundance of a compound, relative to the first sampled harvest week for that variety, i.e. foldchange (FC). FC was not computed for 7 volatiles and 50 non-volatiles due to ≥90 % missing values. In the heatmap, compounds (rows) were ordered according to the HCA outcome, while varieties and harvest weeks within a variety (columns) were placed in chronological order. Additional to HCA, the variation between the metabolite profiles was explored by principal component analysis (PCA) of the Pareto-scaled data matrix. HCA and PCA were performed using R packages dendextend, gplots, ggplot2 and basic R functions.

To investigate the temporal patterns for each compound in more detail, a subset of the data (varieties harvested at least 6 times in 2019) was studied by applying linear regression models. The linear model comprised fixed effects for genetic background (variety), harvest moment (time) and the interaction between variety and time. Time-trends were fitted for each variety using a natural spline with 3 degrees of freedom. For each variety, the temporal profiles were expressed in the model as logFC from the starting harvest time-point onwards and were labelled based on sampling week i.e. week 1-8. The significance of the effects
(variety, time, interaction) was assessed by moderated $F$-tests, taking into account the relationship between the mean and the variance of the metabolite abundances (Ritchie et al., 2015). A False Discovery Rate (FDR) correction was applied to the $p$-values. Adjusted $p$-values $< 0.05$ were considered significant. Linear regression analysis was performed using the R package *limma* (Ritchie et al., 2015).

The estimated temporal profiles of a set of metabolites were grouped by cluster analysis. The set comprised all compounds for which a significant effect of time had been detected by the linear model. Groups of highly correlated metabolites were identified by weighted correlation network analysis (WCNA) using a topological overlap (TOM)-based measure of the dissimilarity between the estimated temporal profiles (that were concatenated across varieties). TOM incorporates information on the direct association (correlation) between pairs of metabolites and information on their neighbourhood (interconnectedness) in a metabolite network that is estimated from the data. Consequently, it is considered a robust approach to assess the association between compounds and counteract the effects of spurious correlations (Yip & Horvath, 2007). Clustering by WCNA was performed in R package *WGCNA* (Langfelder & Horvath, 2008) selecting the 'signed' network option (only positively associated compounds clustering together), with the minimum module (cluster) size set to 10, and deepSplit $= 2$, minKMEtoStay $= 0.5$, and mergeCutThreshold $= 0.25$. Since scale-free-topology could not be achieved, the option softPower was set to a default of 12. Note that similar results were obtained by grouping metabolites using average-linkage HCA with a correlation-based dissimilarity measure (not shown).

To investigate potential influence on the asparagus flavour, the fitted time-trends of compounds that have been previously proposed to be sensory-relevant for white asparagus materials were examined in more detail, independent of their significance in the $F$-test. These were dimethyl sulphide, dimethyl disulphide, methanethiol, methional, hexanal, 2-hexenal, 1-octen-3-ol, 2,3-pentanedione, 2-methoxy-3-isopropyl pyrazine, 2-methoxy-3-isobutyl pyrazine, 2-pentylfuran (Ulrich et al., 2001), asparagusic acid (Dawid & Hofmann, 2012a; Tressl, Holzer, et al., 1977) and its derivative asparaptine (Nakabayashi et al., 2015), protodioscin, shatavarin IX (Dawid & Hofmann, 2014), rutin (Hoberg et al., 1999), and 3-feruloylquinic acid which is derivative of ferulic acid (Rodríguez-Arcos et al., 2004; Tressl, Bahri, et al., 1977).
The influence of the genetic background and cultivation method on the metabolome was studied by a linear model with a fixed effect for each variety and cultivation combination. The model was applied to subsets of the data. Each subset consisted of all varieties harvested at one specific time-point. Significance testing by moderated F-tests was carried out analogously to the procedure described above.

4.3. Results

4.3.1. Composition of the white asparagus metabolome

Following an untargeted metabolomics workflow used to process the acquired GC-MS and LC-MS data, 105 volatile and 261 non-volatile compounds were detected. Among the 89 GC-MS and 58 LC-MS metabolites which could be annotated were compounds from various classes including aldehydes (10 % of the annotated compounds), alcohols (7 %), flavonoid glycosides (18 %), furans (5 %), monoterpenes (10 %), pyrazines (1 %), saponins (10 %), sulphur-containing compounds (16 %) and some amino acids and sugars (12 %) (Tables S2, S3). PCA of the metabolite profiles of all varieties demonstrates that the variation between the QCs was less than the overall variation between the biological samples, indicating good technical reproducibility (Figures S4.1A,B).

To explore the abundance of the detected compounds across the harvest season, the profiles of the volatile and non-volatile metabolites were examined by HCA. To assist in the investigation of potential temporal patterns, the log2-transformed foldchanges (logFC) of the abundances of each metabolite were visualized in a heatmap where each column is one harvest week of one variety and varieties are in chronological order of their harvest period (Figure 4.1). The abundance of most metabolites (rows) appeared to change during the 2019 season as indicated by a colour change between the different harvest moments per variety (columns). The time-trends of the majority of metabolites appeared to differ between varieties throughout the harvest period. The volatile metabolites were distributed across three main clusters based on the formed dendrograms of the cluster analysis (Figure 4.1A). Compounds in cluster V1 (ca. 10 % of the total) changed over time without particularly showing a general or variety-specific trend. The majority of the volatiles in cluster V2 (ca. 40 % of the total), decreased across time for all the early varieties Fortems, Gijnlim, Cumulus and Avalim which were grown in a ‘mini-tunnel’, as demonstrated by the intense green colour (Figure 4.1A; yellow box). In contrast, for the late varieties Grolim, Fortems and
Backlim grown in a regular ‘open field’, these same V2 volatile compounds generally followed an increasing trend towards the end season (but with a fall-off for Backlim). The abundances of the volatile compounds in cluster V3 (ca. 40 % of the total) showed an increasing time-trend for Fortems, Cumulus, and Avalim grown in ‘mini-tunnels’, as indicated by the later intense red colour, while these generally decreased along the harvest period of Gijnlim, Primems and the ‘open field’-cultivated Fortems. (Figure 4.1A; orange dotted-line box).

Heterogeneity in the temporal changes was also observed with respect to the non-volatile metabolites which seemed to be distributed across four main clusters based on the dendrograms (Figure 4.1B).

Figure 4.1 (continues in the next page)
Figure 4.1 Heatmap and HCA of the time-trends of the abundances (rows) of volatile (A) and non-volatile (B) metabolites of white asparagus spears harvested in 2019. The abundance of each metabolite at each harvest week is expressed as a log2-foldchange relative to the first harvest time-point per variety. LogFC values have been truncated to a specific range (-6 to 6) for consistency. Columns represent consecutive harvest weeks per variety and the different varieties are separated by dotted blue lines. Varieties are ordered in chronological order of their overall seasonal harvest period. Bhf: Backlim heated field, Fgh: Fortems greenhouse, F.MT: Fortems mini-tunnel, G.MT: Gijnlim mini-tunnel, C.MT: Cumulus mini-tunnel, A.MT: Avalim mini-tunnel, P.MT: Primems mini-tunnel, T.MT: Tallems mini-tunnel, Gr: Grolim, F: Fortems open field, B: Backlim open field. Numbers indicate the harvest time-point per variety. Green indicates a decrease and red indicates an increase in metabolite abundance compared to the first harvest. Dendrograms show the clustering of the metabolites by HCA using correlation-based distance and average linkage. A Yellow solid line box highlights the majority of clustered compounds in V2. Orange dotted-line box highlights clustered compounds in V3. B Turquoise box highlights clustered compounds in N2. Pink dotted-line box highlights clustered compounds in N4.
The trends of the majority of compounds in clusters N1 and N2 (ca. 20 % of the total) were similar and increased across time specifically for Gijnlim (Figure 4.1B; turquoise box). The abundances of most compounds in N3 (ca. 45 % of the total) showed a mid-season peak for the early ‘mini-tunnel’ varieties Fortems, Gijnlim, Cumulus and Avalim while these metabolites tend to show a gradual decrease during the harvest period for late varieties Primems grown in mini-tunnel and Grolim and Fortems grown in open field (Figure 4.1B). The trends of most compounds in N4 (ca. 20 % of the total) were decreasing for Gijnlim in particular, but varied a lot for the other varieties (Figure 4.1B; pink dotted-line box).

The various trends observed for both volatile (Figure 4.1A) and non-volatile compounds (Figure 4.1B) indicated a complexity with respect to the seasonal dynamics of the secondary metabolome of white asparagus spears. The composition of the volatile profiles appeared to be influenced to some extent by the time of harvest (Figure S4.1C; yellow arrow). Regarding the non-volatile profiles, it appeared that Backlim, grown both in the open field and heated field, had a different composition to the other varieties (Figure S4.1D,E; red circles). To unravel further and assess the significance of the observed complex dynamics, more advanced data mining approaches were followed.

4.3.2. The seasonal dynamics of the asparagus metabolome

The explorative HCA and PCA of the metabolite profiles indicated that the harvest moment during the crop season and the genetic background are of potential relevance regarding the dynamics of the metabolome. The temporal profiles of all detected compounds were modelled for the standard 8-week harvest period. We focused on varieties sampled at least 6 times including the first and final week of their harvest period. These were therefore, Backlim (open field), Fortems (mini-tunnel) and Gijnlim (mini-tunnel) (Table S1a). The rest of the 2019 and the full 2020 data sets were subsequently examined for the potential validation of our findings.

Compounds which significantly changed over time regardless of the genetic background (significant time effect, but non-significant interaction) were examined, as were compounds which changed following variety-specific trends (interaction effect). The metabolites detected at significantly different levels between the three varieties at the start of their harvest are highlighted by the ‘Variety’ effect (Figure 4.2) and these are discussed further in later parts of this article.
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Figure 4.2 Venn diagrams of the number of (A) volatile and (B) non-volatile compounds that were significantly different between the three varieties at the start of their harvest period ('Variety'), the abundance of which was at least at one time-point significantly different compared to the first harvest moment ('Time'), and the time-trends which were significantly varying between the three varieties ('Interaction'). The lists of specific compounds can be found in Table S4a (volatiles) and S4b (non-volatiles).

The first analysis highlighted 28 volatiles (25 % of all volatiles) and 37 non-volatiles (14 % of all non-volatiles) which significantly changed over time throughout the harvest period in all three varieties (Figure 4.2; Time but not Interaction). Among these compounds were carbonyls with 6 – 8 carbon atoms (e.g. hexanal, \((E)\)-2-hexenal, heptanal, 1-octen-3-one), sulphur-containing compounds and two saponins (Table S4). The time-trends of 33 volatiles and 107 non-volatiles were found to be variety-specific (Figure 4.2; Interaction) and these compounds were mainly monoterpenes, benzenoids and saponins (Table S4). The profiles of approximately 40 % of the volatile (42) and non-volatile metabolites (103) were not significantly affected by either the genetic background or harvest time-points. Among these were unsaturated medium-chained aldehydes (e.g. \((E,E)\)-2,4-heptadienal and \((E)\)-2-decenal), two sulphur-containing compounds (i.e. dimethyl disulphide and S-adenosylhomocysteine), 2-pentylfuran, one kaempferol glucoside (772.20662Da), a few sugars and amino acids.

To investigate the prominent temporal patterns in detail, the focus was given to those 61 volatiles and 144 non-volatiles that significantly changed throughout the season for at least one variety (Time and/or Interaction effects in Figure 4.2A,B). The modelled time-trends (expressed as logFC relative to the first harvest) of these compounds were clustered based on TOM by applying WCNA. As, time-trends of the varieties were concatenated before
WCNA, metabolites were clustered based on the similarity of their time-trends per variety, i.e. metabolites 'behaved' the same over time within varieties, but the temporal patterns between varieties were not necessarily the same. This resulted in seven clusters (C1 – C7) across which 169 of the 205 metabolites were distributed. The other 36 metabolites did not end up in any of the clusters, i.e. the time patterns in these metabolites were non-significant according to TOM. Most metabolites ended up in clusters C1 (42 compounds) and C2 (36 compounds) and the time-trends depicted in these clusters changed the most during the season compared to the other clusters (Figure 4.3A,B). Metabolites in C1 including monoterpenes, benzenoids and some lipid products significantly decreased (≤ -4 logFC) towards the end season for the relatively early varieties Fortems and Gijnlim grown in 'mini-tunnels', while they showed a slight increase at the season end for the late variety Backlim grown in open field (Figure 4.3A). Metabolites in C2 comprised mainly saponins but also two volatile compounds (3-methyl-1-butanol and its acetate) and their trends were increasing specifically for Gijnlim (Figure 4.3B). The compounds in the other five clusters (C3 – C7) showed temporal patterns which were sometimes variety-specific, or showed only small changes (±2 logFC) across the season (Figures S4.2A-E). However, the abundances of some of those compounds followed a noticeable variety-specific trend. For example 1-methoxy-2-propanol and its acetate which clustered in C4 both notably decreased (< -4 logFC) across the season for Backlim (Figure S4.2B). The metabolites clustered in C5 included medium-chained volatile carbonyls and although they did not change more than 2-fold compared to the harvest start, their intensities decreased across the season for Gijnlim and Backlim (Figure S4.2C). To help with the interpretation of these clusters, representative metabolites from each cluster and the actual (non-modelled) trends of their intensities in all of the eight studied varieties were examined (line graphs in Figure 4.3A,B and Figure S4.2A-E). These analyses indicated a distinction with respect to temporal patterns of metabolite composition between the early and late varieties and these are summarized in Table 4.1.
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Figure 4.3 (continues in the next page)
Figure 4.3 (continues in the next page)
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Figure 4.3 Weighted correlation network analysis of the modelled time-trends of metabolites detected in white asparagus spears harvested in 2019. Metabolites were clustered based on their fitted time-trends using topological overlap as a distance measure. Two clusters (C1 and C2) of the seven that were formed and the time-trends of the clustered metabolites (rows) have been visualised in a heatmap. Each column represents one harvest week of one variety. Varieties are ordered in chronological order of their overall seasonal harvest period. Vertical blue dotted-lines separate the harvest periods of the varieties (F: Fortems, G: Gijnlim, B: Backlim and numbers indicate the harvest time-point). Clusters C1 (A) and C2 (B) comprised metabolites for which the time-trends showed the largest changes over time. A representative metabolite per cluster was examined with respect to the non-modelled time-trend for all eight varieties labelled same as in Figure 4.1 (line graphs in A and B). LogFC values in y-axis of line graphs have been truncated to a specific range (-4 to 4) for consistency. The other five clusters are presented in Figure S4.2 and all clusters are summarized in Table 4.1. Material from the 2020 crop season was analysed for validation of the findings (C, D). Lines in C and D are coloured according to variety name as in A and B (green: Fortems, black: Gijnlim, purple: Grolim, red: Backlim). Numbers indicate the sampled harvest time-point.

Material from the second harvest season was analysed using GC-MS and the PCA reflected and confirmed the influence of the harvest moment on the volatile profiles of asparagus spears as observed in the previous year’s samples (Figure S4.3A). Most of the observed time-trends could be validated and especially the prominent ones regarding the compounds clustered in C1 (Figure 4.3C) but also the subtle changes (±2 logFC) for the volatiles clustered in C3 – C7 (Figure S4.4). However, the Gijnlim-specific increasing trend for compounds that were clustered in C2 was not observed in the second season (Figure 4.3D).
Table 4.1 Overview of the dynamic character of asparagus metabolites during the 2019 crop season organised in 7 clusters determined by WCNA. The observed temporal patterns of the clustered metabolites are demonstrated by a representative modelled trend for the standard 8-week harvest period. Early season (variety) corresponds to a harvest period starting in March/April and late season corresponds to a harvest period starting in April/May. Trends (solid lines) are expressed as log2 foldchanges (logFC) relative to the first harvest moment. Dotted lines show 95% confidence intervals of the modelled trends.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Metabolites</th>
<th>Early season</th>
<th>Late season</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Monoterpenes, benzenoids, ketones, lipid products</td>
<td>![Early season graph]</td>
<td>![Late season graph]</td>
</tr>
<tr>
<td>C2</td>
<td>Saponins, quinic acid derivatives</td>
<td>![Early season graph]</td>
<td>![Late season graph]</td>
</tr>
<tr>
<td>C3</td>
<td>Quinic acid derivatives, lipid degradation products, asparagusic acid esters, 1-penten-3-ol</td>
<td>![Early season graph]</td>
<td>![Late season graph]</td>
</tr>
<tr>
<td>C4</td>
<td>Asparagusic acid, 1-octen-3-ol, unidentified non-volatiles</td>
<td>![Early season graph]</td>
<td>![Late season graph]</td>
</tr>
<tr>
<td>C5 – C7</td>
<td>C6-9 carbonyls, furans, sulphur-containing metabolites</td>
<td>![Early season graph]</td>
<td>![Late season graph]</td>
</tr>
</tbody>
</table>

*a* Gijnlim showed a distinct pattern (increasing and then levelling off)

*b* Changes <2 fold compared to start of the harvest period
4.3.3. The potential impact of seasonal dynamics on the flavour profile

To obtain some insights into whether the observed overall seasonal changes may have some impact on the flavour of the spears, we also specifically looked into a number of compounds already proposed in the literature to influence the characteristic asparagus flavour. In this way, we aimed to be able to predict the potential impact of the observed changes in metabolite composition on the asparagus flavour dynamics throughout the harvest period. The modelled 8-week trends from Backlim, Fortems and Gijnlim harvested in 2019 were used to follow the temporal patterns of 17 sensory-relevant metabolites (Figure 4.4). The regression analysis showed that dimethyl sulphide, methional, asparagusic acid, asparaptine, hexanal, \((E)\)-2-hexenal, 1-octen-3-ol, 2,3-octanedione, shatavarin IX and 3-feruloylquinic acid significantly change over time for at least one of the varieties (Table S4). The time-trends of these compounds, except for shatavarin IX (C2) and 3-feruloylquinic acid (C3) clustered in either C4, C5 or C7 cluster (Figure S4.2) indicating shifts which were limited within a 2-fold change compared to the start of the harvest period. The changes of most of these potentially sensory-relevant metabolites were indeed ±2FC (Figure 4.4). Moreover, no significant differences between varieties were found specifically for dimethyl sulphide, dimethyl disulphide, asparagusic acid, hexanal, \((E)\)-2-hexenal, 2,3-octanedione, 2-methoxy-3-isopropyl pyrazine and 2-methoxy-3-isobutyl pyrazine (Figure 4.4, Table S4a).

4.3.4. The influence of genetic background on asparagus metabolome

The genetic background of white asparagus spears also appeared to influence the metabolome. We examined the compounds that significantly varied between varieties either at the beginning or throughout the harvest period (Variety and/or Interaction effects in Figure 4.2A,B). These comprised 7 volatiles (methanethiol, 3-methylbutanal, 1,2-dimethoxybenzene and four unidentified) and 43 non-volatiles (including saponins, asparaptine, quinic acid) as well as 16 other compounds which did not significantly change over time (Table S4). We compared the abundances of these compounds between varieties harvested on the same day, and this was repeated for several time-points (calendar weeks 16 – 19). Varietal differences were observed as demonstrated for example by methanethiol (Figure 4.5A) and protodioscin (Figure 4.5B) when comparing the bar graphs of the same colour, while no significant differences were indeed found between the harvest moments of
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Figure 4.4 Modelled time-trends of selected flavour-relevant asparagus metabolites for the standard 8-week harvest period. The abundance of each metabolite at each harvest week is expressed as log2-transformed fold change relative to the first time-point of the harvest period per variety (green: Fortems; black: Gijnlim; red: Backlim). The 95% confidence intervals of the modelled time-trends are depicted (tinted coloured areas above and below time-trends). The horizontal red dotted lines indicate a 2-fold change relative to the first harvest moment.

4.3.5. The potential impact of cultivation method on asparagus metabolome

Modified cultivation methods (e.g. heated field and greenhouse) can be applied to mimic the warm temperature conditions of late spring and thus stimulate the premature development of new spears and enable harvesting earlier in the year (January-March). In the first season, we included samples harvested from a greenhouse (Fortems) and a heated field (Backlim) in early March (Table S1a).
Unravelling the seasonal dynamics of the metabolome of white asparagus spears using untargeted metabolomics

Figure 4.5 Abundance of selected metabolites in different white asparagus varieties harvested in different calendar weeks in 2019 (A, B, C, D) and 2020 (E, F). The selected metabolites were found to be significantly different between varieties but either, without changing over time within each variety (methanethiol, protodioscin) or also changing over time (1,2-dimethoxybenzene, asparaptine). Missing bars in A-D indicate that the variety was not harvested in that week.
The metabolite profiles of these spears appeared to be similar to those of the same varieties harvested in late May from ‘mini-tunnel’ (Fortems) and ‘open fields’ (Backlim) (Figure S4.1C-E; red arrows). During the second harvest season in 2020, we included additional harvest weeks of the heat-enhanced fields (Fortems greenhouse and Backlim heated field). Again we observed that the volatile profiles of the spears grown under heat-enhanced conditions were similar to those of spears harvested later in the season from ‘mini-tunnel’ (Figure S4.3; yellow circles) or ‘open field’ plots (Figure S4.3; blue circles). Although, some variation could be observed when examining individual compounds (Figure S4.4), the overall profile (Figure S4.3) and in particular metabolites which have previously been proposed to contribute to asparagus flavour did not significantly vary despite the interval of more than two months between the harvests of spears grown in heat-enhanced conditions and those in the regular fields.

4.4. Discussion

White asparagus is quite a unique crop as the vegetable is harvested while the developing spears are still fully submerged underground. The dynamics of the asparagus metabolome, in association with the growing conditions and the moment of harvest, has to our knowledge never been fully investigated. A valuable start was recently made for green asparagus where changes in levels of specific primary metabolites, total phenolics and minerals throughout the season were monitored (Soteriou et al., 2021). Asparagus chemical composition with regards to flavour and specific health benefits has also been the focus of a small number of studies. Some of these focused on specific aroma compounds (Hoberg et al., 2003, 2008) and bitter, non-volatile compounds (Dawid & Hofmann, 2012a, 2014) while others specifically studied the properties of the sensory-relevant sulphur-containing metabolites (Miyoshi et al., 2018; Nakabayashi et al., 2015, 2021; Yanagawa et al., 1972). However, all these reports only partly discussed, if at all, the potential influence of genetic background or harvest history of the asparagus materials on their chemical composition.

In the study presented here, we aimed to explore which chemical shifts occur during the harvest season with respect to the profiles of both the volatile and non-volatile secondary metabolites, given that the majority of these compounds can be relevant for quality traits - and in particular - for the typical asparagus flavour (Pegiou et al., 2020). Our findings highlight the dynamic character of the chemistry of the asparagus crop. This metabolic
dynamism during the crop season, reflected by the varying composition of monoterpenes, benzenoids and saponins, might be explained by the rate of spear development, conditions at the precise moment of harvest and the genetic background. However, with respect to flavour quality the overall picture is mainly one of stability regarding the composition of key odorants, suggesting the production of quite a uniform crop, irrespective of genotype or environment. Subtle variations in bitterness is however possible and subtle effects on flavour due to perturbations in individual sensory-relevant compounds cannot be excluded.

4.4.1. The composition of monoterpenes, volatile benzenoids and saponins during the asparagus season

While the asparagus season lasts almost four months, the actual harvest period of each production field is just eight weeks. Asparagus has been bred to create both early and late varieties with the former being able to start producing spears in the early months of the season while the latter performs best in the later months when temperature fluctuations are usually less extreme and average temperatures are higher (Pegiou et al., 2020). Here, we have shown that monoterpenes and benzenoids (clustered in C1) in white asparagus spears harvested between late March – early May (early season) decrease along the harvest period, while the same compounds slightly increased towards the end of the season for late varieties harvested between May – mid June (Figure 4.3A, Table 4.1). Interestingly, these monoterpenes and benzenoids also clustered in the V2 of the HCA where a clear differentiation between all the analysed early and late varieties was observed (Figure 4.1A). Previous studies have investigated the composition of these secondary plant metabolites with respect to plant growth and development as well as in response to environmental conditions such as light and temperature. Regarding plant monoterpene composition, most studies monitor the emissions of these volatile compounds, thus a direct conclusion for accumulation in the plants cannot be driven. Zawilak (2013) actually studied the content of monoterpenes and sesquiterpenes in essential oil of *Hyssopus officinalis* L. at various developmental stages, i.e. vegetative stage in June, beginning of flowering in July and full flowering in August. Monoterpenes were found to significantly decrease in the latest plant growth stage compared to vegetative stage. Whether this was due to increasing temperature, it was not examined, but in our specific case, the average air temperature in mid-May 2019, during the last 3 weeks of the harvest of early varieties was actually significantly lower.
compared to the previous weeks (11 °C compared to 21 °C) (source: timeanddate.com, weather in Helden, the Netherlands). Similarly in 2020, the average temperature in calendar week 18 was 10 °C and significantly lower than in week 15 when it was 22 °C (source: timeanddate.com) and again a decrease in the abundance of monoterpenes was observed (Figure 4.3C). This fall in temperature might therefore explain the observed decrease in monoterpene content.

Cheng et al. (2016) used petunia flowers, which is a model to study the regulation of the phenylpropanoid pathway and the biosynthesis of benzenoids in plants and they investigated the sensitivity of volatile benzenoids to varying light and temperature regimes. They showed that both short (10 h) and long-term (1 month) exposure of the plants to high temperatures (i.e. 22-28 °C instead of 16 °C) reduced the content of volatile benzenoids as analysed by SPME GC-MS using similar settings as used in this study. Therefore, the lower content of these compounds observed in asparagus at specific times might be explained by the varying temperatures that can occur during the harvest season. Although practically challenging, a controlled lab-type experiment would be needed to confirm this.

Saponins, which are widely distributed in the plant kingdom and are especially prevalent in the Asparagaceae, are products of isopentenyl pyrophosphate synthesis via the mevalonate pathway. Their composition in plants can be influenced by genetic background, developmental stage and spatial differential expression (Upadhyay et al., 2018). In Asparagus species, steroidal saponins are generally found to be more prevalent in the roots (Srivastava et al., 2018) and the basal parts of the spears (Lee et al., 2010). Dawid & Hofmann, (2014) also reported a decreasing gradient of saponin levels from the base to the tip of asparagus spears and these observations are likely related to their proposed function as underground protectants. The biosynthesis of asparagus saponins has not yet been investigated, but in this study the levels of saponins detected in white spears were found to decrease as the harvest season progressed (Figure 4.3B, Table 4.1). This might suggest that the plant need for saponins decreases later in the season. Alternatively, it may indicate that synthesis of saponins lags behind growth as the rate of the latter increases with rising temperature. Phrompittayarat et al. (2011) studied the content of saponins in Bacopa monnieri which is also a perennial plant, and they detected higher saponin content in older (4-month old) plants compared to plants that were 1-month old, while all were grown in
summer months. Our findings may also indicate a relationship between the developmental age of the harvested asparagus spear and the saponin content.

The observed patterns for the saponin content in the asparagus spear and all other metabolites clustered in C2 (which also clustered in N2 of Figure 4.1B – turquoise box) were notably differentiating for Gijnlim compared to the other varieties examined (Figure 4.3B, Table 4.1). However, this Gijnlim-specific pattern was not observed in the second season (Figure 4.3C,D and S4) even though the other prominent temporal (Figure 4.3, S4.2, S4.3, S4.4) and varietal (Figure 4.5) patterns were again visible. Variation between seasons of some asparagus varieties has been discussed previously with respect to spear physiology and yield (Creydt et al., 2018; Heißner et al., 2006), and our results therefore suggest that the synthesis / accumulation of metabolites may be differentially affected. Variation between seasons might occur due to e.g. environmental factors or varying growing conditions and this may impact certain synthetic pathways differently. However, climatically, both seasons (2019 and 2020) were essentially representative and involved no extreme weather conditions and contrasts between them.

4.4.2. Weighted correlation network analysis to unravel complex biological relationships

The temporal changes of the detected compounds during the harvest period have illustrated the complexity and dynamic character of the metabolome of white asparagus spears. No group of metabolites from all tested varieties was found to follow a similar general pattern along the season (Figure 4.1). To unravel this heterogeneity and potential compositional shifts, we applied WCNA to cluster the fitted trends that significantly changed over time and used a subset of data from the three varieties which had been harvested at least at 6 time-points. WCNA (or WGCNA) has often been used in ‘omics’ studies, where it has been shown to have great potential for clustering features (e.g. genes, transcripts, peptides, metabolites) which are of biological relevance (Dekker et al., 2022; DiLeo et al., 2011; Ding et al., 2019). This is due to the topological overlap being used for dissimilarity measure in addition to correlation (Langfelder & Horvath, 2008; Pei et al., 2017) and thus indirect relationships between traits, which are common in biological systems, are also taken into account. WCNA of the significant seasonal metabolite trends highlighted the dynamic character of the asparagus metabolome. The most prominent seasonal changes were found in metabolites
assigned to either clusters 1 or 2 (which together comprised ca. 20 % of all originally detected compounds) as discussed above in Section 4.4.1. Ninety-one compounds were clustered in the other five clusters of WCNA (C3 – C7). The changes for the majority of these compounds were small - close to or less than 2-fold relative to the start of the harvest (Figure S4.2). These relatively small temporal changes imply a small impact of time on the composition of these specific asparagus metabolites (Figure S4.2), as was also suggested previously (Pegiou et al., 2021). These metabolites are chemically quite diverse and comprised medium-chain carbonyls, furans, phenolics and sulphur-containing compounds (Table 4.1). Several of these are relevant for specific asparagus biochemistry, such as asparagusic acid (Mitchell & Waring, 2014) and particularly its flavour, including for example dimethyl sulphide, methional and hexanal (Ulrich et al., 2001).

4.4.3. Potential flavour dynamics during the asparagus season
With regard to white asparagus flavour which is one of the most important quality traits, focus in the past has been placed mainly on cooked materials. This work has helped identify a list of potentially key asparagus odorants (Hoberg et al., 2003; Ulrich et al., 2001). However, there has been no previous study investigating the impact of cultivation dynamics on the volatile profile of the raw vegetable, even though this is determinant to the flavour of the final cooked products. For this reason, we specifically looked at a subset of data for the known sensory-relevant asparagus metabolites. Most changes in individual metabolites were ≤ 2-fold compared to the first harvest (Figure 4.4). Moreover, high similarities between the three genotypes studied were found for the key sulphur and nitrogen containing aroma compounds. These findings suggest that there is likely to be no large seasonal or varietal differentiation with regards to asparagus flavour. However with respect to one specific sensory attribute, bitterness, our findings do suggest some variation during the season and potentially also between varieties may occur. The detected bitter compounds shatavarin IX (Onlom et al., 2017), furostane-3,22,26-triol (Dawid & Hofmann, 2012a) as well as other saponins did significantly change throughout the season and varied across varieties (Figure 4.4, Table S4).

4.4.4. The impact of heat-enhanced cultivation
Asparagus growers have developed innovative cultivation methods to expand the crop season and allow asparagus spears to develop and enter the market earlier. For white
asparagus, which is harvested while still below ground, opaque plastic foil is used to cover the ridges to avoid any exposure of the young spears to sunlight. Furthermore, the use of an additional plastic cover is also often used especially during the first half of the standard production season to create what is known as a mini-tunnel (Pegiou et al., 2020). This mini-tunnel creates a greenhouse-type microclimate which keeps the temperature around the asparagus bed more stable and slightly higher than the ambient weather conditions. This protects the plants during cold days and generally stimulates growth. Greenhouses can also be used where temperatures are controlled - again to allow the harvest of standard early varieties, even earlier, already in the winter. Following similar principles, circulating warm water using a network of tubing under the root crowns is also exploited in heated fields to cultivate selected varieties and again enable harvesting artificially early in the season. However, following standard cultivation practices, May - June is considered to be the ‘high asparagus season’ characterized by higher yields of better quality spears when compared to those obtained at the first harvest in March (Heißner et al., 2006).

To investigate the comparability of asparagus spears obtained via standard and heat-enhanced cultivation methods a range of materials was assessed. Heat-enhanced cultivation methods (Fortems greenhouse, Backlim heated field) were seen to yield asparagus spears with similar metabolite compositions to spears harvested from regular fields during the ‘high asparagus season’ (Figure S4.1C-E). Similar conclusions could be made for the second season’s materials (Figure S4.3). Flavour-relevant compounds and typical asparagus metabolites were observed to be present at similar levels from both cultivation methods. It is therefore proposed that enhancing the cultivation temperature is able not only to speed up the development process effectively, to deliver spears earlier, but also this delivers spears of equivalent quality to those obtained later in the season using standard field cultivation conditions.
4.5. Supplementary Figures

**Figure S4.1** Principal component analysis of the metabolite profiles of white asparagus spears harvested in 2019. The explained variance per principal component (PC) is shown in parentheses on the axes. Scores of PC1 against PC2 of log2-transformed and pareto-scaled GC-MS (A) and LC-MS data (B) including the Quality Control (QC) (red). In the remaining PCA plots after excluding the QCs, data points are colored according to the calendar week throughout the harvest season (C), according to variety name (D) and according to the cultivation method applied on the fields (E). The yellow dotted-line arrow in C indicates a possible time-trend in the volatile profiles. Red dotted-line circles in D and E indicate a possible effect of the variety and cultivation method. Red arrows in C, D, and E highlight the data points of spears grown in heat-enhanced conditions (greenhouse: green, heated field: red) harvested in February/March and of spears grown in mini-tunnels (purple) and open fields (magenta) harvested in May.

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Figure S4.1 (continued from previous page)
Figure S4. 2 Weighted correlation network analysis of the modelled time-trends of metabolites detected in white asparagus spears harvested in 2019. Metabolites were clustered based on their fitted time-trends using topological overlap as a distance measure. Seven clusters were formed (C1 – C7). C1 and C2 are presented in Figure 4.3. The time-trends of the clustered metabolites (rows) are visualised in a heatmap (C3: A, C4: B, C5: C, C6: D, C7: E). Each column represents one harvest week of one variety and varieties are ordered in chronological order of their overall seasonal harvest period. Vertical blue dotted lines separate the harvest periods of the varieties (F: Fortems, G: Gijnlim, B: Backlim). A representative metabolite per cluster was examined with respect to the non-modelled time-trend for all eight varieties which are labelled same as in Figure 4.1 (line graphs). All clusters are summarized in Table 4.1.

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Figure S4.2 (continued from the previous page)
Figure S4.3 Scores of principal component 1 (PC1) against principal component 2 (PC2) of log2-transformed and pareto-scaled GC-MS data of the 2020 harvest season. The explained variance per PC is shown in parentheses on the axes. A Data points are colored according to the calendar week throughout the season. B Data points are colored according to variety name. C Data points are colored according to the cultivation method applied on the fields. Yellow circles indicate possible similarities of the metabolite composition of Fortems harvested early in the season in greenhouse and late in the season from mini-tunnel. Blue circles indicate possible similarities of the metabolite composition of Backlim spears harvested early in the season from heated field and late in the season from regular open field.
Figure S4. 4 Time-trends of selected metabolites detected in white asparagus spears harvested in 2020. The selected metabolites are representatives of the WCNA clusters presented in Figure S4.2. Labels on the x axes indicate the variety name and cultivation method applied in the fields (gh: greenhouse, hf: heated field, MT: mini-tunnel) and numbers indicate the sampled harvest time-point.

Supplementary Tables are available online at
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Chapter 5

Elucidating the flavour of cooked white asparagus by combining metabolomics and taste panel analysis

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A modified version of this chapter is submitted to *LWT - Food Science and Technology*
Abstract

A typical feature of cooked white spears of *Asparagus officinalis* is their specific flavour. This has been the focus of several recent instrumental and panel studies. Few of these report on the comparison of raw and cooked materials which may lead to a deeper understanding of the flavour chemistry and compositional shifts occurring during food preparation. Here, solid-phase microextraction GC-MS and LC-MS analyses of raw and cooked spears were performed to profile asparagus metabolites. A taste panel evaluated flavour attributes of the same materials. Unsupervised and supervised statistical approaches were applied to investigate the profiles and link metabolomics and sensory results. Flavonoids, saponins and medium-chain volatile carbonyls were more abundant in the raw spears while sulphur-containing volatile compounds, oxidized fatty acids and phenolic acids were more abundant in the cooked spears. GC-Olfactometry-MS was also performed and revealed 35 odour-active volatile flavour compounds. We have identified the key biochemical pathways and chemical transformations relevant to asparagus 'flavour'.

Keywords White asparagus; GC-O-MS; metabolomics; flavour; Random Forest
5.1. Introduction

Asparagus (\textit{A. officinalis}) is a nutritious perennial crop, the spears of which grow and are harvested in the spring and early summer (March – June in N. Europe) (Pegiou et al., 2020). In N. Europe, white spears are preferentially cultivated, particularly in Germany where ca. 115,000 tons are produced per year (top producer in Europe) and in the Netherlands where production reaches ca. 17,000 tons per year (fifth in the top-producers in Europe) (\textit{Areaal Asperges per Gemeente in 2018, 2019}). Once in season, fresh white spears are highly appreciated and can be found on menu’s either as the main ingredient or as a vegetable side dish. A typical feature of white asparagus is its unique flavour. This is characterized by bitter, earthy, buttery and sulphury flavour attributes (Brueckner et al., 2010; Hoberg et al., 2008). There are many ways to consume the fresh shoots, for example raw and marinated, boiled, steamed, or grilled. However, the traditional way is to boil the top 10 cm without peeling for 9-10 minutes to get tender spears with the characteristic cooked asparagus flavour (Maeda et al., 2012). Using longer spears (ca. 20 cm) is also popular but requires the lower half of the spears to be peeled prior to cooking.

The heat treatment applied during cooking undoubtedly leads to alteration of the chemical composition of food accompanied by the formation of new flavours. Flavour is primarily the interaction of aroma and taste, and volatile and non-volatile secondary metabolites play critical roles in framing the flavour profile of food. Volatile carbonyls with a 5-9 carbon backbone, which have been shown to significantly contribute to fresh, green, grassy and earthy flavours of vegetables and mushrooms (Assaf et al., 1997; Chambers IV & Koppel, 2013), are derived from the enzymatic degradation of linoleic and linolenic acids (Matsui, 2006; Stolterfoht et al., 2019). Other flavour-relevant metabolites come from enzymatic degradation of amino acids. For example, valine and leucine are precursors of volatile methoxypyrazines (Sidhu et al., 2015) which are responsible for the earthy and beany aromas found in grapes (Lei et al., 2018) and bell pepper (Eggink et al., 2012). Methionine and S-methylmethionine are involved in the formation of various sulphur-containing aroma compounds, which requires enzymes (Bourgis et al., 1999) or heat (Scherb et al., 2009). This group of volatile compounds are typically perceived as onion, cabbage and garlic odours (Le Bon & Siess, 2000; Segurel et al., 2005). Taste-related attributes such as bitter, sweet and sour have been correlated more with the presence of non-volatile secondary metabolites.
such as flavonoids, saponins and terpenoids (Bayer et al., 2021; Dawid & Hofmann, 2014; Schwab et al., 2008).

Mass spectrometry (MS) - based metabolomics techniques are now being widely applied to evaluate the flavour chemistry and composition of food (Diez-Simon et al., 2019). Common techniques for analysing the volatile profiles include gas chromatography (GC), while liquid chromatography (LC) techniques are commonly used for examining non-volatile compounds. Ideally the combination of outcomes from both platforms leads to a better elucidation of flavour profiles and importantly, their associated pathways. It is then possible to further uncover the flavour of food by performing taste panel studies in combination with instrumental approaches.

In the case of white asparagus, the flavour of cooked spears has been previously studied by performing both instrumental and panel studies. Dimethyl sulphide has been characterized as the most critical asparagus odorant (Ulrich et al., 2001) and its heat-induced synthesis from S-methylmethionine has been shown upon cooking of vegetables (Scherb et al., 2009). Protodioscin, the saponin with highest intensity found in white spears (M. Wang et al., 2003), has been suggested to be the principle component responsible for the bitterness characterizing cooked asparagus (Brueckner et al., 2010). Ulrich et al. (2001) focused on the composition of volatile compounds that frame the aroma of cooked white spears which were analysed by performing GC Olfactometry (GC-O). According to the highlighted volatile compounds in this and other studies focusing on volatile flavour compounds in asparagus (Hoberg et al., 2003, 2008; R. Sun et al., 2002), a summarized list of proposed ‘key asparagus odorants’ has previously been reported (Pegiou et al., 2020). This list includes sulphur-containing metabolites (e.g. dimethyl sulphide, methional), medium-chained carbonyls (e.g. hexanal, 2,3-butanedione, 1-octen-3-ol) and pyrazines (e.g. 2-methoxy-3-isopropyl pyrazine) (Pegiou et al., 2020). However, only few studies report on the comparison of raw and cooked spears while one could argue that such an investigation may assist in unravelling the chemistry behind flavour.

The main goal of the study presented here was to evaluate the influence of thermal treatment on the composition of secondary metabolome of white asparagus by cooking the fresh spears by boiling in water. Solid-phase microextraction (SPME) GC-MS and LC-MS analyses of both
raw and cooked spears were performed to profile the volatile and semi-polar non-volatile compounds, respectively, given their link to flavour traits. We, also, aimed to discover which secondary metabolites frame typical ‘raw’ or ‘cooked’ asparagus flavours. Therefore, the same samples were evaluated by a taste panel and the data were fused with the metabolomics results to elucidate flavour-relevant metabolites. Analyses were repeated a second year / season using the same varieties and also from the same fields to validate our findings. GC-O-MS was also performed to detect the odour-active volatile metabolites in raw and cooked asparagus and to confirm their relevance.

5.2. Materials and Methods

5.2.1. Asparagus material
White spears of four varieties (Backlim, Cumulus, Fortems, Gijnlim) were kindly provided by the commercial grower Teboza BV (Helden, The Netherlands). All varieties were grown and harvested following standard commercial cultivation practices. Material used for this study was harvested on the same day (May 18th 2021 in first year and May 9th 2022 in the second year). Spears were stored in cold water (4 °C) in dark room overnight which is standard commercial practice. The day after, they were transported on ice to the laboratory and stored at 4 °C for ca. 18 h until further handling.

Spears were cooked in stainless steel pans in unsalted boiling water for 9 minutes with the lid on. Each replicate per variety was cooked in clean fresh water and a clean pot. For the metabolomics analyses, three pooled biological replicates of three spears per variety per condition (raw, cooked) were chopped and frozen in liquid nitrogen as described previously (Pegiou et al., 2021). Samples of raw asparagus were stored at -80 °C until grinding. After cooking, samples of cooked asparagus were put directly on ice for approximately 10 minutes, chopped into pieces and stored at -80 °C. All frozen samples were subsequently ground in liquid nitrogen to fine powder and the powder was stored at -80 °C until further analyses. In the second year five replicates per variety were prepared as aforementioned and analysed to increase statistical power, and the same analytical procedures were followed.

5.2.2. Metabolomics
Headspace SPME GC-MS was employed to trap and analyse the volatile metabolites as described previously (Pegiou et al., 2021). In short, 0.5 g frozen asparagus powder per
sample was weighed in a pre-cooled 10-ml ND18 headspace glass vial (BGB Analytik®, Germany) and mixed with 0.73 g CaCl$_2$ dihydrate (Sigma-Aldrich, The Netherlands) and 0.5 ml 0.09 M EDTA-NaOH (Sigma-Aldrich, The Netherlands) (pH 7.5). Vials were closed with magnetic screw caps (8 mm opening) with Silicone/PTFE septa (BGB Analytik®, Germany). Preconditioning of samples, trapping of volatiles and injection were fully automated using a Gerstel MPS-2 autosampler and operated using Gerstel MAESTRO software version 3.2. Each sample was preconditioned at 50 °C for 15 min, agitating at 300 rpm to release and equilibrate the volatiles to the headspace. Volatiles present in the headspace were absorbing onto a Polydimethylsiloxane/Divinylbenzene/Carboxen 50/30 μm diameter, 1 cm length fibre (Supelco, PA, USA) for 15 min at 50 °C without agitation. The SPME fibre was then thermally desorbed in split-less mode by heating the fibre at 250 °C for 2 min onto the GC column (Zebron ZB-5MSplus with dimensions 30 m × 0.25 mm × 1.00 μm, Phenomenex, The Netherlands) via a cooled injection system (CIS4, Gerstel, Germany) containing a glass liner with a constant helium flow of 1 ml/min. The GC-MS analysis settings for profiling were as described previously (Pegiou et al., 2021). A mixture of samples was used for each quality control (QC) sample and QCs were distributed along the analysis series and analysed in the same way as all individual samples.

Ultra-performance LC-MS was employed to analyse the semi-polar secondary non-volatile compounds. These were extracted by mixing 0.3 g frozen powder per sample with 0.9 ml 32.04 M methanol (Sigma-Aldrich, The Netherlands) and 0.035 M formic acid (Sigma-Aldrich, The Netherlands) in a 2-ml Eppendorf tube followed by sonication for 15 min and centrifugation for 15 min (Pegiou et al., 2023). The LC-MS calibration and analysis settings were as previously described (Pegiou et al., 2021). Chromatographic separation was done using an Acquity UPLC module (Waters) using a reversed Luna C18 column with dimensions 2.0 × 150 mm and 3 μm (Phenomenex, The Netherlands), at 40 °C, using a linear gradient from 5 to 75 % acetonitrile acidified with 0.1 % FA at a flow rate of 0.19 ml/min in 45 min. The injection volume was 5 μl. Compounds eluting were detected both in negative and positive ionization modes of a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific™, Germany) (Pegiou et al., 2023).

Chromatograms of total ion count (TIC) and specific mass ranges were explored using vendor software. GC-MS and LC-MS data were processed for each year following our standard
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Untargeted metabolomics workflow using the packages MetAlign and MSClust (Lommen, 2009; Y. M. Tikunov et al., 2012). For volatile compounds, retention indices (RIs) were calculated using a third order polynomial function and based on a series of n-alkanes (C<sub>6</sub>-C<sub>21</sub>) which was prepared from a set of stock solutions of the individual alkanes (Sigma-Aldrich, the Netherlands) and analysed using the same SPME GC-MS settings. Volatile metabolites were identified by matching the reconstructed mass spectra and calculated RIs with authentic reference standards and with data in the NIST17 and in-house mass spectral libraries. Non-volatile metabolites were annotated by matching the molecular ion mass and in-source fragments with those in available databases KnaPSAck (http://www.knapsackfamily.com/), and mzCloud (https://www.mzcloud.org/) and using data from previous reports on asparagus materials (Bergamasco et al., 2022; Dawid & Hofmann, 2012a, 2012b, 2014; Nakabayashi et al., 2015; Pegiou et al., 2020, 2021). Levels of identification (LOI) were assigned following the Metabolomics Standards Initiative guidelines (Sumner et al., 2007).

5.2.3. Team tasting panel

Team tasting was conducted at Wageningen University (Wageningen, The Netherlands) complying to 2020/2021 national measures and restrictions against the spread of COVID19 (e.g. mouth-masks, 1.5 m distance). The taste panel, in the first year, consisted of four members who each evaluated all materials from the four varieties, both raw (thin slices made using a vegetable peeler) and cooked. Cooked material was prepared as described above for the metabolomics analyses, but in a food-grade kitchen and served to the panellists immediately after cooking. The taste panel was repeated a year later by an extended panel of ten members (including those from year 1) who evaluated cooked materials of the same four varieties. All panel members were fully acquainted with white asparagus. All samples were labelled with a unique code and were thus served blind and evaluated one-by-one in randomized order. Panel members had ca. 8 minutes to evaluate each sample and after having tasted all samples, they discussed their experience and preferred samples within the panel. The list of attributes scored for each sample can be found in Table S5.1.

5.2.4. Statistical analyses and visualization tools

Statistical analyses and combining the outputs for visualization of data were performed using the R packages ropls (Thevenot et al., 2015), randomForest (Liaw & Wiener, 2002), pheatmap,
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`ggplot2` and `gplots` in RStudio with R version 4.0.3 (2022.02.3+492), MetaboAnalyst 5.0 (Pang et al., 2021), Microsoft Excel and PowerPoint for Microsoft 365 (version 2210).

To explore the processed GC-MS and LC-MS profiles, zero values were replaced by a value 1/5 of the smallest non-zero value per metabolite across all samples. Log10-transformed and Pareto-scaled data were used for unsupervised multivariate statistics. To explore the variation and main patterns based on the volatile and non-volatile profiles, Hierarchical clustering analysis (HCA) using Euclidean distance was performed. Observations were clustered using Ward's linkage method and metabolites were clustered using average linkage (Ward & Joe, 1963). HCA outcomes were visualized in heatmaps. To highlight the significantly different compounds between raw and cooked spears one-way Analysis of variance (ANOVA) was performed followed by correction of the \( p \) values for False Discovery Rate due to multiple comparisons (Benjamini & Hochberg, 1995). Volcano plots were constructed depicting the significant differences between raw and cooked materials (\( p \) adjusted < 0.05). To examine further important metabolites for the discrimination between raw and cooked materials, orthogonal partial least squares discriminant analysis (OPLS-DA) was performed for each of the two metabolomics datasets, as this is an approach which has a good potential for comparing two groups of samples with minimal risk of overfitting. The \( R^2_Y, Q^2_Y \) metrics and the \( p \) value after 100 permutation tests were assessed to evaluate the statistical significance of the models and check for overfitting (Pinto et al., 2012). Variable importance in projection (VIP) values >1.50 were considered relevant for feature selection. Specifically for the LC-MS data, due to the large number of metabolites, VIP > 1.90 were considered relevant.

Random Forest classification analyses were performed to highlight the compounds that were potentially specific for each variety. Analyses were thus executed per metabolomics dataset per condition (raw, cooked) and were done with 50 trees (ntree) and the predefined variable cut-off (mtry) of 15, as it is close to the square root of the total number of variables (metabolites), which is the recommended value by Breiman (2001). The performance of each Random Forest model was assessed by the out-of-bag (OOB) error rate (Breiman, 2001). Given that four varieties were studied, OOB < 0.25 was considered acceptable.
Regarding the outcomes of the taste panel, the average scores were visualized in a spider web plot. Student’s t-test was performed to assess the significant differences between raw and cooked spears as evaluated in 2021 and to assess pairwise the varietal differences based on the scored flavour attributes (significant \( p \text{ value} < 0.05 \)) (Table S5.1). In the second repetition, two replicates of one sample were offered and evaluated per panel member in a randomized order. The variation between the replicates of one sample was also assessed and no significant difference was found.

Random Forest regression models were generated to link the metabolomics and taste panel results and examine which of the metabolites correlate with specific flavour attributes. The GC-MS and LC-MS data from the first year (2021) were used and data of the flavour attributes that were assigned significantly different scores (Table S5.1) meaning those were distinguishable by the panel members. The analyses were done with 50 trees (ntree) and the predefined variable cut-off (mtry) of 15. Training and test datasets consisted of 14 and 11 observations, respectively, which were randomly selected. The mean of square residuals and % explained variance were assessed to evaluate the statistical significance of each model. Models with \( \leq 0 \% \) explained variance were considered unreliable and were not investigated further. Concerning the other models, variable importance was estimated by the increase in mean square error (%IncMSE) which expresses how much the percentage of prediction error increases by excluding each variable. Compounds with %IncMSE \( \geq 1.50 \) were considered relevant.

5.2.5. GC-Olfactometry-MS

Materials of raw and cooked white asparagus spears were analysed by SPME GC Olfactometry MS (GC-O-MS), to determine the odour-active volatile compounds. Samples from one variety (i.e. Cumulus) were evaluated as no significant varietal differences were found based on the previous analyses of the material included in this study. A GC column splitter outlet was used to split 1:1 towards the MS and the olfactory detection port (ODP2, Gerstel, The Netherlands). The SPME GC-MS conditions used were as described above for the metabolomics analyses. Four assessors were recruited within the laboratory to do the sniffing and assignment of specific aroma attributes (if any) to the individual peaks. Assessors were first trained by smelling the GC-O profile of a mixture of raw and cooked
material. Each assessor smelled the GC-O profile of one raw and one cooked sample and noted down the perceived aroma, the related retention time and the intensity.

5.3. Results

5.3.1. Metabolite profiles of raw and cooked spears of white asparagus

To examine the chemical shifts that may occur upon cooking, raw and cooked materials were analysed by SPME GC-MS and LC-MS to profile the secondary volatile and semi-polar non-volatile metabolites, respectively. Following our well-established untargeted metabolomics workflow, the GC-MS and LC-MS profiles obtained comprised 130 volatile and 1625 non-volatile compounds. These metabolite profiles were investigated using HCA. The two main clusters based on the dendrograms of the samples revealed that both the volatile (Figure 5.1A) and the non-volatile profiles (Figure 5.1B) changed considerably upon cooking of the spears. The abundances of most volatile compounds were more abundant in the raw spears while those of a few were more abundant in the cooked spears, as depicted by the dark brown colours in the heatmap (Figure 5.1A).

Figure 5.1 (continues in the next page)
Elucidating the flavour of cooked white asparagus by combining metabolomics and taste panel analysis

Figure 5.1 Hierarchical clustering analysis of raw and cooked white asparagus spear data for the volatile (A) and semi-polar non-volatile (B) metabolites detected by SPME GC-MS and LC-MS, respectively. GC-MS and LC-MS data were log-transformed and Pareto-scaled prior to clustering. Varieties are indicated by colours (red: Backlim, yellow: Cumulus, green: Fortems, black: Gijnlim, blue: QCs). Colour grades in the heatmap indicate high (dark brown) or low (light beige) relative metabolite abundance.

Regarding the composition of non-volatile metabolites, it appeared that some compounds were more abundant in the cooked and some in the raw materials (Figure 5.1B). These quantitative differences between the raw and cooked materials could already be seen just by inspecting the GC-MS and LC-MS chromatograms by eye (Figure S5.1). Certain highly volatile compounds appeared to have higher abundance in the cooked material (Figure S5.1A) while most of the other volatile compounds, eluting later, appeared to have lower abundance in the cooked material (Figure S5.1B). With respect to the non-volatile profiles, the major differentiation was in the retention time windows where flavonoids (Figure S5.1C), saponins (Figure S5.1D) and lipid oxidation products (Figure S5.1E) are expected. Samples of the water after cooking were also analysed and this indicated that the cooking water did not contain a high abundance of volatile compounds (Figure S5.2).
To examine those compounds that significantly changed after cooking, one-way ANOVA and OPLS-DA were performed. The levels of ca. 45 % of all detected compounds (82 volatile and 704 non-volatile) were found to be significantly different (ANOVA, \( p \) adjusted < 0.05) between raw and cooked materials. In particular, 11 volatiles were significantly more abundant in the cooked spears and 71 were more abundant in the raw spears (Figure 5.2A). Dimethyl sulphide, methional, 2,3-butanedione and acetic acid were ≥ 2-fold significantly more abundant in the cooked materials (Figure 5.2A) and specifically \( C_6-9 \) ketones and aldehydes and furans were ≥ 2-fold significantly more abundant in the raw ones (Figure 5.2A).

![Figure 5.2](continues in the next page)
Elucidating the flavour of cooked white asparagus by combining metabolomics and taste panel analysis

**Figure 5.2** Volcano plots of volatile (A) and non-volatile (B) metabolites in white asparagus spears that significantly changed after cooking (p adjusted < 0.05). The relative abundance of selected characteristic asparagus metabolites that did not significantly change after cooking (C) are presented in bar graphs where colours indicate the samples analysed (red: Backlim, yellow: Cumulus, green: Fortems, black: Gijnlim, blue: QCs). Error bars show standard deviation (n=3 and for QCs n=4).

For the non-volatiles, 344 metabolites were more abundant in the cooked materials, while 360 others were more abundant in the raw materials (Figure 5.2B). Non-volatile oxidized fatty acids, derivatives of linoleic, linolenic acid, and phenolic acids were ≥ 2-fold significantly more abundant in the cooked spears. Steroidal saponins, flavonoid glucosides, asparaptine, S-adenosylhomocysteine and a number of others were significantly more abundant (≥ 2-fold) in the raw spears (Figure 5.2B). The discriminatory metabolites for the raw (e.g. hexanal, 2-octenal, 1-octen-3-ol, 2-butylfuran), and cooked spears (i.e. dimethyl sulphide, phenolics and fatty acid derivatives) were confirmed by inspecting the VIP scores of the OPLS-DA (Figure S5.3). Some of the annotated compounds the abundances of which were not significantly different between raw and cooked spears, were methanethiol, S-methylmethionine ([M+2H]+ : 166.0864 at 3.18 min)), asparagusic acid ([M-H]− : 148.9737 at 16.69 min), and protodioscin ([M+HCOOH]− : 1093.5447 at 23.95 min) (Figure 5.2C). Results regarding the characteristic metabolites for the raw and cooked materials were in agreement with the duplicated analyses performed on the second-year samples (Figure S5.4A,B).

The genetic background did not appear to have a strong influence on the metabolite composition of the spears (Figure 5.1; coloured blocks per variety), especially in the case of the volatile profiles (Figure 5.1A). Random Forest classification analyses were performed separately for the GC-MS and LC-MS data to compare the metabolite profiles of the varieties studied. The OOB error of the models was 0.49 ±0.23 (Table S5.2) indicating that the overall
composition of secondary metabolites did not significantly differ between the four studied varieties, even though differences might be observed in the case of individual compounds (Figure 5.2C, S5.4).

5.3.2. Flavour differences between raw and cooked spears and between varieties
The flavour attributes that were evaluated during the team tasting were decided on beforehand based on the typical flavour notes of asparagus and were described to the panel members before the tasting session (Table S5.1). Both raw and cooked spears were evaluated by four taste panel members each of whom was able to clearly perceive flavour differences between the two materials. The raw spears were typically characterized by intense ‘green’ and ‘fresh’ flavour attributes but with low ‘buttery’ and no ‘baked potato’ flavours (Figure 5.3A). In contrast, cooked spears were characterized by ‘baked potato’, ‘buttery’ and ‘sulphury’ flavour attributes (Figure 5.3B). Significant differences between the two materials were perceived with respect to ‘grassy/fresh’, ‘baked potato’ and sweet flavours as well as regarding the smooth texture which characterized specifically the cooked spears (Figure 5.3, Table S5.1). Similar but not identical patterns for the flavour of the cooked materials were observed in the second year when ten panellists were involved (Figure S5.5, Table S5.1).

**Figure 5.3** Spider web plots presenting the average scores on the flavour attributes of raw (A) and cooked (B) white spears of four varieties of *Asparagus officinalis* (red: Backlim, yellow: Cumulus, green: Fortems, black: Gijnlim) evaluated by taste panel in 2021. Attributes that received significantly different scores between varieties (*p* < 0.05) are indicated with an asterisk (*).
The panel members could also perceive flavour differences between the four varieties and in particular regarding bitterness, sweetness and texture (fibrous, smooth) but also subtle variation with respect to ‘asparagus’, ‘grassy/fresh’ and ‘baked potato’ attributes (Figure 5.3, Table S5.1). The perceived dissimilarities between varieties regarding the cooked spears, although subtle based on the comments of the panel members, were partly in accordance with the second repetition (Figure S5.5). At the end of the tasting session, panellists were asked to note down their most and least preferred samples. In both years, Cumulus was distinguished by most panel members as being the most preferred while Fortems was distinguished by everyone as being the most bitter and due to this bitterness, it was the least preferred variety by most of the panellists.

5.3.3. Linking metabolomics and taste panel data
The results of the metabolomics analyses and the outcome of the taste panel evaluations have indicated a clear distinction with regards to certain flavour attributes of raw and cooked white asparagus. To investigate possible correlations between (individual) metabolites and the sensory attributes evaluated, the data were linked by applying Random Forest regression analysis. Only results of the flavour attributes that received a significantly different score ($p < 0.05$) between raw and cooked materials (Table S5.1) were included in these analyses (Table S5.3). The ‘baked potato’ flavour can be predicted (>65% explained variance and MSE 0.33) by 2,3-butanedione and two oxidized glycerophospholipids ($C_{27}H_{50}NO_{9}P$ and $C_{23}H_{42}NO_{7}P$) (Figure S5.6, S5.7). Based on the data available from this study, the sweet flavour and smooth texture were not significantly highly correlated with any of the metabolites (low % explained variance and no metabolites with >1.5 &IncMSE in Table S5.3), while the grassy/fresh flavours could not be explained/predicted by the metabolite profiles (Table S5.3).

5.3.4. Odour-active volatile metabolites in raw and cooked white spears
To examine the odour-active volatile compounds, samples of both raw and cooked spears from the second-year experiment were analysed using GC-O-MS and 35 unique aromas were perceived by the sniffers. Based on the time window that the aromas were perceived by the assessors, and information from the MS, certain compounds were related to the aroma attributes (Table 5.1).
Table 5.1 List of odour-active volatile compounds detected in raw and cooked white spears of asparagus (A. officinalis). Aroma attributes as perceived at the olfactory detection port (ODP) by the panellists are provided. Annotated compounds detected by GC-MS are of LOI 1 or 2. When LOI>2, compounds are characterised as unknown. The average intensity (and relative standard deviation of n=12) of each compound when detected by GC-MS is provided (LOD: limit of detection).

<table>
<thead>
<tr>
<th>Aroma attributes perceived at the ODP</th>
<th>Detected by panellists in</th>
<th>Compound detected by GC-MS</th>
<th>Compound Intensity detected by GC-MS in Raw spears</th>
<th>Compound Intensity detected by GC-MS in Cooked spears</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cabbage, sulphery</td>
<td>Cooked</td>
<td>Dimethyl sulphide</td>
<td>8.5E+04 (41%)</td>
<td>1.8E+07 (9%)</td>
</tr>
<tr>
<td>2 sweet, floral, buttery</td>
<td>Cooked</td>
<td>2,3-Butanedione</td>
<td>1.9E+04 (51%)</td>
<td>2.9E+05 (27%)</td>
</tr>
<tr>
<td>3 sweet, fruity, ethereal</td>
<td>Both</td>
<td>2-Methylfuran</td>
<td>1.2E+05 (16%)</td>
<td>6.9E+04 (20%)</td>
</tr>
<tr>
<td>4 banana</td>
<td>Raw</td>
<td>Ethyl acetate</td>
<td>2.3E+05 (25%)</td>
<td>1.9E+05 (18%)</td>
</tr>
<tr>
<td>5 something burnt, pungent</td>
<td>Both</td>
<td>2,3-Pentanedione</td>
<td>3.1E+04 (22%)</td>
<td>8.6E+03 (51%)</td>
</tr>
<tr>
<td>6 sour, floral, pineapple</td>
<td>Both</td>
<td>Pentanal</td>
<td>3.9E+06 (15%)</td>
<td>4.2E+06 (22%)</td>
</tr>
<tr>
<td>7 floral, earthy</td>
<td>Raw</td>
<td>Methyl isobutyl ketone</td>
<td>5.2E+05 (10%)</td>
<td>5.3E+05 (14%)</td>
</tr>
<tr>
<td>8 something cooked</td>
<td>Raw</td>
<td>Dimethyl disulphide</td>
<td>4.0E+04 (19%)</td>
<td>3.1E+04 (16%)</td>
</tr>
<tr>
<td>9 sour, floral</td>
<td>Raw</td>
<td>Unknown</td>
<td>6.7E+04 (18%)</td>
<td>5.5E+04 (27%)</td>
</tr>
<tr>
<td>10 chemical, baked</td>
<td>Both</td>
<td>Toluene</td>
<td>1.3E+03 (43%)</td>
<td>3.7E+03 (56%)</td>
</tr>
<tr>
<td>11 something</td>
<td>Cooked</td>
<td>&lt;LOD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 fruity, pear, grassy, green</td>
<td>Both</td>
<td>Hexanal</td>
<td>3.8E+07 (6%)</td>
<td>1.0E+07 (10%)</td>
</tr>
<tr>
<td>13 strong floral, candy, fruity</td>
<td>Both</td>
<td>3-Methylbutanoic acid ethyl ester</td>
<td>5.6E+04 (35%)</td>
<td>2.9E+02 (40%)</td>
</tr>
<tr>
<td>14 sour</td>
<td>Raw</td>
<td>2-Hexenal</td>
<td>2.5E+05 (15%)</td>
<td>6.2E+04 (13%)</td>
</tr>
<tr>
<td>15 sour</td>
<td>Both</td>
<td>2-Butylfuran</td>
<td>2.0E+05 (23%)</td>
<td>3.6E+04 (20%)</td>
</tr>
<tr>
<td>16 paint, chemical</td>
<td>Both</td>
<td>Styrene</td>
<td>3.1E+06 (30%)</td>
<td>3.3E+06 (36%)</td>
</tr>
<tr>
<td>17 cooked potato</td>
<td>Both</td>
<td>Methional</td>
<td>4.0E+04 (25%)</td>
<td>1.2E+05 (20%)</td>
</tr>
<tr>
<td>18 earthy, sour</td>
<td>Both</td>
<td>2-Heptenal</td>
<td>3.2E+06 (13%)</td>
<td>1.0E+06 (18%)</td>
</tr>
<tr>
<td>19 mushroom, earthy, cabbage</td>
<td>Both</td>
<td>1-Octen-3-ol</td>
<td>1.0E+06 (15%)</td>
<td>2.0E+05 (19%)</td>
</tr>
<tr>
<td>20 sour, fruity, green</td>
<td>Both</td>
<td>2,3-Octandione</td>
<td>2.7E+05 (20%)</td>
<td>8.1E+04 (16%)</td>
</tr>
<tr>
<td>21 something pleasant</td>
<td>Both</td>
<td>Octanal</td>
<td>6.5E+05 (6%)</td>
<td>4.3E+05 (15%)</td>
</tr>
<tr>
<td>22 something chemical</td>
<td>Both</td>
<td>2-Ethyl-1-hexanol</td>
<td>1.9E+06 (14%)</td>
<td>1.4E+06 (20%)</td>
</tr>
<tr>
<td>23 earthy, soil, cocoa</td>
<td>Both</td>
<td>Benzenacetaldehyde</td>
<td>9.3E+04 (34%)</td>
<td>9.6E+03 (33%)</td>
</tr>
<tr>
<td>24 earthy</td>
<td>Both</td>
<td>1,3-Diethylbenzene</td>
<td>1.5E+05 (24%)</td>
<td>1.6E+05 (23%)</td>
</tr>
<tr>
<td>25 potato, mushroom, herbal</td>
<td>Both</td>
<td>2-Octenal</td>
<td>2.9E+06 (13%)</td>
<td>5.2E+05 (20%)</td>
</tr>
<tr>
<td>26 vegetable, potato</td>
<td>Cooked</td>
<td>1,4-Diethylbenzene</td>
<td>4.4E+04 (32%)</td>
<td>4.8E+04 (28%)</td>
</tr>
<tr>
<td>27 raw potato, vegetable, asparagus</td>
<td>Both</td>
<td>2-Methoxy-3-isopropyl pyrazine</td>
<td>7.9E+04 (26%)</td>
<td>4.8E+04 (17%)</td>
</tr>
<tr>
<td>28 fruity, melon, pineapple</td>
<td>Both</td>
<td>Benzene, 1-methyl-3-(1-methylethenyl)-</td>
<td>2.9E+04 (18%)</td>
<td>2.2E+04 (19%)</td>
</tr>
<tr>
<td>29 grassy, vegetable, earthy</td>
<td>Both</td>
<td>Nonanal</td>
<td>2.0E+06 (7%)</td>
<td>1.5E+06 (15%)</td>
</tr>
<tr>
<td>30 earthy, musty, pine</td>
<td>Both</td>
<td>Unknown</td>
<td>8.1E+03 (28%)</td>
<td>1.5E+03 (48%)</td>
</tr>
<tr>
<td>31 something unpleasant</td>
<td>Raw</td>
<td>1,2-Dimethoxybenzene</td>
<td>4.6E+04 (19%)</td>
<td>3.3E+04 (20%)</td>
</tr>
<tr>
<td>32 earthy, fatty, fresh</td>
<td>Both</td>
<td>2-Nonenal</td>
<td>2.0E+05 (12%)</td>
<td>5.5E+04 (40%)</td>
</tr>
<tr>
<td>33 earthy, sour, grilled bell pepper</td>
<td>Both</td>
<td>2-Methoxy-3-isobutyl pyrazine</td>
<td>5.8E+03 (26%)</td>
<td>3.0E+03 (22%)</td>
</tr>
<tr>
<td>34 earthy, sour</td>
<td>Raw</td>
<td>2,4-Nonenial</td>
<td>1.1E+05 (25%)</td>
<td>7.2E+03 (33%)</td>
</tr>
<tr>
<td>35 earthy, nutty, sour</td>
<td>Both</td>
<td>2,4-Decadienal</td>
<td>8.5E+04 (29%)</td>
<td>3.4E+03 (43%)</td>
</tr>
</tbody>
</table>

Four compounds were detected only in the cooked spears and were described as having sulphury, sweet and potato odours. Seven compounds were detected only in the raw materials and were mainly characterized as having floral, earthy and sour aromas (Table 5.1). Although quantitative differences in compound intensities were detected in the MS, the remaining 24 compounds were smelled in both materials including e.g. 2-methylfuran, methional, 2,3-octanone (Table 5.1).
5.4. Discussion

White asparagus is an important crop for the economy of N. Europe and is often discussed thanks to its culinary value and typical flavour. There are however, few studies that have investigated the metabolite composition of asparagus using cooked fresh material (Brueckner et al., 2010; Hoberg et al., 1999, 2003; Scherb et al., 2009; Tressl, Bahri, et al., 1977; Tressl, Holzer, et al., 1977; Ulrich et al., 2001), where only two of which have actually compared raw and cooked materials. One used white asparagus to study the bitter-related saponins and showed compositional shifts after cooking (Dawid & Hofmann, 2014) while the other used green asparagus and showed that the content of most amino acids decreased after cooking while methionine and cysteine increased (Słupski et al., 2010).

In the study presented here, both raw and cooked white spears with a complete set of metadata (precise known origin, cultivation history, etc.) were evaluated by combining metabolomics and taste panel studies. Clear discrimination was found between raw and cooked spears both regarding the composition of secondary metabolites (Figure 5.1, 5.2) and flavour experience (Figure 5.3). No significant differences with respect to the metabolome were found between varieties (Table S5.2), as has been observed previously (Pegiou et al., 2021), although subtle differences were perceived regarding certain flavour attributes (Table S5.1). For these subtle differences a fully-trained taste panel might be valuable to help decipher potential links between minor flavour differences and individual quantitative / qualitative metabolite differences. Nevertheless, the panellists did distinguish, in both years, that Cumulus was considered the tastiest overall variety and Fortems was the least tasty one primarily due to its higher bitterness. Cumulus was sweeter but also had a smoother texture than Fortems (Figure 5.3, Table S5.1), both of which can be relevant from an overall sensory perspective. It could be proposed that sweeter and less bitter varieties producing tender spears are preferred by consumers and this is partly in agreement with Hoberg, Ulrich, and Wonneberger (2008). They performed a large panel study comparing varieties of high and low consumer acceptability and showed that highly bitter varieties were less preferred (Hoberg et al., 2008). Based on the findings presented here (Figure 5.3), we additionally confirm that texture may also play a critical role for consumer acceptance of white asparagus spears (Jaramillo et al., 2007).
Processing and analysing the data obtained from the untargeted metabolomics (GC-MS and LC-MS) and the information from the GC-O-MS analysis gave valuable insights into the chemistry of asparagus flavour. In raw fresh material, compositional shifts occur via chemical conversions dependent on oxygen or light but also via pathways which require specific enzymatic activities. Most enzymatic activities are inhibited in temperatures < 4 °C and > 80 °C (Chakraverty & Singh, 2014) and thus also during cooking. However, oxidation of metabolites can still occur during cooking. Moreover, heat may induce thermal degradation of metabolites and the formation of others. Using specific examples from the findings of the study presented here and previous reports on asparagus and other vegetables, we aim to be able to begin to explain the chemical shifts that the asparagus metabolome undergoes upon cooking.

Dimethyl sulphide was the most discriminatory compound for the cooked materials (Figure 5.2A, S5.4C), and was described as having a cabbage and sulphury aroma with GC-O (Table 5.1). These flavour descriptors agree with those previously described for dimethyl sulphide detected in cooked vegetables including asparagus (Segurel et al., 2005; Ulrich et al., 2001). Therefore, being clearly odour-active in the cooked material, dimethyl sulphide is confirmed to contribute to cooked asparagus flavour. S-methylmethionine which is the main proposed precursor of dimethyl sulphide (Scherb et al., 2009), would be expected to decrease after cooking. However, here the abundance of this compound was not significantly different between raw and cooked materials (Figure 5.2C). S-adenosylhomocysteine, which is a side-product of the biosynthetic pathway of S-methylmethionine (Tagmount, 2002), was however, less abundant in the cooked spears (Figure 5.2B) suggesting the possible deactivation of this pathway. These observations may imply that S-methylmethionine was not used to form dimethyl sulphide during cooking or perhaps that the conversion of methionine to S-methylmethionine (Amir, 2010) can continue on heating. This would agree with the observation that methionine levels can be increased in cooked asparagus as reported previously (Słupski et al., 2010). Taken together, we therefore have no evidence to confirm that S-methylmethionine is the main precursor of dimethyl sulphide in asparagus, or at least - is not the only one, as was previously suggested (Scherb et al., 2009). There are likely more complicated dynamics and kinetics involved during heating that leads to the formation of dimethyl sulphide. For instance, dimethyl trisulphide, which was more
Elucidating the flavour of cooked white asparagus by combining metabolomics and taste panel analysis

abundant in the raw spears (Figure 5.2A), may also be involved in the formation of dimethyl disulphide and dimethyl sulphide (Yeretzian et al., 2017). Therefore, it should be investigated whether dimethyl trisulphide also participates in the heat-induced formation of dimethyl sulphide.

Carbonyls with 6-9 carbon backbone including hexanal, 2,3-octanedione 1-octen-3-ol and 2,4-decadienal as well as 2-butylfuran and 2-pentylfuran (Figure 5.2A, S5.4) were characteristic for the raw spears. These are all volatile and can be released (and partially lost) at the temperatures applied during cooking. All these odour-active carbonyls and furans were detected and characterized by GC-O and described as having ‘fresh’, ‘fruity’, ‘earthy’, ‘grass’, ‘mushroom’ and ‘floral’ aroma notes (Table 5.1). These descriptors have previously been used to describe those volatile compounds detected in different food matrices, such as apple (Fellman et al., 2000), rice (Limpawattana et al., 2008) as well as asparagus (Ulrich et al., 2001). Here, they were characteristic for raw materials (Figure 5.3A). Precursor compounds of these volatile aroma compounds are oxidized fatty acids, derivatives of linoleic and linolenic acids (Matsui, 2006; Stolterfoht et al., 2019). These non-volatile compounds were interestingly found to be significantly more abundant in the cooked asparagus (Figure 5.2B). Lipoxygenase and peroxidase enzymes are required to catalyse the degradation of linoleic and linolenic acids leading to the formation of C6-9 volatile aroma compounds (Stolterfoht et al., 2019). Previous studies have demonstrated that these enzymes can be sensitive to temperatures above 80 °C (Chakraverty & Singh, 2014), thus also during boiling. This means that the related volatile aroma compounds are unlikely to be further formed during cooking hence, their lower abundance in the cooked materials, presumably through their being driven off at higher temperatures. In contrast, oxidation of linoleic and linolenic acids continues at higher temperatures, which could explain the higher abundances of the oxidised free fatty acids as observed in the cooked materials (Figure 5.2B).

The two detected methoxypyrazines, which are synthesised from the amino acids valine and leucine (Sidhu et al., 2015), were both smelled and characterized as giving ‘earthy’, ‘soil’, and ‘vegetable’ notes (Table 5.1). These aroma compounds were already found in raw asparagus materials which confirms that methoxypyrazines are naturally synthesized/present in plants as has previously been observed for grapes (Lei et al., 2018) and asparagus (Pegiou et al., 2021) and are not necessarily the result of heat treatments. Other pyrazines which are
known to be formed upon heating via Maillard reactions (Diez-Simon et al., 2019), such as diethyl-pyrazines, were not detected in the materials analysed here. Maillard reactions generally start at temperatures above 140 °C (Cremer & Eichner, 2000; X. Li et al., 2022), so potentially a different preparation of the spears through e.g. grilling, may still result in the generation of these aroma compounds, but this would then also be predicted to lead to a different flavour profile.

The impact of cooking on the composition of non-volatile phenolic acids (e.g. caffeic acid) in vegetables varies based on the duration and manner of cooking (e.g. boil or microwave; peeled or unpeeled, etc.) (Rashmi & Negi, 2020). In this study, phenolic acids were seen to be more abundant in the cooked unpeeled white spears (Figure 5.2B), indicating that boiling did not result in an overall loss of these compounds. In the case of flavonoid glucosides (e.g. quercetin and kaempferol glucosides), these were significantly found to be more abundant in the raw spears (Figure 5.2B). The same flavonoid glucosides have previously been found to decrease after boiling vegetables such as broccoli (Wu et al., 2019) and cauliflower (dos Reis et al., 2015) and it has been suggested that they may dissolve in the cooking water in the case of beans and zucchini (Andlauer et al., 2013). Our findings do confirm that flavonoid glycosides are heat-sensitive and may degrade at temperature above 90 °C (Chaaban et al., 2017; Rohn et al., 2007) or perhaps leach into the cooking water.

Saponins, which are known bitter compounds believed to have a defence role in e.g. plant roots, were also more abundant in the raw materials (Figure 5.2B). This is consistent with studies on other vegetables including onion (Lombard et al., 2005), carrot and kale (Maqbool et al., 2021) and indeed, asparagus (Dawid & Hofmann, 2014). However, no significant difference regarding specifically the levels of the bidesmosidic saponin, protodioscin, was found here (Figure 5.2C), contradicting previous findings where protodioscin was found to be increased after cooking white spears for 14 minutes (Dawid & Hofmann, 2014). This difference may be due to the longer cooking time used (14 versus 9 minutes). Varying cooking times have been shown to impact the saponin composition of chickpeas and lentils which usually have much longer cooking times and which results in decreased saponin contents (Ruiz et al., 1996).
Bringing the focus to the volatile flavour compounds, the list of 35 odour-active volatile metabolites detected here (28 of which are detected in cooked materials) (Table 5.1) is not completely the same as previously presented by Ulrich et al. (2001) who also performed GC-O of boiled white spears. They reported a list of 12 odour-active volatile compounds (Ulrich et al., 2001), of which, 2,3-pentanedione, hexanal, 2,3-octanedione, 2-methoxy-3-isopropyl pyrazine, methional and benzeneacetaldehyde are in agreement with the findings presented here (Table 5.1). They also detected 2,3-butanedione and dimethyl sulphide by performing GC-MS but these were unexpectedly not detected by GC-O (Ulrich et al., 2001). This may have been due to the challenging set-up of GC-O where some aromas may be very subtle or may pass through only very briefly or follow too close to another odour-active compound making it hard for the assessors to detect them. Nevertheless, both our study and that by Ulrich et al. (2001) highlight and confirm the critical role of sulphur- and nitrogen-containing volatile compounds in the flavour of white asparagus. Especially, methional and 2-methoxy-3-isobutyl pyrazine were correlated with ‘baked potato’ and sweet flavours, respectively (Figure S5.6) and were also detected during GC-O and were linked there directly to baked potato and bell pepper aromas (Table 5.1). These both confirm their odour-activity and their relevance to asparagus flavour. The aroma experience of ‘baked potato’ was also highly correlated with the levels of 2,3-butanedione (Figure S5.6A). Previously, 2,3-butanedione (diacetyl) together with methional were found to contribute to the typical flavour of cooked potatoes (Ulrich et al., 2000) but also the buttery, caramel-like ‘cooked’ flavour in cheese (Boscaini et al., 2003). In the study presented here, 2,3-butanedione and methional were detected in the both the raw and cooked materials but only 2,3-butanedione was smelled after cooking (Table 5.1). Considering that only when tasting the cooked spears, the panel members perceived ‘baked potato’ aromas, it is suggested here that, in asparagus both of these volatile compounds are necessary to experience the cooked-related ‘baked potato’ flavour notes.

Combining information from untargeted metabolomics, taste panel and GC-O has given valuable insights into the flavour composition of white asparagus spears. Compositional shifts of diverse compound classes upon cooking have been demonstrated indicating which metabolites frame the typical flavour of cooked white spears and which metabolites are of relevance when developing strategies to produce processed asparagus ingredients. Key
precursors in raw materials were also highlighted which may deserve particular focus in future breeding programmes focusing on quality aspects of asparagus. Heat applied during cooking induces thermal formation of dimethyl sulphide but also the degradation of methoxypyrazines. The high temperatures also appears to have resulted in inhibition of biosynthesis of C$_6$-9 volatile carbonyls and thus the accumulation of oxidised free fatty acids. Finally, during cooking, there might also be some leaching of flavonoid glucosides and saponins into the cooking water but also retention of phenolic acids thanks perhaps to the peel of the spears. Our findings function as a starting point for follow-up targeted investigations to assess the nuances in the flavour of white asparagus and related (processed) products in the context of the development of new genotypes, cultivation strategies and post-harvest treatments.
Elucidating the flavour of cooked white asparagus by combining metabolomics and taste panel analysis

5.5. Supplementary Material

Figure S5.1 Raw chromatograms obtained from the GC-MS (upper) and LC-MS both negative (middle) and positive ionization mode (lower) analysis of raw and cooked white asparagus spears. Purple boxes (A-E) indicate areas of the chromatograms with observed differences between raw and cooked materials.
Figure S5. 2 Raw chromatograms obtained from the GC-MS analysis of raw (upper) and cooked (middle) white asparagus spears and the cooking water (lower). Peaks present in water are the background peaks of the siloxanes released from the fibre and GC column.

Figure S5. 3 Volatile (A) and non-volatile (B) metabolites selected based on VIP scores calculated from OPLS-DA discriminating raw and cooked white asparagus spears. Base peaks and retention time (min) of non-volatiles: neg_570 (231.0447 at 7.69), neg_896 (300.1925 at 12.58), neg_990 (306.9378 at 13.60), neg_2873 (243.1966 at 46.64), neg_1046 (534.2936 at 14.40), pos_1165 (424.2066 at 15.70), pos_1906 (718.3317 at 19.52), neg_516 (489.0556 at 7.06), neg_1632 (319.1222 at 21.06), neg_189 (489.5569 at 3.69), neg_2935 (504.3097 at 47.95), pos_783 (416.2505 at 12.10), neg_164 (242.0132 at 3.46), neg_168 (477.0502 at 4.09), neg_2893 (504.3096 at 47.03).
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Figure S5. 4 Selected metabolites detected in raw and cooked white asparagus spears analysed in 2021 (A) and 2022 (B). Colours indicate the samples analysed. Error bars depict standard deviation (n=3 and for QCs n=4 in A; n=5 in B).
Figure S5. 5 Spider web diagram presenting the average scores on the flavour attributes of cooked white spears of four varieties of *Asparagus officinalis* (red: Backlim, yellow: Cumulus, green: Fortems, black: Gijnlim) evaluated by taste panel in 2022. Attributes that received significantly different scores between varieties ($p < 0.05$) are indicated with asterisk (*).

Figure S5. 6 (continues in the next page)
Elucidating the flavour of cooked white asparagus by combining metabolomics and taste panel analysis

Figure S5.6 Correlated metabolites with baked potato flavour (A, B), sweet flavour (C) and smooth texture (D) based on Random Forest regression analysis linking GC-MS (in A, C and D) and LC-MS datasets with the taste panel scores. Performance metrics of the generated models are provided in Table S5.3.

Figure S5.7 (continues in the next page)
**Figure S5.** Baseline corrected LC-MS chromatograms (A) and mass spectra of two non-volatile oxidised phospholipids $C_{27}H_{50}NO_9P$ (B) and $C_{23}H_{42}NO_7P$ (C) that were discriminatory for the cooked materials and correlated with baked potato flavour.

**Table S5.** List of flavour attributes based on which the taste panel evaluated raw and cooked white spears of asparagus (*A. officinalis*). The description of each flavour attribute is provided. Attributes that got significantly different scores between raw and cooked materials in 2021 are indicated with * (Student’s t-test $p < 0.05$). The last three columns list the pairs of varieties between which a significant difference was found ($p < 0.05$) based on the taste panel evaluation (B: Backlim, C: Cumulus, F: Fortems, G: Gijnlim, ns: not significant).

<table>
<thead>
<tr>
<th>Flavour Attributes</th>
<th>Description</th>
<th>Significantly different score between raw and cooked spears 2021</th>
<th>Significantly different score between varieties</th>
<th>Raw spears 2021</th>
<th>Cooked spears 2021</th>
<th>Cooked spears 2022</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagus</td>
<td>Like white asparagus</td>
<td>ns</td>
<td>F-G, F-B</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Sulphury</td>
<td>Like cooked cabbage</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grassy/Fresh</td>
<td>Like raw vegetables, grass</td>
<td>*</td>
<td>-</td>
<td>C-G, C-B</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Baked potato</td>
<td>Like baked potato/butter</td>
<td>*</td>
<td>-</td>
<td>C-G, C-B</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Bitter</td>
<td>ns</td>
<td>F-C, C-G, C-B</td>
<td>F-B</td>
<td>F-C, F-G, F-B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet</td>
<td>*</td>
<td>C-G</td>
<td>F-B</td>
<td>F-C, F-B, C-B, C-G, G-B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buttery</td>
<td>Like natural salted butter</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrous</td>
<td>Woody/fibrous</td>
<td>ns</td>
<td>C-B, G-B</td>
<td>F-G</td>
<td>F-C, F-B</td>
<td></td>
</tr>
<tr>
<td>Smooth</td>
<td>Tender/smooth</td>
<td>*</td>
<td>ns</td>
<td>F-G</td>
<td>F-C, F-B</td>
<td></td>
</tr>
</tbody>
</table>
Table S5. 2 Results of the Random Forest classification analyses comparing the volatile (GC-MS) and non-volatile (LC-MS) profiles of the four studied varieties (Backlim, Cumulus, Fortems, Gijnlim). Per model, the classification error is given for each variety. The OOB error per model is the average of the error for classifying each of the varieties.

<table>
<thead>
<tr>
<th></th>
<th>Backlim</th>
<th>Cumulus</th>
<th>Fortems</th>
<th>Gijnlim</th>
<th>OOB</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-MS 2021</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>0.333</td>
<td>0.667</td>
<td>1.0</td>
<td>0.333</td>
<td>0.583</td>
</tr>
<tr>
<td>Cooked</td>
<td>1.0</td>
<td>0.333</td>
<td>1.0</td>
<td>1.0</td>
<td>0.833</td>
</tr>
<tr>
<td>LC-MS 2021</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>0.0</td>
<td>0.333</td>
<td>0.0</td>
<td>1.0</td>
<td>0.333</td>
</tr>
<tr>
<td>Cooked</td>
<td>0.333</td>
<td>0.667</td>
<td>1.0</td>
<td>0.333</td>
<td>0.583</td>
</tr>
<tr>
<td>GC-MS 2022</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>0.2</td>
<td>0.333</td>
<td>0.167</td>
<td>0.333</td>
<td>0.261</td>
</tr>
<tr>
<td>Cooked</td>
<td>0.167</td>
<td>0.4</td>
<td>0.333</td>
<td>0.5</td>
<td>0.348</td>
</tr>
</tbody>
</table>

Table S5. 3 Results of the Random Forest regression analyses to generate predictive models for the evaluated flavour attributes based on the volatile (GC-MS) or non-volatile (LC-MS) profiles of the spears analysed in 2021. The mean of square residuals and % of explained variance per model are provided. For models with % explained variance >0 the highlighted variables were further investigated and those with %IncMSE > 1.5 were considered relevant and the number of them is given here; n.a: not applicable. The lists of selected metabolites are provided in Figure S5.5.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Flavour Attribute</th>
<th>Mean od square residuals</th>
<th>% Explained variance</th>
<th>Variables with %IncMSE&gt;1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-MS</td>
<td>Baked potato</td>
<td>0.33</td>
<td>66.1%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sweet</td>
<td>0.43</td>
<td>18.7%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Smooth</td>
<td>0.88</td>
<td>6.7%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Grassy/fresh</td>
<td>0.76</td>
<td>&lt;0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baked potato</td>
<td>0.61</td>
<td>&lt;0</td>
<td></td>
</tr>
<tr>
<td>LC-MS</td>
<td>Sweet</td>
<td>0.34</td>
<td>65.4%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Smooth</td>
<td>0.77</td>
<td>18.5%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Grassy/fresh</td>
<td>0.72</td>
<td>&lt;0</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 6

*Maltodextrin improves physical properties and volatile compound retention of spray-dried asparagus concentrate*

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Abstract
Traditional hot air drying of asparagus is known to lead to a powder with a poor aroma profile. We here concentrated asparagus juice into asparagus concentrate (21.7 % w/w) and spray-dried it with maltodextrin DE12 as carrier agent to improve the volatile profiles of asparagus powder and to valorise fresh asparagus side-streams. We performed headspace GC-MS with untargeted metabolomics to assess the overall metabolite profile of the spray-dried asparagus powders and identified 70 volatile compounds. The maltodextrin content was positively correlated to the retention of an asparagus key odorant 1-octen-3-ol, as well as other alcohols and aldehydes. Nevertheless, drying conditions had limited effect on the volatile retention of the powders. Moreover, higher outlet temperatures increased the presence of volatiles that were formed during drying, such as 3-methylthio-propanal. From our analyses, it was further found that an increased concentration of maltodextrin was correlated to a lower moisture content, a higher glass transition temperature (Tg) and a narrower size distribution of the spray-dried powders. The Tg of all powders was described with the Gordon-Taylor equation for multicomponent mixtures, and we found a minimum weight fraction of 0.67 (w/dw) maltodextrin required to obtain glassy asparagus powder for storing at ambient conditions.

Keywords: Asparagus; aroma encapsulation; spray drying; GC-MS; maltodextrin
6.1. Introduction

Asparagus (*Asparagus officinalis*) is a popular vegetable consumed all over the world. The production of asparagus comprised 9.1 million tons globally in 2018 (Knoema, 2021). While the green form is most common, the white form is appreciated by its consumers because of its structure and flavour profile. White asparagus flavour has been the topic of several studies in the past (Tressl, Bahri, et al., 1977; Tressl, Holzer, et al., 1977; Ulrich et al., 2001), which has led to the identification of some key aroma components in cooked asparagus (Pegiou et al., 2020). After harvesting, approximately one-third of the lower part of the white asparagus spear is cut off because of its woody texture and to standardize the length of the spears for the market (W. Zhang et al., 2014). A significant waste stream is thus generated, which could potentially be utilised as a food ingredient, for example in the form of asparagus powder for instant soups. Conventionally, commercial asparagus powder is made by air-drying (i.e. tray drying) small pieces of asparagus spear followed by milling (Karam et al., 2016; Nindo et al., 2003). However, this drying process alters the volatile profile of asparagus powder. Some aroma compounds of asparagus might be lost during drying, whereas some other asparagus key odorants may be formed upon drying, including sulphur-containing compounds such as dimethyl sulphide (Nijhuis et al., 1998; Ulrich et al., 2001). Ideally, the formation of those key odorants should be suppressed during drying to prevent aroma loss upon storage. To ensure flavour stability, artificial flavouring agents are added to the asparagus powders. These flavouring agents may not fit in a formulation targeted to be perceived as natural and healthy by consumers (Bearth et al., 2014; Eiser et al., 2002; Shim et al., 2011). Therefore, to obtain asparagus powders with better aroma profile, new drying strategies need to be developed. These strategies include the selection of a drying method as well as the optimisation of this drying method.

Spray drying has been widely used to encapsulate flavour compounds in vegetable powders (Verma & Vir Singh, 2015). Thus, asparagus powder with improved flavour profile may be obtained by using spray drying (J. Siccama et al., 2019). More specifically, asparagus juice can be pressed and concentrated from the side stream and then spray-dried to produce asparagus powder. However, direct spray drying of this concentrate is problematic due to stickiness issues leading to fouling in the drying chamber. This is because asparagus
concentrate is rich in small sugars that have a low glass transition temperature (T_g). Hence, the addition of carrier agents with a high T_g, e.g. maltodextrins with a low dextrose equivalent (DE), to the concentrate can be used to avoid fouling and to improve powder quality (Verma & Vir Singh, 2015).

During spray drying, the liquid feed is atomized into small droplets and exposed to hot air. Initially, the drying is externally limited, but an internal moisture gradient evolves when the surface of the droplet reaches a critical moisture concentration. After this, a semi-permeable ‘skin’ is formed on the droplet surface, which allows further removal of water but hinders the release of larger volatile aroma compounds (Coumans et al., 1994). Loss of volatile compounds mainly occurs before this ‘skin’ is formed, i.e. during the constant drying rate period (Thijssen, 1971). Hence, a shorter constant rate period is favourable for aroma retention during spray drying. Specifically, addition of hydrolysed starches (i.e. maltodextrins) can encapsulate flavours by faster skin formation and increase the T_g of the mixture (Madene et al., 2006; Zuidam & Heinrich, 2010). Adding high molecular weight carrier agents could reduce the diffusion of flavour compounds from within the drying matrix to the droplet surface until the semi-permeable skin is formed (Anandharamakrishnan & Padma Ishwarya, 2015). In general, carriers with low viscosity at high solids content such as maltodextrin are desired, because it allows for relative higher concentrations (Reineccius, 2004). Furthermore, the drying conditions (e.g. inlet temperature) may influence volatile retention during spray drying. For example, a higher inlet temperature may improve aroma retention because it shortens the constant rate period (Coumans et al., 1994; Reineccius, 2004). Nevertheless, a too high inlet temperature can lead to worse aroma retention by inducing excessive bubble formation and surface cracks (King, 1995). Thus, both carrier choice and drying conditions affect the volatile profile of spray-dried powders.

In this study we employ mass spectrometry-based metabolomics, being an emerging technique for the characterization of volatile profiles in foods (Diez-Simon et al., 2019). To the best of our knowledge, the combination of spray drying experiments and gas chromatography-mass spectrometry (GC-MS) analysis of the overall volatile profile of spray-dried powders following an untargeted metabolomics approach has not been reported before. Therefore, we here aim to assess the volatile profile and the retention of key volatiles
Maltodextrin improves physical properties and volatile compound retention of spray-dried asparagus concentrate

in spray-dried asparagus concentrate as influenced by the addition of maltodextrin DE12 and drying conditions. Maltodextrin DE12 was selected since Bangs and Reineccius (1982) found that the overall retention of twelve volatile compounds was maximised for maltodextrins with DE 10-15.

Here, asparagus juice was extracted from a white asparagus waste stream (i.e. bottom of the spears), concentrated with reverse osmosis and mixed with maltodextrin DE12 at different solids ratios. Subsequently, the solutions were spray-dried at different inlet and outlet temperature combinations. The powders were evaluated in terms of physical properties (i.e. moisture content, $T_g$, particle size distribution and particle morphology) and their volatile profile. The volatile profiles were analysed by headspace solid-phase microextraction (HS-SPME), followed by GC-MS.

6.2. Materials & methods

6.2.1. Sample preparation

Raw fresh asparagus cut-offs (*Asparagus officinalis*) were kindly provided by Teboza BV (Helden, the Netherlands). Concentrated asparagus juice was prepared from these asparagus cut-offs by Wageningen Food & Biobased Research (Wageningen, the Netherlands). Specifically, asparagus juice was pressed and centrifuged to remove any fibres. The juice was concentrated to a factor of 5.6 with reverse osmosis into a final dry matter content of 21.7 % w/w. The sugar composition of the asparagus concentrate consists of 78 mg/ml fructose, 72 mg/ml glucose and 8 mg/ml sucrose, which was determined with high-performance liquid chromatography (HPLC) using a Shodex KS-802 8.0 × 300 (mm) column. The column was operated at 50 °C and connected to a refractive index detector (Shodex RI-501). Milli-Q water was used as eluent with a flow rate of 1 ml/min. The concentrate was aliquoted into test tubes and stored at -20 °C before the experiments. For every experiment, a new tube was taken from the freezer and the concentrate was defrosted in the fridge at 4 °C for 18-20 hours.

Maltodextrin DE12 (MD12, Roquette, France) was added to the concentrated asparagus juice to formulate the mixtures for the spray drying experiments shown in Table 6.1. The mass ratios between asparagus solids and maltodextrin in the mixtures were adjusted to 2:1, 1:1 and 1:2, respectively. In addition, one sample with a 1:2 ratio was diluted to obtain the same total solids content as the 1:1 ratio sample. This sample will be referred to as 1:2*. 

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Pure concentrated asparagus juice was used as a reference sample (i.e. 1:0). All the samples were stirred at room temperature at 400 rpm for 1 hour before each spray drying experiment.

**Table 6.1** Overview of the experimental design for spray drying of asparagus juice concentrate and maltodextrin mixtures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Asparagus solids (w/w)</th>
<th>Maltodextrin (w/w)</th>
<th>Total solids (w/w)</th>
<th>Inlet (°C)</th>
<th>Outlet (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>22</td>
<td>0</td>
<td>22</td>
<td>160</td>
<td>90-95</td>
</tr>
<tr>
<td>2:1</td>
<td>20</td>
<td>10</td>
<td>29</td>
<td>160</td>
<td>90-95</td>
</tr>
<tr>
<td>1:1</td>
<td>18</td>
<td>18</td>
<td>36</td>
<td>160</td>
<td>90-95</td>
</tr>
<tr>
<td>1:2</td>
<td>15</td>
<td>30</td>
<td>45</td>
<td>160</td>
<td>90-95</td>
</tr>
<tr>
<td>1:2*</td>
<td>12</td>
<td>24</td>
<td>36</td>
<td>160</td>
<td>90-95</td>
</tr>
<tr>
<td>1:1</td>
<td>18</td>
<td>18</td>
<td>36</td>
<td>180</td>
<td>90</td>
</tr>
<tr>
<td>1:1</td>
<td>18</td>
<td>18</td>
<td>36</td>
<td>180</td>
<td>105</td>
</tr>
</tbody>
</table>

6.2.2. Spray drying experiment

The maltodextrin-asparagus concentrate mixtures were spray-dried with a Model B-290 mini spray dryer (BÜCHI Labortechnik AG, Flawil, Switzerland). The aspirator rate was 90% which corresponded to an airflow of 35 m³/h. To investigate the effect of the maltodextrin concentration on the properties of spray-dried powders, samples (1:0, 2:1, 1:1 and 1:2) were dried under the same conditions, i.e. the inlet air temperature ($T_{in}$) was 160 °C and the outlet air temperature ($T_{out}$) was 90-95 °C. The speed of the peristaltic pump was adjusted to 3 – 10.5 ml feed/min to ensure the desired outlet air temperature. In addition, the sample with a ratio of 1:1 was dried in two inlet/outlet combination experiments (i.e. $T_{in}/T_{out}$=180 °C/90 °C and 180 °C/105 °C), to investigate the effect of drying conditions. For both combinations, the feed rate was adjusted to reach the desired $T_{out}$. For every condition (i.e. ratio and drying condition), three independent spray drying experiments were performed and the asparagus powders were collected at the outlet of the spray dryer for further analysis.

6.2.3. Moisture content

Spray-dried powder (ca. 0.5 g) was placed in a hot air oven (Binder, Tuttingen, Germany) to determine its moisture content. The powder was weighed before and after drying at 105 °C.
overnight, and the moisture content of the powder was calculated on total basis. Measurements were carried out in triplicate.

6.2.4. Glass transition temperature (Tg)
The T_g of the spray-dried powders was determined using differential scanning calorimetry (DSC) (DSC-250, TA Instruments, New Castle, England). In addition, the T_g of asparagus concentrate was determined after pre-drying the concentrate in a climate chamber (Memmert, Germany) at 25 °C and relative humidity of 10 % for 24 hours. Samples of about 5 mg were weighed in aluminium Tzero pans which were then hermetically sealed. Temperature ramp measurements were carried out at a rate of 10 °C/min, and the temperature range was set between -60 °C and 140 °C depending on the moisture content of the sample. The DSC thermograms were analysed using Trios software (TA Instruments, New Castle, England). The T_g was determined from the midpoint, which was based on the inflection of the endothermic shift due to glass transition, i.e. the peak of the derivative. After the temperature ramp measurements, a hole was punched in the lid of the pans and the moisture content of the sample was determined by drying the samples overnight at 105 °C. All measurements were in duplicate and the mean values of the measured results were reported.

6.2.5. Particle size distribution and morphology
The particle size distribution of the spray-dried powders was measured using a Mastersizer 3000 analyser (Malvern Inc, Worcestershire, UK) with the dry powder disperser Aero S. The particle size distribution was determined with the Mastersizer 3000 software and presented on a volume basis. Dried particles were visualised using scanning electron microscopy (SEM). The powders were attached to SEM stubs using carbon adhesive tabs, sputter-coated with gold under vacuum, and examined using a Neoscope JCM-7000 (JEOL, USA). SEM was carried out at 10 kV with a magnification of x1600.

6.2.6. Preparation of liquid samples
Before headspace analysis of the spray-dried powders, the influence of maltodextrin on the gas/liquid-equilibrium in liquid samples was evaluated. Samples were prepared by dissolving maltodextrin DE12 in water at different concentrations, followed by mixing of the maltodextrin solutions with concentrated asparagus juice. The resulting mixtures contained maltodextrin concentrations of 0, 15, 25, 34 and 44 %. The pH of all mixtures was measured.
The pH-values obtained were the same for all mixtures, thus pH could not influence the
gas/liquid partition coefficient in this study. All samples contained the same amount of
asparagus solids, i.e. 30 mg, and had similar total weight. The samples were stored at -80 °C
before analysis of volatile compounds. The volatile compounds were measured according to
the method described below.

6.2.7. Headspace analysis of volatile compounds

Volatile compounds in the defrosted asparagus concentrate, maltodextrin-asparagus
concentrate mixtures and spray-dried asparagus powders were analysed by GC-MS. Samples
were weighed (mass in mg) based on equation 6.1, i.e. all the samples contained 30 mg dry
weight of asparagus solids.

\[
\text{mass} = \frac{30}{X_s X_{a(db)}}
\]  

(6.1)

where \(X_s\) (kg solids/kg total) is the total solids content of the sample determined via
moisture content analysis. \(X_{a(db)}\) (kg asparagus solids/kg solids) is the fraction of asparagus
solids in the solids content of the sample, which can be derived from the mass ratio between
asparagus solids and maltodextrin. For example, \(X_a\) of 2:1 ratio equals 0.67. For a 2:1 ratio
dry sample with a moisture content of 10 % w/w (i.e. \(X_s = 0.90\)), the sample weight for GC-
MS analysis was 50 mg.

In addition, quality controls (QCs) were prepared from a mix of the spray-dried powders,
except for the 1:0 ratio since there was too little sample. All samples were transferred to 10-
ml glass vials which were subsequently stored at -80 °C until analysis. Before analysis, EDTA
and CaCl\(_2\) were added to the samples to a final concentration of 50 mM and 5 M, respectively.

In the case of liquid samples (asparagus concentrate, mix) solid CaCl\(_2\) was added first and
subsequently the EDTA solution to a final volume of 1 ml. In the case of the dry samples
(spray-dried powders), a saturated solution of 50 mM EDTA/5M CaCl\(_2\) was added to a final
volume of 1 ml.

Volatile compounds were extracted from the headspace using solid-phase microextraction
(SPME). A PDMS/DVB/CAR (Polydimethylsiloxane / Divinylbenzene / Carboxen) 50/30 μm
diameter, 1 cm length (Supelco, PA, USA) fibre was used. The samples were incubated at
50 °C for 15 min with agitation. Subsequently, volatiles were trapped by exposing the fibre
to the headspace of the vial for 15 min at 50 °C without agitation. The fibre was then
Maltodextrin improves physical properties and volatile compound retention of spray-dried asparagus concentrate

thermally desorbed in the injector containing an empty glass liner (1 mm ID) (CIS4, Gerstel, Germany) at 250 °C for 2 min with a helium flow of 1 ml/min onto the GC column, in split-less mode. Sample handling was fully automated using a Gerstel MPS-2 autosampler using Gerstel MAESTRO software version 3.2. Analysis of the trapped volatiles was carried out on an Agilent GC7890A coupled to a 5975C quadrupole mass spectrometer. The column used was a Zebron ZB-5MSplus with dimensions 30 m x 0.25 mm x 1.00 μm (Phenomenex). The GC oven temperature was programmed starting at 45 °C for 2 min, then increased at a rate of 8 °C/min to 250 °C and then at a rate of 15 °C/min to 280 °C and maintained at 280 °C for 3 min. The carrier gas was helium, at a constant flow rate of 1 ml/min. The column effluent was ionised by electron impact at 70 eV, in the scan range m/z 33–330. The interface temperature was set to 280 °C. The retention indices (RIs) were calculated based on a series of n-alkanes (C₆-C₂₁) injected at the same conditions as the samples.

GC-MS raw data were processed using an untargeted metabolomics workflow. MetAlign software was used for baseline correction and alignment of the mass signals (S/N>3) (Lommen, 2009). Mass spectra were reconstructed to potential clusters using MSClust (Y. M. Tikunov et al., 2012). Metabolites were putatively identified by matching the obtained mass spectra and RIs with those in commercial (e.g. NIST17) and in-house libraries. The level of identification given to the detected compounds follows the guidelines of the Metabolomics Standards Initiative (Sumner et al., 2007). Compounds with level 4 of identification are characterised as ‘unknowns’ and further investigation is required for their identification.

Before the statistical analysis, zero values in the processed data were randomised around the limit of detection as determined by MetAlign. Subsequently, SIMCA 15.0.2. (Umetrics, Sartorius Stedim Data Analytics AB, Umeå, Sweden) was used to perform principal component analysis (PCA) after log10 transformation and Pareto scaling.

6.2.8. Volatile retention

All mixes and respective spray-dried powder were analysed with SPME-GCMS. The volatile retention of each independent sample was calculated based on the ratio of the spray-dried powder with the corresponding mix in equation 6.2. The retention was reported as the average retention of the triplicates with standard deviations.

\[
\text{retention (\%)} = \left( \frac{\text{Peak intensity}_{\text{spray-dried}}}{\text{Peak intensity}_{\text{mix}}} \right) \times 100
\] (6.2)

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The spray-dried powders were reconstituted to the same maltodextrin concentration as the mix for the headspace analysis, therefore we could safely assume the partition coefficients for the volatiles were similar.

6.2.9. Statistical analysis
All experiments were conducted at least in duplicate and results were presented as mean ± standard deviations. One-way analysis of variance (ANOVA) and Tukey's HSD post hoc test were performed, and \( p \leq 0.05 \) meant the difference between groups was statistically significant. In the case of unequal variances, the Games-Howell post hoc test was used. All statistical analyses were performed with SPSS Statistics (SPSS 25; IBM, USA).

6.3. Results and discussion
6.3.1. Moisture content
Moisture contents were measured for the different spray-dried powders (Table 6.2). The moisture content of powder dried from a 2:1 asparagus solids to maltodextrin ratio was found to be higher than that of the 1:2 ratio sample. By increasing the amount of maltodextrin before drying, the total solids content is increased. Both the maltodextrin concentration and the total solid content of the feed solution were anticipated to affect the properties of the spray-dried asparagus powder. To separate these effects, the 1:2* sample was also analysed, which was diluted with water to give a similar total solids content as the 1:1 ratio sample.

Diluting the sample from 45 to 36 % w/w initial dry matter, i.e. 1:2 and 1:2*, did not significantly affect the residual moisture content of the obtained powders (Table 6.2). It can be argued that the 1:2 and 1:2* samples should follow a similar drying curve, in which mostly the constant rate period will be longer for the diluted concentrate. Nevertheless, the equilibrium moisture constant is the same for these powders.

Spray drying of the 1:1 and the 1:2* samples with the same initial dry matter (i.e. 36 % w/w) however resulted in a significant difference in residual moisture content. When maltodextrin is added, the hygroscopicity of the solids is affected, which influences the residual moisture content. These observations are in line with the study of Grabowski, Truong and Daubert (2006), who investigated the influence of varying maltodextrin-matrix ratios in spray drying of sweet potato puree. An increased maltodextrin concentration in their feed resulted in lower residual moisture contents, even at low maltodextrin
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concentrations (0, 10 and 20 % on dry basis). Furthermore, the drying conditions influence the residual moisture content in the powder. Higher inlet air temperatures and smaller temperature differences between the inlet and outlet air \(\Delta T\) (\(T_{\text{in}} - T_{\text{out}}\)) generally result in powders with lower moisture content (Reineccius, 2004). The residual moisture content of the powders spray-dried at 180/105 (\(T_{\text{in}}/T_{\text{out}}\)) seems somewhat lower compared to 180/90 (Table 6.2), however, this difference was not statistically significant.

Table 6.2 Effects of asparagus solids to maltodextrin ratio and spray drying conditions on the moisture content and the glass transition temperature of the asparagus powder.

<table>
<thead>
<tr>
<th>Solids ratio</th>
<th>T(<em>{\text{in}})/T(</em>{\text{out}}) (({}^\circ\text{C} / {}^\circ\text{C}))</th>
<th>Residual moisture content ((\ % \text{ w/w}))</th>
<th>Glass transition temperature (({}^\circ\text{C}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>160/90-95</td>
<td>20.00(^{\dagger})</td>
<td>-</td>
</tr>
<tr>
<td>2:1</td>
<td>160/90-95</td>
<td>15.03 ± 0.15 (^{a})</td>
<td>4.18 ± 0.76 (^{a})</td>
</tr>
<tr>
<td>1:1</td>
<td>160/90-95</td>
<td>8.91 ± 0.24 (^{b})</td>
<td>28.07 ± 0.24 (^{b})</td>
</tr>
<tr>
<td>1:2</td>
<td>160/90-95</td>
<td>5.24 ± 0.17 (^{c})</td>
<td>44.47 ± 14.14 (^{abc})</td>
</tr>
<tr>
<td>1:2(^{*})</td>
<td>160/90-95</td>
<td>5.49 ± 0.51 (^{c})</td>
<td>55.01 ± 0.11 (^{c})</td>
</tr>
<tr>
<td>1:1</td>
<td>180/105</td>
<td>8.84 ± 0.18 (^{b})</td>
<td>28.61 ± 0.73 (^{b})</td>
</tr>
<tr>
<td>1:1</td>
<td>180/90</td>
<td>9.33 ± 0.22 (^{b})</td>
<td>24.10 ± 1.17 (^{b})</td>
</tr>
</tbody>
</table>

Note: The values followed by different lowercase letters (a-c) within a column are significantly different \((p \leq 0.05)\).
The samples 1:2\(^{*}\) were diluted to obtain a similar total solids content before spray drying as the 1:1 ratio.
\(^{\dagger}\)The same quantity was too low to analyse the moisture content thus an estimated value is reported.

6.3.2. Glass transition temperature

The formation of glassy powder particles is necessary to effectively encapsulate volatile compounds, to avoid fouling in the equipment and to obtain a free-flowing powder (Y. H. Roos, 2010). Therefore, the glass transition temperatures of the different spray-dried powders were measured (Table 6.2). The \(T_g\) of a powder is influenced by both its solids composition and its moisture content, and it is difficult to decouple those. With larger moisture content or less maltodextrin, lower \(T_g\) values are found. The \(T_g\) values of the 1:1 ratio powders spray-dried at different conditions were not significantly different. This was expected because the composition of those samples was the same and the residual moisture content in the powders was similar.
The effects of solid composition and residual moisture content on the $T_g$ were evaluated with the Couchman-Karasz equation for multicomponent mixtures (equation 6.3), previously described by (Bhandari & Howes, 1999).

$$T_g = \frac{x_w \Delta C_{p,w} T_{g,w} + x_m \Delta C_{p,m} T_{g,m} + x_a \Delta C_{p,a} T_{g,a}}{x_w \Delta C_{p,w} + x_m \Delta C_{p,m} + x_a \Delta C_{p,a}}$$ (6.3)

where $x_w$, $x_m$ and $x_a$ are the weight fractions of water, maltodextrin and asparagus solids on wet basis. $\Delta C_{p,w}$, $\Delta C_{p,m}$ and $\Delta C_{p,a}$ represent the heat capacity change at glass transition of water, maltodextrin and asparagus solids, respectively. $T_{g,w}$, $T_{g,m}$ and $T_{g,a}$ are the glass transition temperatures of water (138 K), maltodextrin DE12 and the asparagus solids, respectively. Equation 6.3 can be rewritten to 6.4 with $k_1$ equals $\Delta C_{p,m}/\Delta C_{p,w}$ and $k_2$ equals $\Delta C_{p,a}/\Delta C_{p,w}$, which is often referred to as the Gordon-Taylor equation:

$$T_g = \frac{x_w T_{g,w} + k_1 x_m T_{g,m} + k_2 x_a T_{g,a}}{x_w + k_1 x_m + k_2 x_a}$$ (6.4)

Two coefficients $k_2$ and $T_{g,a}$ were estimated based on Gordon-Taylor equation (6.4) using experimental data, whereas other parameter values were obtained from literature. Specifically, for water a $\Delta C_{p,w}$ value of 1.91 kJ·kg$^{-1}$·K$^{-1}$ was used and for maltodextrin a $\Delta C_{p,m}$ value of 0.425 kJ·kg$^{-1}$·K$^{-1}$ (Siemons et al., 2020). The anhydrous $T_g$ of maltodextrin DE12 ($T_{g,m}$) was 426 K (153 °C) (Siemons et al., 2020). We obtained a good fit ($R^2=0.95$) and estimated a $T_{g,a}$ of 32 °C and a $k_2$ of 0.48 (Figure 6.1). The $T_{g,a}$ can be roughly related to its composition as described in section 6.2.1, i.e. fructose (10 °C), glucose (36 °C) and sucrose (67 °C) (Y. Roos, 1993). The $\Delta C_{p,a}$ of 0.91 kJ·kg$^{-1}$·K$^{-1}$ was derived from the $k_2$ value and is slightly higher than reported values for mono- and disaccharides, e.g. fructose (0.75-0.84 kJ·kg$^{-1}$·K$^{-1}$), glucose (0.63-0.88 kJ·kg$^{-1}$·K$^{-1}$) and sucrose (0.60-0.77 kJ·kg$^{-1}$·K$^{-1}$) (Y. Roos, 1993).

Concerning storage, it is desired to obtain a powder in the glassy state at ambient temperatures (Y. H. Roos, 2010). When the powder shifts from a glassy to a rubbery state, undesired effects may occur, such as sticking, agglomeration and loss of volatile compounds (Bonazzi & Dumoulin, 2011). Based on our findings, only the samples prepared with asparagus solids to maltodextrin ratios of 1:2 or asparagus weight fraction ≤ 0.33 (Figure 6.1) reached a sufficiently high $T_g$ to be stored at ambient conditions.
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This model could potentially be used to estimate the $T_g$ of asparagus powder in future when other carrier agents are used.

![Graph A](image)

**Figure 6.1 (A)** The glass transition temperature of spray-dried asparagus concentrate as a function of the mass fraction of asparagus (dry basis). The experimental data is fitted with the Couchman-Karasz equation for multicomponent mixtures (dotted lines) for different residual moisture contents (0, 5, 10, 15 % w/w). Residual moisture contents of the experimental data can be found in Table 6.1. For the data point with asparagus weight fraction of 0, $T_g$ value of a spray-dried maltodextrin solution (30 % w/w) without asparagus concentrate was reported. For the data point with asparagus weight fraction of 1, $T_g$ value of liquid asparagus concentrate that was pre-dried in a climate chamber at 10 % RH was reported. **(B)** The parity plot of the experimental and the calculated values of $T_g$ based on the model.

### 6.3.3. Particle size distribution

The particle size distribution of all powders was analysed (Figure 6.2). Larger powder particle sizes (100-2000 µm) can be explained by undesired agglomeration. Specifically, agglomeration was observed for the 2:1 ratio of asparagus solids to maltodextrin powder. This sample also had the highest residual moisture content (15.0 % w/w), which stimulated agglomeration of the powder particles. A slight shift towards larger particle sizes can be observed with an increase in maltodextrin content. The highest peaks of the 2:1 ratio, 1:1 ratio and 1:2 ratio samples were found to correspond to a particle size of ca. 8 µm, 10 µm and 15 µm, respectively. This increase in average particle size may be explained by the generation of larger droplets during atomization due to the increase of the feed viscosity with the addition of maltodextrin (Bangs & Reineccius, 1982; Reineccius, 2004). The results of the 1:1 ratio dried at different inlet and outlet temperature combinations were found to
be similar (data not shown) indicating no significant effect of drying conditions on particle size distribution in this study.

**Figure 6.2** Particle size distribution of spray-dried asparagus powders with different asparagus solids to maltodextrin ratios dried at $T_{in}/T_{out}$ of 160/90. The 1:2* samples were diluted to obtain a similar total solids content before spray drying as the 1:1 ratio.

### 6.3.4. Morphology

Different particle morphologies such as smooth round and dented particles were observed for the spray-dried powders (Figure 6.3). Similar dented structures for MD12 were previously observed by Both *et al.* (2018) and Siemons *et al.* (2020). For the 2:1 ratio of asparagus solids to maltodextrin (Figure 6.3A), only small spherical particles with a smooth surface were present in the powder. It is suggested that the high concentration of small sugars from the asparagus concentrate allows more shrinkage of the droplets which results in smoother and more spherical particles. This is also in agreement with Paramita *et al.*, (2010) who observed that replacement of maltodextrin DE 11 by trehalose also provided smoother particles. The influence of the different spray drying conditions on the morphology appeared to be minor (data not shown). All powders had both smooth and dented particles and the particle sizes appear similar based on the SEM images.
Maltodextrin improves physical properties and volatile compound retention of spray-dried asparagus concentrate

Figure 6.3 Powder morphology of spray-dried asparagus powders with asparagus solids to maltodextrin ratios 2:1 (A), 1:1 (B), 1:2 (C), and 1:2* (D). The 1:2* samples were diluted to obtain a similar total solids content before spray drying as the 1:1 ratio. All powders were spray-dried at 160/90 (Tin/Tout). Bar = 10 µm.

6.3.5. Analysis of asparagus volatile compounds

To study the influence of maltodextrin on the volatile compounds after spray drying, first the overall volatile profiles of asparagus concentrate samples before and after spray drying were compared. Processing of the GC-MS raw data and manual filtering of system artefacts resulted in a list of 70 compounds that were further analysed. The focus was brought on the known key asparagus volatiles from literature (Pegiou et al., 2020).

Principal component analysis (PCA) was performed to obtain a general overview of the samples and how their volatiles composition differ. PCA on all the samples showed that PC1 separates the spray-dried powder and the concentrate/mix before drying, indicating a clear difference in the volatile profiles (Figure S6.1A). The QCs consisting of a mix of spray-dried powders were analysed to determine technical variability. The QCs group together in the PCA plot, indicating a low technical variation of the measurements. To get a better view of
the difference between the powders, PCA was performed on all the spray-dried powder samples with and without maltodextrin (Figure S6.1B). The first two PCs explain 48 % and 19.6 % of the total variation, respectively. The variation on PC1 can be attributed to the different ratios of maltodextrin used. This is more obvious from Figure 6.4A, where the PCA was performed on the spray-dried powders containing the different concentrations of maltodextrin. Here, the first PC explains 38.4 % of the variation between powder samples. The black arrow indicates that spray-dried powders with higher maltodextrin concentrations move towards the negative x-axis.

The loading plot (Figure 6.4B) shows that the influence of maltodextrin concentration on the retention of aromas is dependent on their molecular properties. Focusing on the PC1, we can see that mainly alcohols and aldehydes were more abundant in samples with higher concentrations of maltodextrin. Sulphur-containing volatile compounds were more abundant in samples with lower maltodextrin concentrations, while carboxylic acids, ethers and furans were not significantly affected by the maltodextrin concentration.

Among these 70 compounds, some compounds were previously identified as asparagus aromas including 3-methylthio-propanal, 1-octen-3-ol, 1-pentanol, octanal and dimethyl sulphide (Tressl, Bahri, et al., 1977; Ulrich et al., 2001). Some of these compounds, such as 1-octen-3-ol and octanal, are present in the asparagus concentrate and we aim to retain those during spray drying.

Figure 6.4 (continues in the next page)
Maltodextrin improves physical properties and volatile compound retention of spray-dried asparagus concentrate

Figure 6.4 (A) Score plot of the volatile profiles of the spray-dried samples prepared with maltodextrin. The score plot is based on 70 volatiles. The colours indicate the ratio of asparagus solids to maltodextrin. The samples were spray-dried at $T_{in}/T_{out}$ of 160/90, unless stated otherwise. (B) The loading plot corresponding to the PCA score plot.

Other compounds, e.g. dimethyl sulphide and 3-methylthio-propanal, are formed upon drying. For the latter category, higher peak intensity values were found with less or no maltodextrin. Most of the compounds in the spray-dried samples are positively correlated with the increasing concentration of maltodextrin (data not shown), which suggests that maltodextrin is effective as a carrier agent. The aromas already present in asparagus as well as the aromas that are generated during asparagus processing can be of great importance to the final aroma and taste of the powder.

6.3.6. Effect of maltodextrin on 1-octen-3-ol in mixtures

One volatile compound was selected for an in-depth analysis, 1-octen-3-ol. This alcohol is a key aroma compound in cooked asparagus conferring an earthy and mushroom-like aroma and is already present in the raw asparagus (Pegiou et al., 2020). 1-Octen-3-ol is formed via oxidative degradation of linoleic acid, which involves the transformation of hydroperoxides into the volatile 1-octen-3-ol facilitated by cleaving enzymes (Assaf et al., 1997; Tressl, Bahri, et al., 1977). 1-Octen-3-ol was present in the asparagus concentrate at relatively high concentration, indicating that the oxidative degradation might have been initiated during the pressing and concentration of the juice.
The influence of maltodextrin solutions on the partitioning of volatile compounds between the gas/liquid phases has been investigated before. Chung and Villota (1990) found that the concentration of butanol in the gas phase decreased when the maltodextrin content was increased. They suggested that maltodextrin forms aggregates with hydrophobic portions on the inside and these hydrophobic portions interact with butanol and other hydrophobic compounds. Jouquand, Ducruet and Giampaoli (2004) also stated that the retention of aroma compounds by maltodextrin is linked to the hydrophobicity of aroma compounds. In our study, we evaluated the effect of maltodextrin on the partition coefficient of 1-octen-3-ol by analysing the headspace of mixtures with equal amounts of 1-octen-3-ol and different maltodextrin concentrations. The results (Figure 6.5) indicate an inverse correlation between the measured 1-octen-3-ol in the headspace and the concentration of maltodextrin in the liquid phase. This inverse correlation is explained by the relative hydrophobicity of 1-octen-3-ol, i.e. log P = 2.5, and therefore enabled its complexation by maltodextrin.

![Figure 6.5](image)

**Figure 6.5** The peak intensity of 1-octen-3-ol measured by SPME GC-MS in asparagus concentrate prepared with different maltodextrin concentrations (results from two independent experiments are shown).

6.3.7. **Effect of maltodextrin on retention of 1-octen-3-ol in spray-dried powder**

The detected peak intensities of 1-octen-3-ol in the mixes before drying and in the spray-dried powders are shown in Figure 6.6. The peak intensities in the mix were found to decrease for the higher ratios of maltodextrin, this could be explained by the effect of maltodextrin on the gas-liquid partition coefficient as discussed previously.
Maltodextrin improves physical properties and volatile compound retention of spray-dried asparagus concentrate

**Figure 6.6** The peak intensity profiles of 1-octen-3-ol before (white circles) and after spray drying (grey circles) for asparagus concentrate prepared with different asparagus solids to maltodextrin ratios. The triangles indicate the samples 1:2*, which were diluted with water to give a similar initial solids content as the 1:1 ratio sample. All samples were spray-dried at $T_{in}/T_{out}$ of 160/90. The error bars represent the standard deviation of the experimental data ($n = 3$).

The peak intensities of 1-octen-3-ol were lower after spray drying, indicating that 1-octen-3-ol was partially lost during drying. Without maltodextrin (1:0) only a small amount of 1-octen-3-ol could still be detected after drying. Higher concentrations of maltodextrin increased the detection of 1-octen-3-ol. This suggests that maltodextrin DE12 retains part of the 1-octen-3-ol during spray drying, which may be explained by the reduction of the constant rate period. For the samples that were prepared with 1:2 ratios, the diluted samples (triangles in Figure 6.6) have slightly lower peak intensity after drying. The encapsulation of 1-octen-3-ol may have been decreased by the lower initial solids content.

To discuss the role of maltodextrin on aroma retention, we calculated the retention of 1-octen-3-ol based on the ratio of the peak intensities of the mix and the corresponding spray-dried powder. The retention of 1-octen-3-ol increases with higher concentrations of maltodextrin (Figure 6.7). This is in line with the results by Bangs and Reineccius (1982), who evaluated the retention of twelve organic flavour compounds, including 1-octen-3-ol, in a model system with maltodextrin DE10. The overall retention increased from 23 to 80 % when the maltodextrin concentration was increased from 32 % w/w to 50 % w/w. We observe similar trends (Figure 6.7) for two other alcohols in white asparagus, namely 1-pentanol and 1-hexanol (Tressl, Bahri, et al., 1977). To a smaller extent the retention of heptanal, an aldehyde previously detected in green asparagus juice (Chen et al., 2015), benefitted from an increase in maltodextrin (data not shown).
Figure 6.7 The retention after drying of 1-octen-3-ol, 1-pentanol and 1-hexanol. The dashed line is drawn to guide the eye for 1-octen-3-ol. The samples were prepared with different asparagus solids to maltodextrin ratios and spray-dried at $T_{in}/T_{out}$ of 160/90. The retention of the volatile compounds was calculated based on the peak intensities of the mix before drying and the spray-dried powder. The error bars represent the standard deviation of the experimental data ($n = 3$).

6.3.8. Effect of drying conditions

Three combinations of inlet and outlet temperatures were applied during spray drying of 1:1 ratio mixtures. In Figure 6.8 the abundances of selected compounds have been plotted. For 1-octen-3-ol, the influence of the drying conditions tested in this study had a minor influence on the measured abundance profiles. For dimethyl sulphide, there was no significant influence of inlet/outlet temperature combinations on the abundance. For 2-methylpropanal, 3-methylbutanal and 3-methylthio-propanal, however, the temperature combination of 180/105 resulted in larger headspace concentration compared to 160/90 and 180/90. These high values after drying at 180/105 can be related to the high outlet temperature inducing formation of these compounds. Ideally, we aim to minimise the formation of volatile compounds during drying. Volatiles that are formed during drying might get lost during storage due to package permeability, consequently, flavour stability will be reduced. Instead, these volatiles should be formed while the consumer prepares the food product.
Maltodextrin improves physical properties and volatile compound retention of spray-dried asparagus concentrate

Figure 6.8 Abundance of 1-octen-3-ol, dimethyl sulphide, 2-methylpropanal, 3-methylbutanal and 3-methylthio-propanal in spray-dried asparagus powder dried at different inlet and outlet temperatures (T\textsubscript{in}/T\textsubscript{out}). All powders were prepared with 1:1 ratio asparagus solids to maltodextrin. The error bars represent the standard deviation of the experimental data (n = 3). The same letters represent no significant difference (p ≤ 0.05).

6.4. Conclusions

The influence of maltodextrin concentration and drying conditions on the physical properties and aroma retention of spray-dried asparagus was studied. Increasing maltodextrin concentration resulted in spray-dried powders with a lower moisture content, higher T\textsubscript{g} and less undesired agglomeration. A ternary Gordon-Taylor equation reasonably described the effects of the composition of multicomponent mixtures and residual moisture content on the T\textsubscript{g} of asparagus powder. The drying conditions tested in this study had a minor effect on the physical properties of asparagus powder. Moreover, maltodextrin concentration in carrier formulations influenced the retention of a key flavour compound 1-octen-3-ol. Increasing maltodextrin concentration increased the retention of 1-octen-3-ol, as well as other alcohols and an aldehyde. Nevertheless, the drying conditions studied did not have a significant influence on volatile retention. Interestingly, higher outlet air temperature resulted in a higher amount of several asparagus volatiles that were formed during drying. To minimise the formation of these compounds, but also to still have enough drying capacity and avoid fouling issues, the outlet temperature should not be too high, preferably not above 90 °C.
In conclusion, this study showed that maltodextrin can be used to yield maximum volatile retention after spray drying of asparagus concentrate. This is because firstly aroma compounds can form a complex with maltodextrin, and secondly, a shorter constant rate period and fast skin formation during drying could hinder evaporation of aromas when increasing maltodextrin concentration.
6.5. **Supplementary Figures**

![Supplementary Figures](image)

**Figure S6.1** Score plots of the volatile profiles of (A) juice, mix, spray-dried samples and quality controls (QC) and (B) spray-dried powders only. The score plots are based on 70 volatiles. The colours indicate the ratio of asparagus solids to maltodextrin. The samples were spray-dried at $T_{in}/T_{out}$ of 160/90, unless stated otherwise.
Chapter 7

Metabolomics and sensory evaluation of white asparagus ingredients in instant soups unveil important (off-)flavours

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Abstract
Split-stream processing of asparagus waste stream is a novel approach to produce spray-dried powder and fibre. Asparagus ingredients processed by this method and a commercial asparagus powder were compared by evaluating their flavour profile in a soup formulation. Professional sensory panel and untargeted metabolomics approaches using GC-MS and LC-MS were carried out. Unsupervised and supervised statistical analyses were performed to highlight discriminatory metabolites and correlate these to sensory attributes. The spray-dried powder scored higher on asparagus flavour compared to the commercial powder. The fibre negatively impacted the taste and mouthfeel of the soups. GC-O-MS confirmed the role of dimethyl sulphide, 2-methoxy-3-isopropyl pyrazine and 2-methoxy-3-isobutyl pyrazine in asparagus flavour. Seven new volatile compounds are here also proposed to contribute to asparagus flavour notes, most of which were more abundant in the spray-dried powder. This research demonstrates the feasibility of upcycling asparagus waste streams into flavour-rich ingredients with good sensorial properties.

Keywords: Spray-dried powder; Metabolomics; Sensory perception; Asparagus flavour; Asparagus fibre; Random Forest
7.1. Introduction

Instant vegetable soup is primarily composed of dried vegetable powders. Commercial vegetable powders are commonly made by oven-drying (i.e. hot-air drying) small vegetable pieces which are then milled into a fine powder (Kamiloglu et al., 2016; Karam et al., 2016). This drying process is known to irreversibly alter the flavour profile of the vegetable (Nijhuis et al., 1998; Sagar et al., 2010). Consequently, supplementary flavour components often need to be added to the dried vegetable powders to obtain the desired sensory complexity of the final product (e.g. instant soups) and to ensure flavour stability. To produce products that are perceived as natural and healthy, the addition of these flavourings should ideally be avoided (Román et al., 2017).

A novel split-stream process has recently been developed to produce ingredients from vegetable waste streams generated during or after harvesting (J. W. Siccama, Pegiou, Zhang, et al., 2021). In this process, vegetable juice is separated from the fibre fraction and both streams are processed and dried separately, after which they can be recombined to yield vegetable powder. White asparagus (*Asparagus officinalis*) was selected as the model crop for two reasons. First, because the flavour of the commercial asparagus powders does not meet the standards of the industry and thus flavour supplements are required and second, due to the large waste volumes generated within a harvest season (ca. 30% of all harvested material). A significant part of this waste stream comprises the basal parts of each asparagus spear (ca. 5 cm) which are cut off and discarded (Pegiou et al., 2020). Within the scope of a more sustainable and circular food system, it is of interest to investigate the potential of exploiting these vegetable waste streams to generate dried powders for use as food ingredients in products such as instant soups (J. W. Siccama, Pegiou, Zhang, et al., 2021). The composition of volatile compounds of the obtained spray-dried asparagus powder has previously been investigated and it was suggested as a potential new natural food ingredient with high retention of volatile flavour compounds (J. W. Siccama, Pegiou, Eijkelboom, et al., 2021), but yet its flavour profile still needs to be assessed concerning sensory attributes like aroma, taste and mouthfeel.

It is well-known that combining sensory evaluation and metabolomics may give a deeper insights into the flavour composition of food products (Jacobs et al., 2021; Utpott et al., 2022). Recently, few studies have used the potential of both sensory evaluation and...
metabolomics for examining the flavour of, e.g. strawberry (Buvé et al., 2018), beer (Bettenhausen et al., 2020), tomato soups (Davarzani et al., 2021), cocoa powder (Greño et al., 2023) and soy sauce (Diez-Simon et al., 2021). There have been three studies combining sensory and metabolomics techniques to study cooked white asparagus (Dawid & Hofmann, 2012a; Hoberg et al., 2003; Ulrich et al., 2001). It is thanks to these three studies that we can link specific steroidal saponins to the bitter asparagus notes (Dawid & Hofmann, 2012a) and refer to several volatiles (e.g. dimethyl sulphide, 2-methoxy-3-isopropylpyrazine, hexanal) as being ‘key odorants’ of cooked asparagus (Hoberg et al., 2003; Ulrich et al., 2001). Specifically, dimethyl sulphide (DMS), is considered to be the aroma compound giving the typical ‘cooked asparagus’ flavour (Tressl, Bahri, et al., 1977; Tressl, Holzer, et al., 1977; Ulrich et al., 2001). However, it is of relevance to confirm the contribution of the known asparagus flavour compounds to the sensory profile of the processed asparagus materials as it is likely that multiple compounds are required to give the true asparagus experience.

The main objective of the study described here was to compare the in-house processed asparagus ingredients (i.e. concentrate and spray-dried powder) to a commercial asparagus powder with and without a flavour supplement, by evaluating the flavour profile in an instant soup formulation. The soup formulations were prepared based on the composition of a commercial instant asparagus soup only differing in the asparagus ingredients. We hypothesized that the variation in the ingredient composition of the soups would be reflected by differences in both the metabolite composition and the sensory profiles. The spray-dried powder was hypothesized to have a richer flavour profile compared to the commercial powder which is oven-dried. Moreover, the asparagus fibre, which remains after the extraction of the juice from the asparagus pieces, was processed and added to some of the soup prototypes. We hypothesized that the fibre would provide more structure and thickness to the soups, influencing the presumed mouthfeel sensation.

To investigate these hypotheses, the soup prototypes were assessed by a trained expert panel and were analysed using advanced metabolomics platforms. Solid phase microextraction gas chromatography mass spectrometry (SPME GC-MS) and liquid chromatography mass spectrometry (LC-MS) systems were employed to profile the volatile and non-volatile metabolites, respectively. Metabolite data were processed using an untargeted metabolomics approach. Random Forest techniques were used to link the
sensory and metabolomics data. The odour-activity and aroma attributes of the highlighted volatile compounds were confirmed by GC Olfactometry MS (GC-O-MS). Physical properties (particle size distribution, viscosity and morphology) of the samples were also monitored in relation to the mouthfeel of the soups.

7.2. Materials & Methods

7.2.1. Chemicals and reagents
Maltodextrin DE12 (GLUCIDEX® 12, Roquette, Lestrem, France) was used as carrier for the spray drying. A mix of n-alkanes (C₆ – C₂₁) was prepared. All alkanes were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The analytical standards used for metabolite identification had a purity between 96-99 %. All standards were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) except for methanethiol, 1,3-diethylbenzene, styrene and 1,4-diethylebnzene which were purchased from Greyhound Chromatography (Wallasey, UK). All standards were dissolved in methanol (Biosolve BV, Valkenswaard, The Netherlands). Methanol, formic acid and acetonitrile (Sigma-Aldrich, Zwijndrecht, The Netherlands) were used for the extraction and analysis of semi-polar non-volatile metabolites present in the soup samples.

7.2.2. Production of asparagus ingredients
Raw fresh asparagus cut-offs were kindly provided by Teboza BV (Helden, The Netherlands). The concentrated asparagus juice (concentrate) and spray-dried powder were prepared under hygienic conditions and following the split-stream processing method as described previously (J. W. Siccama, Pegiou, Eijkelboom, et al., 2021). In brief, concentrated juice was prepared from asparagus cut-offs by pressing them, followed by centrifugation of the juice to remove any solids. The juice was concentrated using reverse osmosis. The remaining asparagus fibre after pressing was dried with hot air and milled to fine powder. Aliquots of the concentrate (21.7 % w/w solids) were transferred to 1 L autoclaved glass bottles and stored at -20 °C until further analyses. The remainder of the concentrate was used for spray drying after adding maltodextrin DE12 in a 1:2 mass ratio (asparagus solids : maltodextrin). A Mobile Minor spray dryer (GEA, Dusseldorf, Germany) was used for spray drying with inlet and outlet temperatures of 196±3°C and 85±1°C, respectively. The spray-dried powders were stored in sealed aluminium bags at ambient temperature.
The asparagus fibres were stored in sealed plastic bags at -20 °C until further processing. After storage, the fibres were thawed in boiling water and washed at least three times at 70 °C to remove traces of sand. Subsequently, they were blanched at 90 °C for 3 min and dried in a food dehydrator (Sedona, Tribest, USA) at 68 °C for 20 h. After drying, the fibres were milled using a Pulverisette-14 RotorMill (Fritsch, Idar-Oberstein, Germany) using a 0.2 mm sieve and at 10,000 rpm. The milled fibres were manually sieved using a 0.25 mm sieve and the fine fraction was stored in sealed aluminium bags at ambient temperature for later use as one of the ingredients for two soup prototypes.

To ensure food safe processing of the asparagus ingredients, a risk assessment was performed. Hygiene indicators were tested by taking samples during different steps in the process. The analysed indicators were total viable count, Enterobacteriaceae, Lactobacilli, yeast, moulds, Bacillus cereus and Staphylococcus aureus. The results are reported in the Table S7.1. Lastly, we ensured the elimination of microorganisms by heating the asparagus soups for at least 2 min at 70 °C before consumption (Cebrián et al., 2017).

### 7.2.3. Preparation of asparagus soup prototypes

Six asparagus soup prototypes were prepared for evaluation of the sensory and metabolite profiles. Their composition was based on the recipe of a commercial asparagus instant soup. All prototypes included the same concentration of potato starch, salt, asparagus solids and final maltodextrin content. The six prototypes differed regarding the asparagus ingredients: the commercial dried asparagus powder with (CF) and without (C) asparagus flavour mix, the juice concentrate with (JA) and without (J) asparagus fibre and the spray-dried powder with (SA) and without (S) asparagus fibre (Table 7.1). The commercial powder and asparagus flavour mix were kindly provided by Unilever (R&D Unilever BV, Wageningen, The Netherlands). Potato starch and sea salt were purchased from a local supermarket (Wageningen, The Netherlands). The dry ingredients, i.e. starch, maltodextrin, salt and asparagus powders, were weighed according to Table 7.1 and mixed four days before the first sensory evaluation training session and stored in sealed plastic opaque containers at room temperature. The concentrate (1 L flask) was aliquoted into 50-ml polypropylene screw cap test tubes (Sarstedt AG & Co, Germany) after thawing overnight at 4 °C and aliquots were stored at -20 °C until use.
For each sensory evaluation, the required number of test tubes with concentrate were transferred to the fridge the day before and thawed to 4 °C.

**Table 7.1** Recipe for the preparation of the six asparagus soup prototypes in 1 L water. The labels indicate the asparagus ingredients of the soups. The amount of the asparagus ingredient is on total weight basis, maintaining the same asparagus solids content per soup. The salt, starch and final maltodextrin content were the same for all prototypes and based on the composition of a commercial product. The volume mean particle diameter (D[4,3]) and the viscosity of the soups measured at 50 s\(^{-1}\) and 40 °C (n=2) are presented. The letters show the significant differences based on pairwise comparisons after one-way ANOVA with the Games-Howell post hoc test for unequal variances (significant \(p\)-value < 0.05).

<table>
<thead>
<tr>
<th>Label</th>
<th>Asparagus ingredients</th>
<th>Primary asparagus component (powder or concentrate) (g)</th>
<th>Asparagus fibre (g)</th>
<th>Maltodextrin (g)</th>
<th>D[4,3] (μm)</th>
<th>Viscosity (mPa∙s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF</td>
<td>Commercial powder and added flavour mix</td>
<td>7.15</td>
<td>0</td>
<td>41.54</td>
<td>214 ± 7 (^{a})</td>
<td>7.3 ± 4 (^{a})</td>
</tr>
<tr>
<td>C</td>
<td>Commercial powder Concentrate and asparagus fibre</td>
<td>7.15</td>
<td>0</td>
<td>41.54</td>
<td>186 ± 48 (^{a})</td>
<td>8.4 ± 4 (^{a})</td>
</tr>
<tr>
<td>JA</td>
<td>Concentrate Spray-dried powder and asparagus fibre</td>
<td>14.31</td>
<td>3.58</td>
<td>41.54</td>
<td>227 ± 19 (^{a})</td>
<td>8.4 ± 0.5 (^{a})</td>
</tr>
<tr>
<td>J</td>
<td>Spray-dried powder</td>
<td>28.62</td>
<td>0</td>
<td>41.54</td>
<td>184 ± 0 (^{a})</td>
<td>11.8 ± 6 (^{a})</td>
</tr>
<tr>
<td>SA</td>
<td>Spray-dried powder</td>
<td>10.84</td>
<td>3.58</td>
<td>35.55</td>
<td>223 ± 18 (^{a})</td>
<td>8.4 ±1 (^{a})</td>
</tr>
<tr>
<td>S</td>
<td>Spray-dried powder</td>
<td>21.68</td>
<td>0</td>
<td>29.43</td>
<td>199 ± 32 (^{a})</td>
<td>5.8 ± 0.7 (^{a})</td>
</tr>
</tbody>
</table>

Note: 23.08 g of potato starch and 5.85 g of salt were added to 1 L of all soups.

Shortly before evaluation, 1 L of boiling water per prototype was added to the dry ingredients. In the case of 'J' and 'JA', the aliquoted concentrate was added to the dry ingredients immediately before adding the water. All samples were individually mixed using an electric hand-blender (Kenwood kMix Triblade Hand Blender, Kenwood Corporation, Tokyo, Japan) for 30 seconds. The samples were kept warm in Thermos flasks until the evaluation. When serving to the panellists, one additional sample (15 ml) of each soup was taken and stored in a 50-ml polypropylene screw cap test tube (Sarstedt AG & Co, Germany) at -20 °C for the metabolomics analysis.
7.2.4. Sensory evaluation

The sensory evaluation was carried out at the facilities of Essensor BV (Wageningen, The Netherlands). The sensory panel was selected from the top 10% of the population after screening on sensory abilities and sensitivities following the ISO 8586 criteria. Ten selected professional panellists first became fully acquainted with the six soup prototypes during four training sessions, to be acquainted with the samples without knowing the composition of ingredients, before the final descriptive evaluation. The sessions were carried out on separate days and each started at 10:00 AM. A set of 24 attributes was determined for the soups during the four training sessions, which covered odour, taste, mouthfeel and aftertaste attributes (Table S7.2).

During the final descriptive sensory evaluation, the six soup prototypes were served to the panellists twice in randomized order. Each sample was labelled with a unique three-digit code. The serving temperature was 65-70 °C. The panellists first evaluated the soups on the odour attributes. Subsequently, each sample was tasted at 60 °C to evaluate the taste, mouthfeel and after-feel attributes. Each panel member used thermometers to confirm and monitor the temperature. Evaluation scores were within the range of 0-100.

For the analysis of the sensory profiling data, the SenPAQ© software (QIStatistics, UK) was used. Principal component analysis (PCA) was performed to investigate the variation in the sensory profiles of the soup prototypes, after normalisation of the raw data (mean-centred and divided by the standard deviation). Two-way Analysis of variance (ANOVA) was performed per attribute and p-values were adjusted for multiple comparisons using the Benjamin-Hochberg approach. The Quality Index (QI) technique (Hyldig & Green-Petersen, 2004) was applied as a measurement of whether the panellists could reliably distinguish the soup prototypes regarding each sensory attribute.

7.2.5. Metabolomics

To profile the volatile and non-volatile chemical composition of the six soup prototypes, 15 ml were taken from each soup on each training sensory session day as well as the final descriptive evaluation and stored at -20 °C. After the final descriptive evaluation was complete, all samples were transported to the lab on ice. Once fully defrosted, these were placed in a water-bath at 70 °C to mimic the temperature as served to the panellists. Afterwards, they were respectively aliquoted in glass vials and Eppendorf tubes for further
analysis using SPME GC-MS and LC-MS as described below. All samples were analysed in a single sequence per metabolomics platform, in randomized order.

To profile the volatile compounds, 1 ml per soup replicate was pipetted in a 10-ml ND18 headspace screw glass vial (BGB®, Germany) and vials were closed with ND18 magnetic screw caps (8 mm hole) with Silicone/PTFE septa (BGB®, Germany). Before extraction, each sample was preconditioned at 65 °C for 10 min agitating at 350 rpm, to release the volatiles to the headspace mimicking how the panellists smelled the samples. Volatiles in the headspace were extracted at 65 °C for 10 min without agitation and absorbed onto a Polydimethylsiloxane/Divinylbenzene/Carboxen 50/30 μm diameter, 1 cm length fibre (Supelco, PA, USA). After extraction, SPME fibres were desorbed onto the GC-MS by heating the fibre at 250 °C for 2 min. The GC-MS analysis settings were as previously described (J. W. Siccama, Pegiou, Eijkelboom, et al., 2021). A mixture of all samples was used for each quality control (QC) sample. QCs were analysed in the same way as all biological samples and were distributed along the analysis series. A range of n-alkanes (C$_6$ – C$_{21}$), prepared from a set of stock solutions of the individual alkanes, was analysed in the same way to calculate retention indices.

To profile the non-volatile compounds, ultra-performance LC-MS was performed. The semi-polar compounds were extracted by mixing 0.3 ml of each soup replicate with 0.9 ml 32.04 M methanol and 0.035 M formic acid followed by sonication and centrifugation, as described previously by De Vos et al. (2007). The LC-MS calibration and analysis settings were as previously described (Pegiou et al., 2021) using both negative and positive ionization modes of the Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermos Fisher Scientific™, Germany). 0.3 ml from the same mixture of all samples as prepared and mentioned above, was used for each QC sample and analysed in the same way as the biological samples.

All GC-MS and LC-MS data were processed following the untargeted metabolomics workflow centred around the software packages MetAlign and MSClust as described before (Pegiou et al., 2021). The obtained relative abundances of the reconstructed metabolites in the processed data were log-transformed and a correction for signal drift was carried out, based on the QC samples (Wehrens et al., 2016).
Volatile metabolites were identified based on matching the reconstructed mass spectra and calculated RIs with authentic reference standards and those present in the NIST17 Mass Spectral Library and in-house databases. Non-volatile compounds were putatively identified based on matching their molecular ion mass and associated in-source fragments with the detected LC-MS asparagus compounds described by (Pegiou et al., 2021) and the online databases KnaPSAck (http://www.knapsackfamily.com/) and mzCloud (https://www.mzcloud.org/). The given level of identification (LOI) follows the guidelines of the Metabolomics Standards Initiative (Sumner et al., 2007).

7.2.6. Multivariate statistical analysis

The log-transformed metabolomics data were mean-centred, Pareto-scaled and variation between samples was initially explored by applying PCA. PCA analyses were performed using the R package ropls (Thevenot et al., 2015). Random Forest was applied for the supervised analysis and variable selection, considering the composition of the data matrices obtained. The bootstrapping method, which is applied during Random Forest analyses, converges to the leave-one-out cross-validation method which is suitable for datasets with a limited number of observations, enabling a higher prediction performance. For the Random Forest analyses, log-transformed data were used and analyses were performed using the R package randomForest (Liaw & Wiener, 2002). The number of trees (ntree) and the number of variables (mtry) for each decision rule were optimized for the minimum prediction error for each model. The Random Forest classification approach was followed to determine those GC-MS and LC-MS compounds indicating the ingredient composition of a specific soup prototype. Two classification analyses were performed per dataset; one based on the primary asparagus component (3 classes: commercial, concentrate, spray-dried) and one based on the presence of asparagus fibre (2 classes: yes, no) (Table 7.1). The commercial powder was classified as fibre-containing as it is produced from whole asparagus pieces, thus, including the fibres. The performance of each model was assessed by the out-of-bag (OOB) error rate which corresponds to the prediction error after leave-one-out cross-validation. Variable importance was estimated by the mean decreased accuracy which expresses how much accuracy the model loses by excluding each variable. Compounds with Mean Decrease Accuracy > 1 were considered relevant. Hierarchical clustering (HCA) of the soups was subsequently performed focusing on the selected variables after log-transforming
and autoscaling their abundances, and this was visualised in a heatmap. The HCA analysis
and visualisation were performed using the R packages pheatmap and ggplot2 in RStudio
with R version 4.1.1 (2021-08-10). The Random Forest regression approach was followed
to determine which individual compounds have a predicting relevance to sensory attributes
having a QI > 0.65 and p-adjusted < 0.05 (Table S7.2). The performance of each model was
assessed by the mean square error (MSE). Variable importance was estimated by the
increase in mean square error (%IncMSE) which expresses how much the percentage of
prediction error increases by excluding each variable. Compounds with %IncMSE > 1.50
were considered relevant. Microsoft Excel and PowerPoint (version 2104, 2021) were used
for additional data analysis and visualisation.

7.2.7. GC-Olfactometry-MS
The regression analyses highlighted 18 volatile compounds as being correlated to specific
sensory attributes of which eight could be unambiguously identified (level 1). A mixture of
the reference standards of these eight compounds was analysed with GC-O-MS, to determine
whether these volatiles are odour-active within the concentration range detected in the
soups without the extra addition of the flavour mix. A GC column splitter outlet was used to
split 1:1 towards the MS and the olfactory detection port (ODP2, Gerstel, The Netherlands).
The reference standards were dissolved in methanol and were used to prepare a solution in
water comprising dimethyl sulphide (5 µg/ml), pentanal (5 µg/ml), 1-hexanol (5 µg/ml),
1,3-dimethylbenzene (0.2 µg/ml), 1-octen-3-ol (0.5 µg/ml), octanal (0.05 µg/ml), (E)-2-
heptenal (0.2 µg/ml) and 2-methoxy-3-isopropyl pyrazine (0.2 µg/ml). The same SPME GC-
MS conditions as described in section 7.2.5 were used except that the GC oven temperature
program was adjusted to shorten total run time to 24 min by increasing the ramp to 20
°C/min after 15 min. For sniffing and assigning the individual peaks to a specific aroma
attribute (if any), three assessors were recruited within the laboratory. Each assessor
smelled the GC-O profile of the prepared standard solution and noted down the perceived
aroma per compound at the given retention times without knowing which compound
corresponds to which peak.

7.2.8. Physical characteristics
Selective physical properties of the dry powders and soup prototypes were analysed. The
particle size distributions of the commercial asparagus powder, spray-dried powder and
asparagus fibre were measured using a Mastersizer 3000 analyser (Malvern Inc, Malvern, UK) with the dry powder disperser Aero S. The size distributions of the commercial asparagus powder and asparagus fibre were analysed in the non-spherical analysis mode using the refractive index (RI) of cellulose, i.e. 1.468, since cellulose was considered the most abundant compound. The spray-dried powders were analysed with the spherical analysis mode using the RI of maltodextrin, i.e. 1.670. Furthermore, the particle size distributions of the soup prototypes were analysed in the Hydro MV module and the Mastersizer 3000 with the non-spherical analysis mode. The RI of cellulose (1.468) was used for the dispersed phase and 1.330 for the continuous phase (water). In addition, the particle size distribution of pure starch dissolved in hot water was measured using the spherical analysis mode and the RI of starch (1.450).

The viscosity of the soups was determined with the Anton Paar rheometer (MCR301, Anton Paar GmbH, Graz, Austria) with a concentric cylinder geometry (CC-27). A shear rate sweep with a logarithmic increasing shear rate from 1 to 1000 s\(^{-1}\) was performed. The rheology measurements were performed at 40 °C, which is assumed to be the relevant temperature inside the mouth before swallowing the soup (Deblais et al., 2021). The viscosities of the soups at 50 s\(^{-1}\) are reported (Table 7.1). This shear rate has been adopted as the oral shear rate standard by the National Dysphagia Diet task force and is considered a reasonable order of magnitude for swallowing liquids (Ong et al., 2018; Popa et al., 2013).

Microscopy images of the soups were taken using a light microscope (Carl Zeiss AxioScope, Jena, Germany) after the soup samples were vortexed to ensure homogeneity. A drop of the sample was placed on a microscopic slide under a coverslip. The images were captured (AxioCam MRc 5 camera) at a 20x magnification.

7.3. Results

7.3.1. The sensory profiles of the asparagus soups

Ten professional panellists evaluated the soup prototypes on 24 sensory attributes (Table S7.2). PCA was performed on the sensory profiles and >79 % of the overall variance was explained by the first two principal components (Figure 7.1A). The soup containing the commercial powder (C) had a similar sensory profile as the soups containing the concentrate or the spray-dried powder with fibre (JA and SA), as these are located close to each other on the PCA plot. These three prototypes had a contrasting sensory profile to the soups with the
concentrate or the spray-dried powder without fibre (J and S), as well as the one with the flavour-supplemented commercial powder (CF), which, along PC2, was separated from all the other soups (Figure 7.1A). The loadings (sensory attributes) being close to each other were positively correlated while attributes being located at opposite directions in Figure 7.1A were negatively correlated. Likewise, soups located close to specific attributes obtained a high score for those sensory characteristics. For instance, the ‘CF’ soups scored high on the asparagus odour (O_Asparagus), asparagus taste (T_Asparagus) and the overall aroma intensity (O_Intensity) (Figure 7.1A). These scores were significantly higher (p adjusted < 0.05) compared to all soups except for the ‘S’ prototype (Figure 7.1B). In contrast, the soups containing the commercial powder (C) or the in-house processed ingredients with asparagus fibre (SA, JA) had a significantly higher score on the cardboard taste (T_Cardboard) compared to the other soups (Figure 7.1A,B). Significant differences between the soup prototypes were also observed regarding the thickness (M_Thickness), powdery mouthfeel and after-feel (M_Powdery, AF_Powdery) (Figure 7.1B, Table S7.2). The samples without asparagus fibre, i.e. ‘J’ and ‘S’, scored significantly lower on the powdery mouthfeel and after-feel compared to all the other soups. The ‘J’ prototype scored also significantly lower on thickness (Figure 7.1B).

The sensory attributes that had a significantly different score between any of the soup prototypes (p adjusted < 0.05 and QI > 0.65) (Table S7.2) were further investigated by combining the sensory with the metabolomics results as indicated below.

**7.3.2. The metabolite profiles of the asparagus soups**

The chemical composition of the soups was studied regarding the secondary metabolites using SPME GC-MS (volatiles) and LC-MS (non-volatiles). Following an untargeted metabolomics workflow for processing the raw data the compounds retained for further examination were 85 volatiles and 951 non-volatiles (both ionisation modes). Sulphur-containing compounds, furans, pyrazines, aldehydes, alcohols, ketones, aromatics, hydrocarbons, flavonoid glucosides and saponins were among the main groups of annotated compounds (Tables S3, S4). All compounds had a lower relative standard deviation (RSD) across the QCs than the biological samples and the mean RSD across the QCs was lower than 0.25, indicating good technical reproducibility.
Figure 7.1 Statistical analysis of the sensory evaluation data of the six soup prototypes from ten trained professional panellists. Score range: 0 – 100 with min=0 and max=82.7. (A): PCA biplot of the six different soups and the 24 sensory attributes that were scored during the evaluation which are labelled as in Table S7.2. Data points representing the soups are based on the average of all scores from all panellists and ellipses show the standard deviation. Data points are coloured based on the asparagus ingredients of the soups as presented in Table 7.1. The % of explained variance per PC is provided in parentheses on the x and y axes. (B): Bar graphs of the average score for seven selected sensory attributes that showed significant differences between the six soup prototypes related to odour (O), taste (T) mouthfeel (M) and after-feel (AF). The error bars show the 95 % confidence intervals based on the scores of 10 panellists who evaluated each prototype twice (n=20). Bars are coloured based on the asparagus ingredients of the soups as in shown in (A). The letters indicate significant differences between the soup prototypes per attribute based on pairwise comparisons after two-way ANOVA. P-values were adjusted with the Benjamin Hochberg FDR procedure (significant p-value < 0.05).
The GC-MS and LC-MS profiles were studied separately by PCA to explore the variation between the soup prototypes (Figure 7.2A,B). The main differentiation was detected along PC1, explaining 41% and 21% of the overall variation of the volatiles and non-volatiles, respectively, and was between the soups containing the commercial powder and those containing the in-house processed ingredients (Figure 7.2A,B). The metabolites with the highest contribution to the observed separation of the soups are depicted in Figures 7.2C and 7.2D. These were one thiazole, acetoin, ethanol, one nitrogen-containing volatile compound (Figure 7.2C) and three LC-MS compounds (pos_120, neg_334 and neg_309 in Figure 7.2D) being detected at high levels in the in-house processed ingredients, and dimethyl disulphide (DMDS), (E)-2-heptenal, decanal, 2-butyl-2-octenal (Figure 7.2C) and four LC-MS compounds (neg_71, neg_575, pos_521 and pos_727 in Figure 7.2D) being detected at high levels in the commercial powder. Among the prototypes with the in-house ingredients, the presence of asparagus fibre appeared to influence the metabolite composition, which is most evident from the non-volatiles (Figure 7.2B). The impact of adding the flavour mix to the commercial powder is only reflected by the volatiles (Figure 7.2A). The three volatiles that were highly abundant in the flavour-supplemented soups were dimethyl sulphide (DMS), 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine (Figure 7.2C).

7.3.3. Compounds specific for the asparagus ingredients of the soups

The metabolite profiles of the soup prototypes varied based on the asparagus ingredients. The addition of asparagus fibre to the in-house prototypes appeared to have a dominant effect on their metabolite profiles (Figure 7.2A,B). The composition of the soups was therefore, examined by performing two Random Forest classification analyses to highlight the discriminatory compounds. One analysis for classifying the soup prototypes based on the primary asparagus component (commercial, concentrate, spray-dried) and one based on the presence of asparagus fibre (CF, C, JA, SA contain fibre) (Table 7.1).
Figure 7.2 Principal component analyses (PCA) of the metabolite profiles of the six soup prototypes. PCA score plots based on (A) 85 volatiles and (B) 951 non-volatiles. The first two PCs are presented, and the explained variance is shown in parentheses on the axes. The black ellipses represent the 95% confidence interval from the Hotelling T2 function. Data points are coloured based on the asparagus ingredients of each soup as presented in Table 7.1. The coloured ellipses show the confidence interval for each soup prototype based on the analysed replicates (n=4 or 5). (C): PCA loading plot of the GC-MS profile with the top 12 variables highlighted. Annotation is given in case of LOI<3. (D): PCA loading plot of the LC-MS profiles with highlighted the variables having high contribution. Analyses and plots were made using the ropls R package.

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Figure 7.2 (continued from the previous page)
The Random Forest classification models highlighted eight compounds which were indicative for the primary asparagus component (mean OOB error rate from the two metabolomics datasets 26.7%) and 12 compounds indicating the presence (six compounds) or absence (six compounds) of asparagus fibre (mean OOB error rate from the two metabolomics datasets 3.6%). HCA of the soups based on the 20 discriminatory compounds demonstrated the grouping of the prototypes according to the asparagus ingredients (Figure 7.3A). The soups without asparagus fibre are in cluster I and the soups with asparagus fibre are in cluster II (Figure 7.3A). The 20 compounds were clustered in three main groups (Figure 7.3A). The compounds in groups 1 and 2 are indicative of the absence or presence of asparagus fibre in the soups, respectively. The compounds in group 3 were highly abundant in the dried powders (spray-dried and commercial), except for DMS and 1-hexanol, which were specifically more abundant in the spray-dried and the flavour-supplemented commercial powder (Figure 7.3A). The five sulphur-containing metabolites were examined in detail considering their reported high relevance to asparagus flavour notes. Asparagusic acid, S-methylmethionine and 1,2-dithiole followed similar patterns and were more abundant in the prototypes without fibre (Figures 7.3B – 7.3D). DMS was detected at high levels in the ‘CF’, but also in the ‘S’ soups (Figure 7.3E) and DMDS was highly abundant in the ‘C’ and ‘CF’ soups (Figure 7.3F).

7.3.4. Compounds relevant for the perception of asparagus flavour notes

To retrieve compounds that are of specific relevance to certain sensory attributes, the outcomes of the descriptive sensory analysis and the metabolomics evaluation of the asparagus soups, were combined using advanced statistical tools.

Of the 24 scored sensory attributes, 13 had significantly different scores between the soups ($p$ adjusted < 0.05 and QI > 0.65) (Table S7.2). Random Forest regression analysis was performed to determine which metabolites potentially contribute to certain flavour characteristics. Five sensory attributes were reliably predicted by GC-MS profiles and three sensory attributes were reliably predicted by LC-MS profiles (MSE<25). In addition, the Pearson’s correlation coefficient between each compound and the linked sensory attribute was calculated to help in the interpretation of their relationship (Tables 7.2A and 7.2B).
Figure 7. Visualisation and inspection of compounds found to be specific for the composition of the asparagus soups based on the Random Forest classification analyses. (A) Heatmap of the 20 highlighted compounds (Mean Decreased Accuracy > 1) and their abundances in the soup prototypes (Fibre-specific: protodioscin, pos_479, asparagusic acid, neg_494, neg_803, neg_310, S-methylmethionine, pos_217, gcms_71, dimethyl disulphide, 1,2-dithiole, pentanal. Ingredient-specific: pos_112, pos_439, pos_82, pos_293, 1-hexanol, 3-methylbutanal, 1-octen-3-ol, dimethyl sulphide). Data were log-transformed and scaled. Dendrograms correspond to the clustering of the soups or the compounds based on HCA using Ward's method. (B – F): The abundances of the five sulphur-compounds shown in (A) in the six soup prototypes are presented as boxplots.

(continues in the next page)
Figure 7.3 (continued from the previous page)
Concerning the GC-MS profiles, in total 18 volatiles were highlighted as significantly correlating to asparagus odour, overall odour and taste intensity and the two powdery attributes (Table 7.2A). The highlighted relationships between volatiles and sensory attributes were mainly positively correlated. Specifically, the volatiles that were found to significantly correlate to high perception of asparagus odour and taste were DMS, 2-methoxy-3-isopropylpyrazine, 3-methyl-1-butanol, 1,3-dimethylbenzene and one unidentified compound (Table 7.2A).

Concerning the LC-MS profiles, six compounds were found to be highly associated with cardboard taste and 12 compounds were linked to the two powdery attributes (Table 7.2B). For each compound, the provided monoisotopic mass was calculated based on the base peak and the Pearson’s correlation coefficient indicates whether the presence (positively correlated) or absence (negatively correlated) of these specific compounds can predict the perception of the linked flavour attribute. For example, S-adenosylhomocysteine was positively correlated with the powdery mouthfeel while protodioscin was negatively correlated with the powdery experience (Table 7.2B).

7.3.5. GC-Olfactory-MS confirms the sensory-activity of asparagus volatiles.

To test whether the volatile compounds that were associated with specific flavour attributes are odour-active, a series of GC-O-MS analyses were executed. A mix of eight identified volatile compounds that were purchased as pure standards was prepared. The mix was then analysed with GC-O-MS and smelled by three assessors in independent runs. Retention times of the eight compounds were such that each peak could be readily evaluated separately. Results showed that 2-methoxy-3-isopropyl pyrazine, 1-octen-3-ol and (E)-2-heptenal were odour-active based on all three assessors who also recorded similar aromas (Table 7.2A). DMS, pentanal, 1-hexanol and octanal were also odour-active and could be detected by two of the three assessors, while 1,3-dimethylbenzene was not perceived by any of the assessors (Table 7.2A). Interestingly, when the three assessors were finally asked to smell the mixture of the standards from the GC vial, two directly described this as being like asparagus.
Table 7.2A List of the volatile compounds that were linked to a sensory attribute based on Random Forest regression (%IncMSE > 1.50). The Pearson’s correlation coefficient of each compound with the related sensory attribute was calculated to interpret the relationship between them. The aroma characteristics per compound based on the Good Scents and FooDB databases are provided. In the final column, the aroma characteristics per compound as perceived by the assessors during the GC-O-MS analysis are listed.

<table>
<thead>
<tr>
<th>Compound / ID</th>
<th>Sensory attribute(s)</th>
<th>Pearson’s correlation coefficient</th>
<th>Aroma attribute based on databases¹</th>
<th>Aroma attribute perceived at the ODP²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulphide</td>
<td>O_Intensity 0.55</td>
<td>onion, cabbage</td>
<td>earthy, asparagus, potato</td>
<td></td>
</tr>
<tr>
<td>2-Methoxy-3-isopropylpyrazine</td>
<td>O_Intensity 0.49</td>
<td>earthy, bean, pea</td>
<td>earthy, vegetable, asparagus</td>
<td></td>
</tr>
<tr>
<td>gcms_112</td>
<td>O_Intensity 0.20</td>
<td>Na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>O_Intensity 0.44</td>
<td>herbal, fruity</td>
<td>herbal, grassy</td>
<td></td>
</tr>
<tr>
<td>2-Methoxy-3-isobutylpyrazine</td>
<td>O_Intensity 0.36</td>
<td>bell pepper, earthy</td>
<td>Na</td>
<td></td>
</tr>
<tr>
<td>gcms_115</td>
<td>O_Asparagus 0.30</td>
<td>Na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methyl-1-butanol</td>
<td>O_Asparagus 0.43</td>
<td>fruity, banana</td>
<td>Na</td>
<td></td>
</tr>
<tr>
<td>1,3-Dimethylbenzene</td>
<td>O_Asparagus 0.01</td>
<td>-</td>
<td>not detectable</td>
<td></td>
</tr>
<tr>
<td>gcms_105</td>
<td>T_Intensity 0.30</td>
<td>Na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octanal</td>
<td>T_Intensity 0.20</td>
<td>waxy, orange peel</td>
<td>fruity, floral</td>
<td></td>
</tr>
<tr>
<td>gcms_66</td>
<td>T_Intensity 0.52</td>
<td>Na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentanal</td>
<td>M_powdery 0.84</td>
<td>fermented, nutty</td>
<td>sweet, floral</td>
<td></td>
</tr>
<tr>
<td>1-Octen-3-ol</td>
<td>M_Powdery 0.80</td>
<td>earthy, mushroom</td>
<td>earthy, mushroom</td>
<td></td>
</tr>
<tr>
<td>gcms_71 (alkane)</td>
<td>M_Powdery 0.65</td>
<td>Na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gcms_43 (nitrogen-containing)</td>
<td>M_Powdery -0.78</td>
<td>Na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E)-2-Heptenal</td>
<td>M_Powdery 0.72</td>
<td>fatty, pungent, intense</td>
<td>strong chemical</td>
<td></td>
</tr>
<tr>
<td>n-Caproic acid vinyl ester</td>
<td>M_Powdery 0.66</td>
<td>-</td>
<td>Na</td>
<td></td>
</tr>
<tr>
<td>gcms_76 (sulphur-containing)</td>
<td>M_Powdery -0.77</td>
<td>Na</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ aroma attribute based on The Good Scents (http://www.thegoodsentscompany.com/) and FooDB (https://foodb.ca/) databases.
² aroma attribute perceived at the ODP by at least two of the three assessors. Na when no reference standard was analysed.

7.3.6. Physical properties and mouthfeel of the soup prototypes

The particle size distributions of the dry asparagus ingredients (i.e. commercial, spray-dried powder and asparagus fibre) were analysed using the dry powder dispenser and different size distributions were observed (Figure S7.1A).
Despite the fine milling and sieving, the asparagus fibre preparation consisted of the largest particles, with a volume mean particle diameter (D[4,3]) of 236 µm, whereas the commercial and the spray-dried powders were characterised with a D[4,3] of 160 and 155 µm, respectively. Therefore, it was anticipated that the presence of fibre might negatively influence the sensory perception of the soups, giving a sandy and thicker mouthfeel. Hence, physical properties of the soup samples were measured and then related to the perceived mouthfeel. Despite the differences in perceived sensorial properties (i.e. powdery mouthfeel) (Figure 7.1B), the D[4,3] values indicated no significant differences among the soup prototypes (Table 7.1). The particle size distributions of the soups were almost the same despite the different asparagus ingredients used, which were also identical to the particle size distribution of pure starch suspended in hot water (Figure S7.1B). The small quantity of asparagus fibre present did not yield changes in the particle size distribution of the soups. In addition, the viscosity of soups measured at 50 s⁻¹ shear rate and 40 °C did not
differ significantly, and all soup samples showed similar shear-thinning behaviour (data not shown).

The soup prototypes containing spray-dried powder with or without asparagus fibre were also observed under the light microscope (Figure S7. 2). As expected, the presence of asparagus fibre was clearly visible in the ‘SA’ soup as a dense and elongated particle. The other particles that were visualised in both ‘S’ and ‘SA’ soups were starch granules given their morphology of swollen and round particles. Other soup prototypes were also analysed and the images of the samples with fibre (CF, C and JA) were similar to the ‘SA’ soup, while the sample without fibre (J) was similar to the image of ‘S’ (data not shown).

7.4. Discussion

7.4.1. A positive impact of spray drying on the flavour of instant asparagus soups

The flavour-supplemented commercial powder scored significantly higher than the commercial powder on its own with regards to asparagus flavour and flavour intensity (Figure 7.1A,B), indicating the effectivity of the added flavour mix on the overall sensory perception. However, the ultimate goal for industry would be to design a product which can avoid this required addition of flavour supplements in food products (Román et al., 2017). This would result in a more natural and sustainable solution.

The sensory and the metabolomics results indicated that the soup prototypes with split-stream processed asparagus ingredients but without asparagus fibre had similarities with the flavour-supplemented commercial powder (Figures 7.1A and 7.2AB). Specifically, the soups containing the spray-dried powder were awarded higher scores on the key attributes asparagus odour and taste than the soups containing the commercial powder without the supplementary flavour mix (Figure 7.1B). This indicated that the sensory profile of the spray-dried powder was richer in asparagus flavours compared to the hot-air dried commercial powder. The soups containing the spray-dried powder had slightly higher scores on the asparagus odour and taste attributes than those containing the concentrate (Figure 7.1B), highlighting a positive impact of spray drying on the flavour profile. This confirms the richer flavour of the spray-dried powder compared to the concentrate as was also suggested based on the chemical composition of the processed asparagus ingredients (J. W. Siccama, Pegiou, Zhang, et al., 2021).
Known asparagus aroma compounds, such as DMS and 2-methoxy-3-isopropylpyrazine (Hoberg et al., 2003; Tressl, Bahri, et al., 1977; Ulrich et al., 2001) were seen to be highly abundant in the flavour-supplemented commercial powder (Figure 7.2C, Table S3) and were also positively correlated with the asparagus flavour attributes (Table 7.2A). However, when comparing the spray-dried to the commercial powder, these key odorants were more abundant in the spray-dried powder and hardly detected in the commercial one (Figure 7.3E, Table S3). This confirms the successful retention of key aroma compounds upon spray-drying, as was predicted previously (J. W. Siccama, Pegiou, Zhang, et al., 2021).

With respect to the sulphur-containing flavour compounds, which have been previously investigated in cooked asparagus spears highlighting their sensory-relevance (Tressl, Bahri, et al., 1977; Ulrich et al., 2001), we detected and monitored two non-volatile precursors (S-methylmethionine and asparagusic acid) and the volatiles DMS, DMDS and 1,2-dithiole (Figures 7.3B-F). The non-volatile precursors which can be converted on heating to DMS and DMDS were more abundant in the spray-dried powder compared to the commercial one (Figures 7.3B,C). This explains the higher levels of DMS in the spray-dried powder compared to the pure commercial powder (Figure 7.3E). Therefore, spray drying which we predict leads to the encapsulation of aroma compounds (J. W. Siccama, Pegiou, Zhang, et al., 2021) may also have a positive impact on retaining key flavour precursors. The fact that DMDS is equally high in both prototypes with the commercial powder indicates that it was already present in the actual commercial powder (and not in the flavour mix). Given that DMDS is also formed during heat treatment (Belitz et al., 2008; Parry et al., 1985; Tressl, Holzer, et al., 1977), the much higher abundance in the commercial powders compared to the others can be explained by the fact that the commercial powder has undergone a longer heat treatment than the in-house produced powders.

7.4.2. The negative impact of asparagus fibre on the flavour and mouthfeel of instant asparagus soups

During the split-stream processing of asparagus waste streams, as a first step, the fibres are separated from the juice (J. W. Siccama, Pegiou, Eijkelboom, et al., 2021). To fully exploit the generated asparagus ‘waste’ in a sustainable and circular manner, it was considered that the asparagus fibre could potentially be reintroduced into the production process to make powders. It was hypothesized, that the addition of the processed fibre in the final soup...
formulation could (positively) affect the mouthfeel sensation by providing more structure and thickness to the soups, thereby having a higher sensory impact. Lyly et al. (2004) evaluated the sensory characteristics and rheological properties of vegetable soups enriched with soluble fibres in the form of oat and barley β-glucans. They found that the concentration of β-glucan positively correlated to the viscosity and perceived thickness of the soups. Alqahtani et al. (2014) investigated the addition of insoluble fibre (i.e. orange, wheat and oat fibre) to beverages and demonstrated that the fibre-enriched beverages are perceived favourably by the panellists. Furthermore, the oat fibre samples received the highest score on overall acceptability, even higher than the commercial prototype. In the study presented here, samples with added asparagus fibres scored higher on cardboard taste, artificial taste, off-taste and powdery mouthfeel (Figure 7.1A), which may suggest that the addition of fibres negatively impacts the overall sensory perception. Interestingly, despite these differences in mouthfeel as perceived by the panellists (Figure 7.1B), the volume mean particle diameter (D[4,3]) and viscosity of the soups were not significantly different (Table 7.1). While examining the morphology of the soup prototypes under a light microscope, we saw clear differences between samples with and without fibre. The asparagus fibre was different in shape compared to the starch granules (Figure S7.2). The textural perception of particles is strongly influenced by the size, shape and deformability of the particles (Appelqvist et al., 2015; Tyle, 1993). Although the starch granules and asparagus fibres were found to be in the same size range, the asparagus fibres were rigid insoluble particles that were less deformable compared to starch granules. In addition, asparagus fibres were more irregular in shape, due to the milling process. Tyle (1993) demonstrated that hard and angular particles result in more perceived grittiness than soft and round particles. Therefore, in this study, the perceived thicker and powdery mouthfeel of the soups containing asparagus fibre was possibly caused by the presence of these rigid and angular fibre particles.

The metabolite profiles of the asparagus instant soups which were affected by the presence of fibre (Figures 7.2A,B and 7.3A) were also linked to the sensory data. Some of the fibre-specific metabolites (Figure 7.3A) were also found to correlate with specific flavour attributes (Tables 7.2A,B). The compound pos_479 which was highly abundant in all the fibre-containing prototypes (Figure 7.3A), was also highly positively correlated with the
cardboard taste of the soups (Table 7.2B), suggesting that this non-volatile compound should be further investigated as an off-flavour in the asparagus soups (Whitson et al., 2010). The volatile compounds, pentanal, 1-octen-3-ol and gcms_71 which were highly abundant in the fibre-containing prototypes (Figure 7.3A), were positively correlated with the powdery mouthfeel (Table 7.2A). The powdery sensation for an instant soup is likely undesirable (Ssepuuya et al., 2018), as it was also indicated by the sensory evaluation of the soups where the powdery attributes were positively correlated with the off-taste and artificial attributes (Figure 7.1A,B). The fibre-containing soups scored higher on powdery mouthfeel compared to the other soups (Figure 7.1A,B), suggesting a negative impact of the asparagus fibre on the flavour sensation. Thus, the fibre-specific metabolites may potentially be related to off-flavours if they were sensory-active.

7.4.3. Proposal of new asparagus odorants and off-flavours

The previously reported key asparagus odorants DMS, 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine (Ulrich et al., 2001) were in this study strongly correlated with important asparagus flavour attributes of the soups (Table 7.2A) and moreover DMS and 2-methoxy-3-isopropyl pyrazine were also perceived to have an earthy and asparagus smell by at least two of the three assessors during the GC-O-MS study (Table 7.2A). Additionally, 3-methyl-1-butanol, 1-hexanol, octanal and four unidentified compounds (gcms_66, gcms_105, gcms_112 and gcms_115) were also highly associated with sensory attributes, specifically with asparagus odour, overall odour and taste intensity. The three annotated compounds have fruity and herbal aroma attributes (Table 7.2A) that resemble fresh asparagus. The aromatic compound 1,3-dimethylbenzene was linked to asparagus odour, but in the GC-O-MS analysis, it was not detected by any of the assessors (Table 7.2A). This is in agreement with the Good Scents database (http://www.thegoodscentcompany.com/) which does not associate it with a detectable aroma. It may be that this compound acts as an aroma enhancer when present in a matrix but this would require additional studies using an aroma matrix dilution series.

The powdery sensation which was evaluated regarding the mouthfeel and after-feel (Table S7.2) is not a preferred feature for soup products (Ssepuuya et al., 2018) and this was confirmed as the powdery attributes were positively correlated with off-taste (Figure 7.1A), although potential causality should be further investigated for their contribution to off-
flavours. Asparagusic acid, two saponins (protodioscin and pos_371) (Table 7.2B) and two volatiles (one nitrogen-containing and one sulphur-containing compound) (Table 7.2A) were negatively correlated with the powdery mouthfeel implying their contribution to ‘positive’ asparagus flavours. The essential role of asparagusic acid in the formation of important sulphur-containing asparagus odorants is known (Parry et al., 1985; Tressl, Bahri, et al., 1977). Further investigation of the two volatiles (gcms_76 and gcms_43), which were highly abundant in the prototype soups without fibre (Table S3) might lead to the discovery of two new asparagus odorants. In contrast, pentanal, (E)-2-heptenal, 1-octen-3-ol and n-caproic acid vinyl ester which were highly positively correlated with the powdery sensation of the soups are potential off-flavours worthy of attention. Blanda et al. (2010) investigated the volatile composition of boiled potatoes and the influence of additives on the flavour. They showed that increased levels of medium-chained aldehydes (e.g. pentanal, 2-heptenal) led to the formation of off-flavours as perceived by the panellists. Pentanal, (E)-2-heptenal and 1-octen-3-ol have been previously reported as key odorants in cooked white asparagus (Ulrich et al., 2001) but here are suggested as causal off-flavours. This contradiction is likely related to the different matrix of the asparagus materials used (cooked spear versus instant soup) as well as the concentration of the specific compounds and the rest of the profile. Regarding the non-volatile profile of the asparagus soups, six compounds were positively correlated with the cardboard taste attribute (Table 7.2B), suggesting that these compounds are also potentially causal off-flavours. Table 7.3 summarizes the outcomes of our study and our proposal with respect to the asparagus flavours and off-flavours.

7.5. Conclusions
This sensory descriptive analysis showed that soup prototypes prepared with spray-dried asparagus powder made from concentrated asparagus juice had similar flavour notes to those prepared from a flavour-supplemented commercial powder. Adding asparagus fibre negatively affected the flavour and mouthfeel of the soup prototypes. Using advanced metabolomics tools, a chemical characterization of the soups was performed and the datasets of the two analyses (sensory and metabolomics) were fused and examined following Random Forest approaches. Not only key known asparagus odorants were highlighted and confirmed, but we also suggest new compounds with potential relevance for the sensory profile of the asparagus ingredients. In conclusion, this study has revealed that
Spray drying of asparagus concentrate is a promising processing method to produce flavour-rich asparagus powder, as compared to the conventional oven-drying process. A similar process may be tested to upcycle other vegetable waste streams to produce flavour-rich food ingredients, in turn contributing to the sustainability of food systems. Ultimately, this could reduce the usage of supplemental flavourings in food products to make them more natural.

**Table 7.3** Summarizing list of the confirmed asparagus sensory-relevant compounds which are known from the literature and the compounds that are proposed as new (off-)flavours based on this study. For the unidentified non-volatiles pos/neg refers to the mode of detection used.

<table>
<thead>
<tr>
<th>Volatiles</th>
<th>Non-volatiles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Known flavour compounds (confirmed)</strong></td>
<td></td>
</tr>
<tr>
<td>Dimethyl sulphide</td>
<td>Asparagusic acid</td>
</tr>
<tr>
<td>2-Methoxy-3-isopropyl pyrazine</td>
<td>Protodioscin</td>
</tr>
<tr>
<td><strong>New flavour compounds (suggested)</strong></td>
<td></td>
</tr>
<tr>
<td>3-Methyl-1-butanol</td>
<td></td>
</tr>
<tr>
<td>1-Hexanol</td>
<td></td>
</tr>
<tr>
<td>Octanal</td>
<td></td>
</tr>
<tr>
<td>gcms_112 (alkane)</td>
<td></td>
</tr>
<tr>
<td>gcms_115 (ether)</td>
<td></td>
</tr>
<tr>
<td>gcms_43 (N-containing compound)</td>
<td></td>
</tr>
<tr>
<td>gcms_76 (S-containing compound)</td>
<td></td>
</tr>
<tr>
<td><strong>Causal off-flavours (suggested)</strong></td>
<td></td>
</tr>
<tr>
<td>Pentanal</td>
<td>neg_204</td>
</tr>
<tr>
<td>(E)-2-Heptenal</td>
<td>neg_411</td>
</tr>
<tr>
<td>1-Octen-3-ol</td>
<td>neg_97</td>
</tr>
<tr>
<td>n-Caproic acid vinyl ester</td>
<td>pos_315</td>
</tr>
<tr>
<td></td>
<td>pos_479</td>
</tr>
<tr>
<td></td>
<td>pos_490</td>
</tr>
</tbody>
</table>
### Chapter 7

#### 7.6. Supplementary Material

**Table S7.1** Microbiological analysis of hygiene indicators after different processing steps of the asparagus juice (J) and asparagus fibre (F). ND: not determined.

<table>
<thead>
<tr>
<th>Label</th>
<th>Processing step</th>
<th>Total viable count (kve/g)</th>
<th><em>Enterobacteriacea</em> (kve/g)</th>
<th><em>Lactobacilli</em> (kve/g)</th>
<th>Yeast (kve/g)</th>
<th>Moulds (kve/g)</th>
<th><em>B. cereus</em> (kve/g)</th>
<th><em>S. aureus</em> (kve/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J-01</td>
<td>Asparagus juice (after pressing)</td>
<td>$&gt;3.0 \cdot 10^5$</td>
<td>$&gt;1.5 \cdot 10^5$</td>
<td>$&gt;3.0 \cdot 10^5$</td>
<td>$1.0 \cdot 10^4$</td>
<td>$3 \cdot 10^4$</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>J-02</td>
<td>Microbial decontaminated juice</td>
<td>20</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>J-03</td>
<td>Concentrated juice</td>
<td>150</td>
<td>10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>J-04</td>
<td>Spray drying feed</td>
<td>230</td>
<td>10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>J-05</td>
<td>Spray-dried powder</td>
<td>$3.6 \cdot 10^3$</td>
<td>60</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>F-01</td>
<td>Asparagus fibre (after pressing)</td>
<td>$6.4 \cdot 10^4$</td>
<td>95</td>
<td>$4.9 \cdot 10^4$</td>
<td>575</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>F-02</td>
<td>Thawed and washed asparagus fibre</td>
<td>1100</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>F-03</td>
<td>Blanched asparagus fibre</td>
<td>100</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>F-04</td>
<td>Dried asparagus fibre</td>
<td>1300</td>
<td>450</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>ND</td>
</tr>
<tr>
<td>F-05</td>
<td>Milled asparagus fibre</td>
<td>$1.4 \cdot 10^4$</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>
Table S7.2 List of the 24 sensory attributes concerning the odour, taste, mouthfeel and after-feel sensation which were scored by the professionally trained panellists during the descriptive analysis of the six soup prototypes. A description is provided per attribute. The \( p \)-values are adjusted for false discovery rate for multiple comparisons after two-way ANOVA comparing the six soup prototypes (significant \( p \)-value < 0.05). The Quality Index (QI) is a measurement of the agreement between panellists concerning the differentiation between soups based on each attribute (QI>0.65 reliable data).

<table>
<thead>
<tr>
<th>Label, Aspect, Attribute</th>
<th>Attribute description</th>
<th>( p )-Value</th>
<th>QI</th>
</tr>
</thead>
<tbody>
<tr>
<td>O_Intensity Odour Intensity</td>
<td>Total odour intensity</td>
<td>&lt;0.01</td>
<td>0.88</td>
</tr>
<tr>
<td>O_Asparagus Odour Asparagus</td>
<td>Total asparagus odour</td>
<td>&lt;0.01</td>
<td>0.93</td>
</tr>
<tr>
<td>O_Potato Odour Potato</td>
<td>Total intensity of potato (all kinds of potato)</td>
<td>0.61</td>
<td>0.02</td>
</tr>
<tr>
<td>O_Maize Odour Maize</td>
<td>Total maize odour</td>
<td>&lt;0.01</td>
<td>0.84</td>
</tr>
<tr>
<td>O_Green Odour Green</td>
<td>Total green odour (e.g. green beans)</td>
<td>0.09</td>
<td>0.48</td>
</tr>
<tr>
<td>T_Intensity Taste Intensity</td>
<td>Total taste intensity</td>
<td>&lt;0.01</td>
<td>0.69</td>
</tr>
<tr>
<td>T_Asparagus Taste Asparagus</td>
<td>Total asparagus taste</td>
<td>&lt;0.01</td>
<td>0.87</td>
</tr>
<tr>
<td>T_Artificial taste</td>
<td>Total artificial taste</td>
<td>&lt;0.01</td>
<td>0.76</td>
</tr>
<tr>
<td>T_Potato Taste Potato</td>
<td>Total potato taste (all kinds of potato)</td>
<td>0.06</td>
<td>0.60</td>
</tr>
<tr>
<td>T_Sweet Taste Sweet</td>
<td>Total sweet taste</td>
<td>0.04</td>
<td>0.65</td>
</tr>
<tr>
<td>T_Sour Taste Sour</td>
<td>Total sour taste</td>
<td>0.60</td>
<td>0.00</td>
</tr>
<tr>
<td>T_Salt Taste Salt</td>
<td>Total salty taste</td>
<td>0.05</td>
<td>0.56</td>
</tr>
<tr>
<td>T_Green Taste Green</td>
<td>Total green taste (e.g. green beans)</td>
<td>0.11</td>
<td>0.49</td>
</tr>
<tr>
<td>T_Grain Taste Wheat/grain</td>
<td>Total taste of Brinta, wheat, grain, bread</td>
<td>0.06</td>
<td>0.62</td>
</tr>
<tr>
<td>T_Maize Taste Maize</td>
<td>Total maize taste</td>
<td>0.21</td>
<td>0.43</td>
</tr>
<tr>
<td>T_Cardboard Taste Cardboard</td>
<td>Total taste of cardboard</td>
<td>&lt;0.01</td>
<td>0.85</td>
</tr>
<tr>
<td>T_Chemical Taste Chemical</td>
<td>Total chemical taste (e.g. glue, chlorine)</td>
<td>0.02</td>
<td>0.69</td>
</tr>
<tr>
<td>T_Off-taste Taste Offtaste</td>
<td>Total off taste (e.g. liquorice, woody/earthy, old garlic)</td>
<td>0.12</td>
<td>0.55</td>
</tr>
<tr>
<td>M_Thickness Mouthfeel Viscosity</td>
<td>The degree to which the product feels viscous</td>
<td>&lt;0.01</td>
<td>0.85</td>
</tr>
<tr>
<td>M_Slimy Mouthfeel Slimy</td>
<td>The degree to which the product feels (s)limy</td>
<td>&lt;0.01</td>
<td>0.63</td>
</tr>
<tr>
<td>M_Powdery Mouthfeel Powdery</td>
<td>The degree to which the product feels powdery</td>
<td>&lt;0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>AF_Powdery After-feel Powdery</td>
<td>The degree to which the product gives a powdery aftertaste</td>
<td>&lt;0.01</td>
<td>0.88</td>
</tr>
<tr>
<td>AF_Dry After-feel Dry</td>
<td>The degree to which the product gives a dry aftertaste</td>
<td>&lt;0.01</td>
<td>0.82</td>
</tr>
<tr>
<td>AF_Tingling After-feel Tingling/stinging sensation</td>
<td>The degree to which the product gives a tingling/stinging sensation</td>
<td>0.04</td>
<td>0.64</td>
</tr>
</tbody>
</table>
Figure S7.1 Analysis of the physical properties of the asparagus ingredients used in the six soup prototypes. (A) Particle size distribution of the dried asparagus ingredients measured in the dry powder disperser Aero S (Mastersizer 3000, Malvern Inc, Malvern, UK). (B) Particle size distribution of the six soup prototypes formulated with different asparagus ingredients and only starch dissolved in hot water. The soups were analysed in the Hydro MV module (Mastersizer 3000, Malvern Inc, Malvern, UK) using water as the continuous phase.
Figure S7. 2 Microscopy images of the soup prototypes prepared with spray-dried powder (A) and spray-dried powder with asparagus fibre added (B). The red rectangle highlights an asparagus fibre particle.

Supplementary Tables S3 and S4 are available online at https://doi.org/10.1016/j.foodchem.2022.134986.
Chapter 8

General Discussion
In this thesis, I have explored the metabolome of asparagus (*A. officinalis*), in both raw and processed forms, focusing on flavour composition by performing both chemo-analytical and sensory studies. Asparagus was chosen as the research subject as this crop is important for the Dutch economy and currently generates a large volume of unutilised waste. A significant part of this waste is actually generated during harvest which implies that its biochemical composition is highly similar, if not identical, to the spears that do finally reach the market and the consumer. This makes asparagus a great case for reassessment of its waste potential within the context of current crop circularity goals to generate valuable (by-)products for use in the food industry. Promising processing strategies to exploit this ‘waste’ are under development aiming to create products with high authentic flavour properties (J. W. Siccama, 2022). A novel approach that has been investigated in this thesis is called split-stream processing. Here, waste material of the crop is first separated into fibre and juice after which the latter is non-thermally concentrated followed by spray drying to produce asparagus powder which was also evaluated with sensory analysis in e.g. soup formulations. However, to be able to assess effectively the impact of split-stream processing and drying of asparagus concentrate on its biochemical composition, we were restricted by the current limited knowledge of the asparagus crop metabolome and how this is potentially impacted by genotype, environment and treatment. It was therefore initially necessary to define better the metabolome of the envisaged source material and determine its natural variability and potential compositional shifts that may occur relating to seasonal changes and genetic background effects. It was then also necessary to investigate the metabolite composition of the processed asparagus materials and compositional shifts that may occur upon heat treatment relating to the desired nature and utility of the waste-derived powders that could become available for food application.

### 8.1. The Metabolome of Asparagus

The plant metabolome is the profile of all metabolites produced, stored and excreted by the different tissues both originating from primary and secondary metabolism. Changes in the metabolome generally occur as a plant develops and grows but also due to external stimuli such as abiotic and biotic stresses. In a food crop, these compositional changes may also impact organoleptic properties which then directly impact its flavour attributes. Figure 8.1 summarizes the knowledge and insights obtained here, with respect to the asparagus
metabolome focusing on secondary metabolites as analysed by SPME GC-MS and LC-MS and on potentially flavour-relevant compounds (indicated in bold). It presents a global overview in a simplified scheme of biochemical pathways which have been found to be involved in the accumulation of asparagus metabolites as highlighted in this thesis (Chapters 3-7). It also includes simplified visualisations of the main findings with respect to spear type-specific compounds (green versus white: green and yellow bars based on Chapter 3), prominent changes during harvest season (pink lines based on Chapter 4) and impact of cooking (blue arrows based on Chapter 5: up when more abundant in cooked and down when more abundant in raw spears) (Figure 8.1).

8.1.1 Flavonoids and Benzenoids

A varying metabolome was anticipated when comparing white and green spears although these can actually be obtained from the same plant. Mode of cultivation and exposure to sunlight, which occurs in the case of green spears, can lead to the activation of certain metabolic pathways relating to e.g. photosynthesis, carotenoids and flavonoid biosynthesis which are (more) active in green asparagus spears during development (Fanasca et al., 2009; Tenorio et al., 2004). Flavonoids were found to be more abundant in the green spears (Chapter 3) but they were also detectable in white spears (Chapters 3-5). This is in agreement with earlier studies suggesting that flavonoids act as protectants against damage caused by sunlight and UV radiation (Petrussa et al., 2013) and that their accumulation is repressed, but importantly not fully inhibited, in darkness (Buer & Muday, 2004). These studies have confirmed the light-dependence of flavonoid biosynthesis and suggested a role in auxin-transport which is highly involved in the production of new cells and thus also in spear elongation (Kojima & Sakurai, 1994; Lill et al., 1990; Matsubayashi et al., 1999). It cannot be excluded that flavonoids might be produced post-harvest in the case of white spears once they are exposed to light but this is unlikely to have happened as light exposure was very limited. With respect to changes in the metabolome of white spears during the harvest period, flavonoids were not found to vary significantly (Chapter 4), perhaps indicating a basic role of a background level of these metabolites in spear development.
Figure 8.1 Simplified scheme of metabolic pathways involved in the accumulation of flavour-relevant asparagus secondary metabolites, indicated in bold, based on the findings presented in this thesis. Simplified visualisations of the main findings with respect to spear type specific (green versus white: green and yellow bars based on Chapter 3), significant prominent changes during harvest season (pink lines based on Chapter 4) and impact of cooking (blue arrows based on Chapter 5: up when more abundant in cooked and down when more abundant in raw spears) are provided.
Volatile benzenoids were found to change significantly during the harvest period and their temporal patterns interestingly were opposite to those observed in the case of non-volatile metabolites from the phenylpropanoid pathway (Figure 8.1). This suggests a differential regulation of the specific metabolic routes involved which might be influenced by the environment, as was previously shown for Petunia (Cheng et al., 2016).

8.1.2 Saponins and Monoterpenes
Steroidal saponins and monoterpenes which are terpenoid products of the mevalonate (MVA) and methyl-erythritol-4-phosphate (MEP) pathways (Figure 8.1), were seen to be more abundant in the white spears (Chapter 3), suggesting that the regulation of these metabolic routes might be light-dependent or specific to the contrasting mode of development of white asparagus. Varying regimes of light and temperature have proven to have certain tissue-specific impacts on the regulation of MVA and MEP pathways in plants, e.g. oak (Quercus ilex L.) (Staudt & Bertin, 1998) and tea leaves (Camellia sinensis) (Xu et al., 2018). These studies have shown variable responses of different monoterpenes to light and temperature (Xu et al., 2018) and that even the period of light / heat exposure plays a role in the content of these metabolites (Staudt & Bertin, 1998). For the monoterpenes in plants, many studies have investigated the emission of these volatile compounds as a response to environmental stimuli and therefore linked the patterns of monoterpene content to mechanisms of plants responding to biotic or abiotic stresses (Isah, 2019). However, what is reported in this thesis relates primarily to the monoterpene content naturally present in the spears and thus not induced by any biotic stress.

There are limited studies investigating the specific role of saponins in plants but a few indicate a potential relationship between the composition of saponins and the developmental stage of tissues, and the role of these compounds again as protectants to pathogens or abiotic stimuli (Faizal & Geelen, 2013; Upadhyay et al., 2018). Saponins have been shown to be more abundant in the basal parts of white spears (Dawid & Hofmann, 2014; Lee et al., 2010), perhaps as part of a mechanism from the plant to protect itself from the soil predators. Lee et al. (2010) also analysed samples from the crown, the roots, and the foliage of asparagus plants and showed that the abundance of protodioscin, the main asparagus saponin (Shao et al., 1999), was increasing from the tip to basal part of spears but it was also
highly produced and accumulated in the crown of the plant. This agrees with observations here that relative abundance of saponins detected in the upper 10 cm of white spears (Chapter 5) was lower compared to samples of the full spears (Chapters 3 and 4). This suggests a potential relationship between saponin accumulation and developmental age of the plant tissue, as asparagus spears grow from the tip (Ku et al., 2007; Lill et al., 1990). Both saponins and monoterpenes were found here to change throughout the season (Figure 8.1) while also temperature and daylight vary throughout the season, suggesting a crosstalk between light and/or temperature and the accumulation of terpenoids (monoterpenes and saponins).

8.1.3 Sulphur-containing metabolites and pyrazines
Asparagus is a rich source of secondary sulphur- and nitrogen-containing compounds (Chapter 2). The aroma properties of these compounds are undoubtedly of high relevance for asparagus spears regarding the organoleptic properties of the vegetable, as has also been shown here (Chapters 5 and 7). No abundance differences between varieties or spear types were found regarding these metabolites (Figure 8.1) and this suggests them as being ‘typical’ asparagus metabolites.

8.2. The genetic background and seasonal dynamics of the asparagus metabolome
The genetic background of asparagus appeared to influence the metabolite composition, but these differences were relatively small and less significant compared to the influence of the harvest moment during the season (Chapters 3 and 4). The main difference between asparagus varieties is the shoot initiation which occurs at relatively cold temperatures for early varieties and at relatively warmer temperatures for late varieties (Limgroup, 2022). Asparagus cultivars have been particularly bred to optimise agronomic traits including morphology and yield (Lill et al., 1994) and to generate a uniform product and thus likely also for resilience to environmental conditions depending on the period within the season when they are meant to be harvested. This suggests that asparagus plants grown in one specific country or region have similar mechanisms to adapt to their environment regardless genotype. Moreover, it is considered that the genetic diversity in the asparagus crop in Europe is relatively low as many varieties have common lineage (Jacobi et al., 2023; Limgroup, 2022). Taken together, these may explain the minor varietal differences that were
observed here with respect to the profile of secondary metabolites. Nevertheless, subtle varietal differences were observed when tasting raw and cooked spears (Chapter 5) although it is not common that consumers eat spears of individual varieties, as asparagus in the market is not categorised based on genetic background.

Given the seasonal changes and minor varietal differences observed (Chapters 3-5), and from the point of view of a consumer or producer, it is relevant to consider that differences regarding the metabolome and thus potentially also flavour, might occur throughout the season. Being at the very beginning or end of the harvest period, the plant is likely to be functioning differently. In the first phase, the asparagus plant needs to switch from a form of dormancy to spear production (Ku et al., 2007). Later in the season, the plant has already used much of its nutrient reserves from its root system and needs to produce foliage and restart photosynthesising to again build up reserves for growth the following spring (Chapter 2). In the early months (already in January/February), asparagus spears can nevertheless still be produced using artificially-controlled heat-enhanced conditions (greenhouse, heated fields). Spears grown in this way and harvested ‘pre-season’ were found to have a similar metabolome to spears harvested in the middle of the asparagus season from plants grown in normal fields (either mini-tunnel or open fields) (Chapter 4). Thanks to the controlled and stable conditions inside a greenhouse or in a heated field, spears grown there are expected to be of high, if not best quality, with respect to morphology (Gąsecka et al., 2009; Heißner et al., 2006) but also with respect to flavour. It was not possible to deliberately test the influence of environmental changes on the metabolome of white asparagus, but changes in the metabolite profiles were found to be less prominent in the middle of the season when the weather, air and soil temperatures were less variable (Chapter 4). These findings strongly suggest that the shoot initiation in asparagus is temperature-dependent rather than to the daylight (photoperiod). This is also in agreement with previous reports (Ku et al., 2007).

The seasonal dynamics as discussed above with respect to the asparagus metabolome of course impacts the generated waste-streams as well. A significant part of asparagus waste consists of the basal parts which are cut off to produce spears of exactly the same length for the market. This waste product was also the starting material for the split-stream processing
and spray drying applied and investigated in Chapter 6. It could be argued in a commercial context that using waste generated at the beginning or end of the season may yield powders of diverging flavour profiles. However, while I did not specifically investigate this by comparing powders produced from such waste-streams, this would be a valuable follow-up study to perform in a commercial / quality control context.

8.3. The Flavour of Asparagus

Focusing now on the flavour of asparagus, the studies reported in this thesis have strongly contributed to the re-definition of a list of the most sensory-relevant metabolites. These are highlighted in Figure 8.1 but are now also included in the sensory wheel in Figure 8.2. Figure 8.2 is the revisited version of the sensory wheel from our published review from 2020 (Pegiou et al., 2020) presented in Chapter 2. The previous version was constructed solely based on literature (Chapter 2). This revised Asparagus Sensory Wheel has now been amended with the findings from the combined metabolomics, taste panel and GC-O-MS approaches reported here (Chapters 5-7). This sensory wheel comprises attributes regarding aroma, taste and texture (mouthfeel) of cooked white spears. In the outer space of the sensory wheel, metabolites have been included which were found to be statistically correlated or linked based on aroma experience, with specific attributes (Figure 8.2).

Volatile sulphur-containing metabolites, two methoxypyrazines, 2,3-butanedione and 2,3-pentanedione were interestingly not significantly changing during the season (Chapter 4) and also did not significantly vary between genotypes or spear types (Chapters 3-5). However, they were seen to change upon cooking (Chapter 5) and also to be odour-active and thus likely sensory-relevant (Chapters 5 and 7). These suggest their relevance for the typical flavour notes of cooked asparagus which is proposed to be confirmed by performing for example spiking experiments. Importantly, these metabolites were present also in the instant soups (Chapter 7), confirming that these compounds or their precursors are successfully concentrated and retained during the split-stream processing and spray drying (Chapter 6 and J. W. Siccama, Pegiou, Eijkelboom, et al., 2021). It is worthy to mention and to realise that to experience a certain flavour, it is not necessary that all ‘key flavour compounds’ are present in high abundance. It is rather the correct balance / ratio between compounds and their sensory thresholds that are important.
Regarding monoterpenoids, benzenoids, flavonoids and saponins which were discriminatory for one asparagus type (Chapter 3) or for a specific period in the harvest season (Chapter 4), they are likely to be the metabolites responsible for the different flavour nuances when comparing green and white spears or spears from the beginning or end of the season (Cuppert et al., 1997; Hoberg et al., 2003). To confirm this, follow-up experiments should be performed. Monoterpenes and benzenoids are often linked with fruity, sweet and nutty aromas (source: www.foodb.ca) and in the studies presented in this thesis they were also linked to similar aroma attributes (Figure 8.2). Moreover, they were detectable in the spray-dried powder materials (Chapter 6) and the instant soup formulations (Chapter 7), which indicates that they are well-retained during split-stream processing and spray drying of the...
waste-streams. Both flavonoids and saponins, when flavour-active, contribute to a bitter perception (Drewnowski & Gomez-Carneros, 2000). The varying levels of these metabolites in asparagus combined with the varying levels of C5 alcohols which were more abundant in the green spears, and levels of medium chain aldehydes which were more abundant in the white ones (Chapter 3), are proposed to contribute to the divergent bitterness of green and white spears (Cuppett et al., 1997). Volatile carbonyls with a 5 carbon backbone are described to have bitter, tropical, and pungent aromas while medium-chain unsaturated aldehydes are linked to fatty, sweet and creamy flavours (source: www.foodb.ca). These potentially flavour-relevant metabolites were again detected in the processed asparagus ingredients of the soup formulations (Chapter 7) which confirms their retention during split-stream processing and spray drying as suggested in Chapter 6. The updated Asparagus Sensory Wheel may therefore function as a starting point for follow-up targeted investigations to assess the precise nuances in the flavour of white asparagus and its related (processed) products.

8.4. Split-stream processing of asparagus waste-streams

When aiming to upscale asparagus (waste) material, it is of value and importance to maintain the starting and intermediate materials (spears, juice, concentrate) below 4 °C and in dark place so that enzymatic activities are minimised and chemical shifts induced by light or heat are hindered. During spray drying, a skin is formed around droplets in seconds helping to ensure encapsulation of metabolites which might then protect them from the hot temperatures applied in the drying chamber. Indeed, no severe heat-induced compositional shifts occurred and the ‘raw asparagus’ profile was essentially maintained in the spray-dried powder. Variation in the metabolite profile was observed between the juice and the spray-dried samples but this can be explained by the addition of maltodextrin and also the different matrix of the samples analysed by SPME GC-MS (Chapter 6). Importantly, volatile alcohols and aldehydes that were found to be more abundant in the raw asparagus compared to cooked (Chapter 5) and they are also proposed to contribute to the formation of the typical asparagus flavour (Chapters 5 and 7) were well retained in the spray-dried powder (Chapter 6). Maintaining the metabolite profile resembling the raw fresh spear enables and potentially ensures that the retained flavour compounds or their precursors can be released/generated as normal when dissolving the powder in hot water. This can then result
in the formation of the cooked asparagus flavour as it was also determined by a professional
taste panel who scored the soup formulations on flavour attributes including ‘asparagus’
flavour (Chapter 7). It is worthy to mention here that samples from intermediate steps in
the split-stream processing have also been analysed with SPME GC-MS. No losses of volatile
compounds were found and the concentrations of the compounds were confirmed (data not
presented). These findings strongly indicate the potential of split-stream processing and
spray drying of vegetable materials, or even better, vegetable waste materials, for
valorisation of agricultural waste-streams.

Compositional heterogeneity was clearly found with respect to secondary metabolites along
the spear (Chapter 3). This did not actually bode well for the ultimate objective of this
project. However, additional to the cut-offs, full or broken spears may also be discarded as
waste and these are called ‘rejects’ (Chapter 1). This suggests that the composition of the
waste-stream regarding the proportion of cut-offs to the broken spears might impact the
metabolite profile and thus potentially the flavour experience of the concentrate and its
spray-dried powder. Nevertheless, the instant soups containing spray-dried powder made
from cut-offs were perceived as ‘asparagus’ by the expert panel, although they did not score
as high for asparagus flavour as the flavour-enhanced soup formulation (Chapter 7). It might
be of relevance to consider adding some ‘rejects’ or wasted upper spear parts in the starting
waste-stream used for the split-stream processing. This may result in spray-dried powder
with even better sensory quality.

In a side-line vegetable split-stream processing validation approach, the same split-stream
processing and spray drying strategy was applied to process waste of red bell peppers (J. W.
Siccama, 2022). The spray dried powder was compared to a commercially available one,
regarding its physical properties and the composition of volatile metabolites with a special
focus on proposed key aroma compounds. It was shown to be feasible to translate the
proposed pipeline for the processing of other vegetable materials. However, adjustments
might be necessary depending on the application of interest, as for example in the case of red
bell pepper, the red colour was lost in the intermediate steps of split-stream processing
although importantly, without affecting the profile of volatile compounds (J. W. Siccama,
2022). Evaluating the flavour profile by sensory panel would have assisted in better
assessing any potential causal effects resulting from colour loss on the sensory characteristics of the powder.

8.5. **Unravelling the flavour of new processed ingredients**

Flavour is a key aspect in the development of food products like instant vegetable soups. The methods applied to process the ingredients can influence mouthfeel and the presence of specific flavours. Flavour is the outcome of chemical interactions and sensory perception and within the hundreds of metabolites characterising a food product, only a fraction of these does eventually likely frame its flavour. It is therefore, valuable to integrate data acquired from different platforms (e.g. instrumental and human senses) to help create the most complete picture. In the case of asparagus materials, important key odorants for cooked asparagus were highlighted by performing GC-O-MS (Chapters 5 and 7). New compounds were thus also positively correlated with key asparagus flavour notes (1-hexanol, 3-methyl-1-butanol, octanal) or with the experience of off-flavours (pentanal, (E)-2-heptenal, 1-octen-3-ol) (Chapter 7). It must be noted that the ratio of levels of certain metabolites in the context of the food matrix are of relevance here. It may be the case that the suggested ‘off-flavours’ in Chapter 7 were correlated with certain attributes (i.e. cardboard taste) because of their abundance. It is possible that if they are present in lower abundance the experience of ‘cardboard’ taste is not enhanced or is masked (Burseg & De Jong, 2009). Such findings should already occur and be validated at an early stage in the development of new processed food ingredients, as this can assist in faster decision-making for the optimization and final design of the product.

Based on the studies described in Chapters 5 and 7, I present in Figure 8.3 a schematic overview of the suggested steps to be planned and performed when developing a food product. Most of the steps are undoubtedly known and already integrated in such projects but what is worthy of highlighting here is the additional use of metabolomics in combination with the sensory studies. Planning and performing both metabolomics and sensory studies for an individual product may require much labour input and different expertise domains. Nevertheless, it can contribute to yielding results of high relevance and it can strongly assist in the decision making and design of the subsequent steps in the processing strategy, as was also done here. Moreover, Figure 8.3 suggests that this process should occur in a circular way
which means that each circle may also lead to additional improvements to a food product which can subsequently be optimised and developed further following the same steps. It is of value to perform trials and pilots to avoid costly mistakes later in the suggested pipeline (Figure 8.3). For instance, when preparing the ingredients for the instant soup formulations (Chapter 7), two team tastings were performed first to verify that the ingredients produced with split-stream and spray drying of asparagus waste would be acceptable to consume, but also to confirm that the recipes were appropriate. Having performed these, we were able to conclude also that the asparagus fibre initially used needed to be milled to a finer powder to avoid an intense powdery mouthfeel. This might have helped to improve the mouthfeel of the soups, but it did not completely solve the issue as the addition of the asparagus fibre negatively impacted the sensory descriptive analysis. The fibre-containing soup formulations scored higher on off-flavours compared to the other soups (Chapter 7). Asparagus fibre, as separated from the waste-stream during split-stream processing may therefore better be further processed and used for alternative applications (J. W. Siccama, 2022; J. W. Siccama et al., 2022). Nevertheless, a data-driven strategy as such (Figure 8.3) has led to a better understanding of the asparagus flavour profile and the definition of potential candidates as being (new) key sensory-relevant compounds or off-flavours.

To confirm the odour-activity and the potential contribution to flavour perception of certain aroma compounds, GC-O-MS is one approach which was also applied in Chapters 5 and 7. Alternatives or potential follow-ups can be to make aroma dilution series using analytical standards, or studies evaluating samples spiked with the specified flavour compounds to confirm or deny the exact relevance of specific compounds on sensory impact. One can argue here that in these cases the contribution/influence of the actual food matrix is disturbed. However, there are methods that can monitor and thus assess flavour of a food matrix in vivo and combined with addition/omission studies (Burseg & De Jong, 2009; de Jong et al., 2019; Hodgson et al., 2003). Individual compounds can be excluded from the actual matrix and recollected for the purpose of directly seeing the impact of their exclusion as well as their combination regarding sensory perception (de Jong et al., 2019). Such approaches are used to study the complexity of flavour and the influence of compositional shifts in the matrix of interest which is of high relevance. These kinds of methods can be employed as a follow-up
to confirm findings and conclusions made in studies combining untargeted metabolomics and sensory panels.

**Figure 8.3** Schematic presentation of proposed pipeline for data-driven investigation of flavour of (new) food products / ingredients under development involving metabolomics approaches.

### 8.6. Final remarks and General Conclusions

Circularity is a term that increasingly enters today's society. It is defined as the economic system that aims for zero (or less) waste and pollution during production, from the starting material to the final consumer (Nobre & Tavares, 2021). To reach such a goal, one first thing to consider is the definition of the word ‘waste’. What is waste? And what are the
prerequisites to call something waste? Focusing on agriculture and food production, the quality profile of raw materials, e.g. vegetables and fruits, is mainly defined based on their appearance. However, one may question whether appearance alone should really comprise an issue, as the consumer will usually (un)consciously ‘grade’ other attributes, such as flavour, nutrients and calories. Appearance attributes such as colour, can of course function as indicator of deterioration, but shape indicators such as length and diameter, which are key quality indicators for asparagus spears (Chapter 1) should be re-assessed as being quality indices with regards to crop products.

Flavour is unambiguously a quality indicator of food products which attracts increasing attention in the food industry. Additionally, consumers are tending to prefer increasingly more natural products and thus, producers and retailers ought to comply with such changes. Therefore, naturalness and natural food ingredients gain attention, interest and are replacing older products containing flavour additives, preservatives and enhancers. To achieve this, the flavour profile of the starting material must be maintained as much as possible during all processing steps, thus resembling the natural flavour and retaining that experience to the user once consumed.

Studying the chemical composition of food materials, either as raw or in any processed form is of great value to assess and evaluate the environmental influence, post-harvest handling, the impact of processing techniques as well as storage conditions on the variability of the starting materials. Metabolomics has numerous showcases and examples of both untargeted and targeted studies, investigating differences between storage conditions, genotypes, growing conditions, and the influence of processing techniques (e.g. Buvé et al., 2018; Creydt et al., 2018; Creydt & Fischer, 2020; Lopez-Sanchez et al., 2015; Romero et al., 2021; van Treuren et al., 2018). In this thesis, metabolomics often combined with sensory analysis have been used to evaluate raw and processed asparagus (waste) materials.
As a result of fruitful collaborations across several disciplines and expertise domains, necessary for a detailed elucidation of the metabolome and flavour composition, the general conclusions of this research are:

1) The metabolomes of conventionally-grown asparagus spears harvested in the beginning and end of season are different compared to those harvested in mid-season.

2) Asparagus tips have a different metabolome compared to the basal parts.

3) Asparagus aroma was seen to be mainly impacted by the presence of dimethyl sulphide, methional, two methoxypyrazines, 2,3-butanedione and C$_6$ carbonyls.

4) Spray drying of concentrated asparagus juice yielded natural asparagus powder which can be used as ingredient for instant soups by having a similar metabolome and flavour profile as fresh cooked asparagus.

5) Metabolomics combined with sensory studies can strongly assist in better designing and decision making during the development of (new) food ingredients.
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**Summary**

Asparagus (*A. officinalis*) is widely known and appreciated for its high nutritional value and the distinctive flavour of its young shoots. These shoots, or spears of asparagus are consumed in either green or white form. In the Netherlands, white spears are preferred and are mainly cultivated in the south of the country, and people refer to these as ‘White Gold’. This can be explained by the laborious work required for cultivating, harvesting and post-harvest processing of the asparagus spears. To deliver a superior product, substantial volumes of waste are however, generated during a harvest season. Hence, there may be potential to exploit these waste-streams to generate a (by-)product of significant value.

A common way to exploit vegetable waste streams is to oven-dry them and generate powders for use as food ingredients. Oven-dried powders are often characterized by having little or no flavour and this is due to the loss of important aroma compounds. Spray drying is a desirable alternative to oven drying, by allowing the encapsulation and thus retention, of flavour compounds. When vegetables are cooked or processed in any way, different compounds may be formed or lost. These compositional shifts may influence the flavour of the final product. To study the flavour, both sensory and instrumental approaches are suggested to be required. Linking outcomes from both may assist in better elucidation and understanding of the composition of flavour and the combination of compounds needed for specific sensory perception. However, before this, it is important to improve our understanding of the composition of the starting materials and how this may vary due to developmental, seasonal and genetic differences.

In this PhD thesis, mass spectrometry (MS) – based techniques hyphenated with advanced liquid chromatography (LC) and gas chromatography (GC) systems were used to investigate the metabolome of raw, cooked and processed asparagus materials.

First, in Chapter 1, I introduce the topic and the aim of this thesis and I present an overview of the following chapters. In Chapter 2, a comprehensive review is presented after we performed a literature study to collect all available information with respect to the metabolite composition of asparagus with a particular focus on flavour. For this review, studies on both green and white asparagus were taken into consideration although distinction between the two was not always made clear in the publications. It was concluded that untargeted metabolomics studies would offer new knowledge on the asparagus metabolome. Chapter 3 describes one of the first untargeted metabolomics studies to compare directly the metabolome of green and white asparagus and the first one to explore the composition of volatile organic compounds in both forms as well as different varieties – also in relation to flavour. Volatile metabolites were profiled using GC-MS and non-volatile metabolites were analysed using ultra-performance LC-MS. Multivariate and univariate statistics were performed and showed the discriminatory compounds between the two forms and revealed minor differences between varieties. Moreover, sections along the spears were profiled individually with respect to the volatile composition and this indicated that the top sections of the spears have different metabolite composition to the basal parts.
Appendix

In Chapter 4, we focus on white asparagus, as this is mainly cultivated in the Netherlands and was envisaged to be the starting material for our waste exploitation concept. Eight varieties were sampled throughout the harvest season and analysed using untargeted metabolomics. Advanced statistical tools were applied and revealed the dynamics of the white asparagus metabolome throughout the season. The harvest moment was found to have a higher impact on the metabolome compared to the genetic background indicating a greater developmental and/or environmental influence on secondary metabolites. Flavour-relevant asparagus metabolites were shown to not significantly change over time. In Chapter 5, GC-MS and LC-MS methods were applied to unravel the changes in the asparagus metabolite composition which occur upon cooking. The results were combined with the outcome from team-tasting analyses and supervised multivariate statistics were used to indicate potential flavour-relevant compounds. The aroma-relevant metabolites in both raw and cooked materials were then identified by GC-Olfactometry-MS (GC-O-MS), partly confirming the so-called 'key odorants' that have been proposed in previous studies. The obtained knowledge regarding the asparagus metabolome and flavour was of value to assess the impact of the process of spray drying asparagus concentrate on the final flavour of the produced powder. In Chapter 6, we focus on specific volatile compounds to assess the impact of split-stream processing and spray drying using maltodextrin as carrier agent. Compounds that were found to contribute to the typical asparagus flavour were found to be better retained in spray-dried powder produced with increasing concentration of maltodextrin. In Chapter 7, asparagus ingredients produced using the split-stream processing method were used in instant soup formulations and were compared to soups made from commercial oven-dried powder with or without flavour supplements. Both untargeted metabolomics and sensory analysis using an expert trained panel were performed. Multivariate chemometric statistics and machine learning approaches confirmed the higher quality of the spray-dried powder compared to the commercial oven-dried asparagus preparation and highlighted key asparagus sensory-relevant metabolites. Moreover, the retention of key flavour asparagus metabolites or their precursors during split-stream processing and spray drying was confirmed. GC-O-MS of reference standards was also performed to confirm the odour activity of many of the proposed odorants. Finally, in Chapter 8, I discuss all the findings of the thesis, with respect to the general biochemistry and flavour composition of asparagus. I also discuss the potential of metabolomics when combined with sensory analyses to examine (new) food ingredients, and I round off with future perspectives and concluding remarks.

All chapters in this thesis have contributed to gaining a better understanding of the composition of the asparagus metabolome and asparagus flavour, and to validate the potential of spray drying to generate flavoursome asparagus powder from waste materials which has superior quality compared to the commercially available ones. All chapters combined have indicated that combining untargeted metabolomics and sensory studies should be a prerequisite in food research related to flavour and fragrance.
Asperges (*A. officinalis*) zijn wereldwijd bekend om de hoge voedingswaarde en de kenmerkende smaak. Er zijn witte en groene asperges, maar in Nederland gaat de voorkeur meestal uit naar de witte asperges. Deze worden geproduceerd in het zuiden van Nederland. De witte asperges worden door Nederlanders ook wel het ‘witte goud’ genoemd, aangezien het een arbeidsintensief proces is om de asperges te telen, te oogsten en te verwerken. Helaas worden er tijdens het oogstseizoen aanzienlijke hoeveelheden groenafval gegenereerd om het ‘witte goud’ te produceren. Er is potentieel om deze stroom groenafval te verminderen door een (bij-)product van significante waarde te genereren.

Een gebruikelijke manier om de afvalstroom van groenten te verminderen is door ze te drogen in de oven en het overgebleven poeder in ander voedsel te verwerken. Oven-droogde poeders hebben vaak weinig of geen smaak, wat wordt veroorzaakt door het verlies van belangrijke aroma’s. Een alternatief voor oven gedroogde groenten is sproeidrogen. Hierbij worden de smaakstoffen in het poeder ingekapseld en blijft de smaak behouden. Als groenten gekookt of op een andere manier bewerkt worden, zijn er altijd chemische verbindingen die ontstaan of verdwijnen in het proces. Deze veranderingen in samenstelling kunnen de smaak van het eindproduct beïnvloeden. Om smaak te kunnen bestuderen is het nodig om zintuiglijke en instrumentele methodes te gebruiken. Het koppelen van zowel de zintuiglijke als de instrumentele resultaten kan een beter inzicht geven van de samenstelling van de smaak en welke chemische samenstellingen nodig is om een bepaalde smaak beleving te kunnen bereiken. Voordat hiermee wordt begonnen is het belangrijk om inzicht te krijgen in de samenstelling van het startmateriaal en hoe dit kan variëren door ontwikkelings-, seizoens- en genetische invloeden.

In dit proefschrift, massaspectrometrie (MS) gekoppeld met vloeistofchromatografie (LC) en gaschromatografie (GC) methodes zijn ze uitgevoerd om de chemische samenstelling (metaboloom) van rauwe, gekookte en verwerkte aspergematerialen te onderzoeken. Eerst introduceer ik in **Hoofdstuk 1** het onderwerp en het doel van dit proefschrift en geef ik een overzicht van de hoofdstukken. In **Hoofdstuk 2** wordt een uitgebreid overzicht gegeven van een literatuurstudie die we hebben uitgevoerd om alle beschikbare informatie te verzamelen met betrekking tot de chemische samenstelling van asperges met in het bijzonder aandacht voor de smaak. Voor deze review hebben we studies over zowel groene als witte asperges gevonden, hoewel het onderscheid tussen beide niet altijd duidelijk werd gemaakt in de publicaties. Geconcludeerd werd dat niet-gerichte metabolomische onderzoeken nieuwe kennis over het asperges-metaboloom zouden opleveren. Niet-gerichte studies helpen bij het ontdekken van bijna de hele samenstelling dan alleen het bestuderen van enkele metaboliiten.

**Hoofdstuk 3** beschrijft een van de eerste niet-gerichte metabolomische studies, waarin het metaboloom van de groene en witte asperges rechtstreeks worden vergeleken evenals het onderzoek naar de samenstelling van vluchtige, organische stoffen in beide asperge soorten, ook in relatie tot smaak. Vluchtige metaboliiten werden geanalyseerd met behulp van GC-
MS en niet-vluchtige metabolieten werden geanalyseerd met behulp van ultraperformance LC-MS. Zowel meerdimensionale en eendimensionale statistische analyses werden uitgevoerd, die de chemische verbindingen toonden die kenmerkend zijn voor de twee vormen van asperges. Ook lieten de resultaten kleine verschillen tussen de verschillende variëteiten zien. Bovendien werden delen langs de scheuten van het product de samenstelling van de vluchtige stoffen geanalyseerd, Dit liet zien dat aan de bovenste delen van asperges een andere chemische samenstelling hebben dan de onderste delen (richting de wortel).

In hoofdstuk 4 richten we ons op witte asperges, omdat deze vooral in Nederland worden geteeld en bedoeld zijn als uitgangsmateriaal voor ons concept om groenafval te exploiteren. Tijdens het oogstseizoen werden acht variëteiten bemonsterd en geanalyseerd met behulp van niet-doelgerichte metabolomica. Geavanceerde statistische methodes werden toegepast en gaven de dynamiek van het metaboloom van witte asperges gedurende het seizoen weer. Het oogstmoment bleek een groter effect te hebben op het metaboloom dan de genetische achtergrond. Dit wijst op een grotere invloed van de omgeving op secundaire metabolieten. Aspergemetabolieten die van invloed zijn op de smaal bleken in de loop van de tijd niet significant te veranderen.

In hoofdstuk 5 worden GC-MS en LC-MS gebruikt om de veranderingen in de samenstelling die tijdens het koken optreden in de aspergemetaboliet uit te pluizen. De resultaten werden gecombineerd met de resultaten van zintuiglijke smaaktesten en meerdimensionale statistiek werd gevruikt om de potentiele chemische verbindingen die relevant zijn voor de smaak te identificeren. De aroma-relevante metabolieten in zowel rauwe als gekookte materialen werden vervolgens geïdentificeerd door GC-Olfactometrie-MS (GC-O-MS), wat gedeeltelijk de belangrijkste smaakmetabolieten bevestigde die in eerdere studies zijn voorgesteld.

De opgedane kennis over het metabolisme en de smaak van rauwe en gekookte witte asperges was waardevol bij het evalueren van het effect van het sproeidroogproces op de uiteindelijke smaak van het poeder. Het plantaardige afval van witte asperges werd verwerkt met behulp van de split-stream verwerkingsmethode. Het sap werd vervolgens geconcentreerd en vervolgens gedroogd met de methode van sproeien. De smaak van gedroogde monsters door de methode van sproeien en oven gedroogde witte asperges werd onderzocht door zowel zintuiglijke testen als door metabolomische analyse uit te voeren.

In hoofdstuk 6 concentreren we ons op specifieke vluchtige verbindingen om de impact van split-stream verwerking en sproeidroog met maltodextrine als draagstof te beoordelen. Chemische verbindingen die bijdragen aan de kenmerkende aspergesmaak bleken beter behouden te worden in gesproeidroogd poeder wanneer meer maltodextrine wordt toegevoegd.

In hoofdstuk 7, werden asperge-ingrediënten die geproduceerd zijn met behulp van de split-stream verwerkingsmethode gebruikt in instant soepen en werden vergeleken met soepen gemaakt van in de oven gedroogd poeder met of zonder smakadditieven. Er werden zowel ongerichte metabolomica als zintuiglijke analyses uitgevoerd met behulp van een door
experts opgeleid smaak panel. Meerdimensionale chemometrische statistieken en machine learning benaderingen bevestigden de hogere kwaliteit van het sproei gedroogde poeder in vergelijking met de commerciële oven gedroogde aspergeverwerking en lieten de belangrijkste zintuiglijk relevante metabolieten van asperges duidelijk zien. Bovendien werd het behoud van belangrijke smaakstofmetabolieten van asperges of hun kiemen tijdens split-stream verwerking en sproeidrogen bevestigd. GC-O-MS werd ook uitgevoerd om de geur activiteit van veel van de voorgestelde geurstoffen te bevestigen.
Ten slotte, in hoofdstuk 8 combineer ik de resultaten van de verschillende studies die in de vorige hoofdstukken zijn beschreven. Dit proefschrift heeft bijgedragen aan een beter begrip van alle biochemische reacties (metabolomica) van witte asperges en aan het begrip van de samenstelling van de smaak ervan. Ook wordt besproken dat door de sproei droogmethode een smakelijk poeder uit groenafval (dat tijdens de oogst wordt geproduceerd) wordt gecreëerd en dat de kwaliteit hiervan supurieur is aan de commercieel beschikbare poeders. Door middel van dit proefschrift is aangetoond dat de combinatie van niet-gerichte metabolomische en zintuiglijke analyses een voorwaarde zou moeten zijn voor het onderzoek van voedingsmiddelen / producten gerelateerd aan smaak en geur.
Περίληψη

Τα σπαράγγια (Asparagus officinalis) είναι από τα υγιεινά και ευρέως γνωστά λαχανικά. Εκτιμώνται για την υψηλή διατροφική τους αξία καθώς και για τη χαρακτηριστική γεύση τους. Τα σπαράγγια τα συναντούμε σε διάφορους τόνους του πράσινου, αλλά και του λευκού. Στην Ολλανδία, τα λευκά σπαράγγια προτιμώνται και καλλιεργούνται κυρίως στις νότιες περιοχές της χώρας, και οι άνθρωποι αναφέρονται σε αυτά με την ονομασία «Λευκός χρυσός». Αυτό μπορεί να εξηγηθεί από την επίπονη εργασία που χρειάζεται για την καλλέργεια τους, για τη συγκομιδή, καθώς και για την αποθήκευση και μεταποίησή τους. Δυστυχώς κατά τη διάρκεια της περιόδου συγκομιδής και της μεταποίησης αυτών, παράγονται σημαντικοί όγκοι φυτικών αποβλήτων. Ο σκοπός μας, με βάση το μοντέλο της κυκλικής οικονομίας που αποβλέπει σε μία πιο βιώσιμη αλυσίδα παραγωγής τροφίμων, είναι η μείωση αυτών των φυτικών αποβλήτων. Με τη χρησιμοποίηση αυτών των φυτικών αποβλήτων, στοχεύουμε στην παραγωγή υψηλής ποιότητας συστατικών τροφίμων, διατηρώντας μάλιστα την μέγιστη δυνατή ποιότητα γεύσης στα τελικά προϊόντα. Αξίζει να σημειωθεί πως παράλληλα με την χρησιμοποίηση των φυτικών αποβλήτων των λευκών σπαραγγιών, συμβάλλουμε και στην μείωση της ρύπανσης του περιβάλλοντος.

Ένας τρόπος εκμετάλλευσης των φυτικών αποβλήτων που παράγονται κατά την περίοδο της συγκομιδής των οπωρολαχανικών, είναι η ξηρασία αυτών σε φούρνο και η παραγωγή ξηράς σκόνης για να χρησιμοποιηθεί ως συστατικό τροφίμων. Οι ξηρές σκόνες που παράγονται κατά τη διάδοση αυτή, συχνά χαρακτηρίζονται από ήπια ή καθόλου γεύση και αυτό οφείλεται στην απώλεια σημαντικών πτητικών χημικών ενώσεων γεύσης. Η ξηρασία με ψεκασμό (spray drying) είναι μια επιθυμητή εναλλακτική λύση αντί της ξηρασίας σε φούρνο, διότι επιτρέπει την ενθυλάκωση και συνεπώς τη διατήρηση των πτητικών χημικών ενώσεων στο τελικό προϊόν, που ενδεχομένως συμβάλλει στο άρωμα και στη γεύση των συστατικών / τροφίμων. Όταν τα λαχανικά μαγειρεύονται ή υποβάλλονται σε οποιαδήποτε μεταποίηση, ήπιες χημικές ενώσεις, οι ονομαζόμενες τροφίμους (metabolites). Αυτές οι αλλαγές στη χημική σύνθεση (metabolome) μπορεί να επηρεάσει τη γεύση του τελικού προϊόντος.

Στο Κεφάλαιο 1, παρουσιάζω το θέμα και το στόχο της συγκεκριμένης διατριβής καθώς και μια σύνοψη των μελετών που περιγράφονται στα επόμενα κεφάλαια. Το Κεφάλαιο 2
περιλαμβάνει μια βιβλιογραφική μελέτη που πραγματοποιήθηκε για τη συλλογή όλων των
dιαθέσιμων πληροφοριών σχετικά με τη χημική σύνθεση των σπαραγγιών, δίνοντας
έμφαση στη γεύση. Ελήφθησαν υπόψη μελέτες τόσο για τα πράσινα όσο και για τα λευκά
σπαράγγια, καθώς διαπιστώσαμε ότι η διάκριση μεταξύ των δύο ειδών δεν ήταν πάντα
σαφής σε προηγούμενες δημοσιεύσεις μελέτες. Το συμπέρασμα και οι παρατηρήσεις μας
ήταν ότι οι μη στοχευόμενες μεταβολομικές αναλύσεις (metabolomics) θα πρόσφεραν νέα
gνώση σχετικά με τη (βιο-)χημική σύσταση των σπαραγγιών.

Στο Κεφάλαιο 3, περιγράφουμε μία από τις πρώτες μη στοχευόμενες μεταβολομικές
μελέτες για να κατανοήσουμε την χημική σύσταση τόσο του πράσινου ωστο και του λευκού
σπαραγγιού, καθώς επίσης και να συγκρίνουμε τα δύο είδη αλλά και διάφορες ποικιλίες
tους. Είναι, μάλιστα, η πρώτη μελέτη που διερεύνησε τη σύνθεση των πτητικών οργανικών
ενώσεων που, καθώς μπορούν να εξατμιστούν, συνεπώς ενδέχεται να διεγείρουν το αίσθημα
tης όσφρησης. Αυτή η διαδικασία είναι ζωτικής σημασίας για τον προσδιορισμό της γεύσης
για τις δύο μορφές σπαραγγιού. Η ανάλυση των πτητικών ενώσεων πραγματοποιήθηκε με
χρήση της μεθόδου GC-MS και η ανάλυση των μη πτητικών χρησιμοποιώντας την μέθοδο
LC-MS. Πραγματοποιήθηκαν πολυμεταβλητές και μονομεταβλητές στατιστικές αναλύσεις,
οι οποίες έδειξαν τις χημικές ενώσεις που είναι χαρακτηριστικές των δύο μορφών
σπαραγγιού. Επίσης, τα αποτελέσματα έδειξαν μικρές διαφορές μεταξύ των διάφορων
ποικιλιών. Επιπλέον, δείγματα από τομές κατά μήκος των βλαστών του προϊόντος,
anαλύθηκαν ως προς την σύνθεση των πτητικών ενώσεων και αυτό έδειξε ότι τα επάνω
μέρη των σπαραγγιών έχουν διαφορετική χημική σύσταση από τα κάτω μέρη (προς τη ρίζα).

Στο Κεφάλαιο 4, επικεντρωνόμαστε στα λευκά σπαράγγια καθώς αυτά καλλιεργούνται
κυρίως στην Ολλανδία και προβλέπεται να αποτελέσουν την πρώτη ύλη μας για την
μεταποίηση των φυτικών τους αποβλήτων. Ελήφθησαν δείγματα από οκτώ ποικιλίες
λευκών σπαραγγιών σε όλη τη διάρκεια της περιόδου συγκομιδής και τα δείγματα αυτά
αναλύθηκαν ως προς την σύνθεση των πτητικών ενώσεων και αυτό έδειξε ότι οι επάνω
μέρη των σπαραγγιών έχουν διαφορετική χημική σύσταση από τα κάτω μέρη (προς τη ρίζα).

Στο Κεφάλαιο 5, παρουσιάζουμε την ανάλυση των πτητικών και μη πτητικών ενώσεων που
πραγματοποιήθηκε με χρήση των μεθόδων GC-MS και LC-MS, με σκοπό την κατανόηση των
αλλαγών στη χημική σύσταση των λευκών σπαραγγιών που συμβαίνουν κατά την
επεξεργασία τους. Φρέσκα, ωμά και μαγειρεμένα λευκά σπαράγγια αποτελούν τα
dείγματά μας σε αυτή τη μελέτη. Τα αποτελέσματα αυτά συγκρίθηκαν και με το αποτέλεσμα
της αισθητηριακής ανάλυσης που είναι η χρήση των ανθρωπίνων αισθήσεων (γευσιγνωσία
- sensory). Εφαρμόζοντας εποπτευόμενες πολυπαραγοντικές στατιστικές αναλύσεις,
αποκαλύφθηκαν οι πιθανές σχέσεις μεταξύ γεύσης και χημικών ενώσεων (μεταβολιτών

Summary
συντηρητική αξιολόγηση. Επίσης, αποδείχθηκε ότι δια της μεθόδου ξήρανσης μέσω ψεκασμού,

Στο Κεφάλαιο 8, συνδυάζω τα αποτελέσματα όλων των μελετών που περιγράφονται και συζητούνται στα προηγούμενα κεφάλαια και εκφέρω την άποψη μου σχετικά με τη σημασία της έρευνας που περιγράφεται στη διατριβή αυτή. Η όλη προσπάθεια της συγκεκριμένης διδακτορικής διατριβής συνέβαλε στη καλύτερη κατανόηση του συνόλου των βιοχημικών αντιδράσεων (μεταβολισμό) του λευκού σπαραγγιού και στην κατανόηση της σύνθεσης της γεύσης αυτού. Επίσης, αποδείχθηκε ότι δια της μεθόδου ξήρανσης με ψεκασμό,
επιτυγχάνεται η δημιουργία γευστικής σκόνης από φυτικά απόβλητα που παράγονται κατά την διάρκεια της συγκομιδής. Μέσω της διατριβής αυτής, έχει αποδειχθεί ότι ο συνδυασμός μη στοχευόμενων μελετών μεταβολισμού (metabolomics) και αισθητηρίων (sensory) θα πρέπει να αποτελεί προϋπόθεση για την έρευνα τροφίμων/προϊόντων που σχετίζεται με τη γεύση (flavour).
Appendix

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Appendix

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About the author

Eirini loves plants and food. To a great extent, this is thanks to the beautiful times she had as a child in Thessaloniki (Greece) with her loving parents, her brother Paraskevas, and later also with their grandpa. At the age of 18, Eirini moved to Alexandroupoli for her BSc studies in Molecular Biology and Genetics. Her adventures in the Netherlands started in March 2014, when she went to Rotterdam for her BSc thesis at Erasmus MC in the department of Biochemistry. During that period, besides practicing basic molecular biology techniques, she realised that she would like to stay in the Netherlands. The Dutch culture, vibes, and Lennaert got her attention and heart.

In April 2015 she was back and started her MSc in Biopharmaceutical Sciences in Leiden University. She performed two thesis projects (at LACDR and BaseClear) and one internship (at DSM), as she was following subjects of two specialisations: Analytical Biosciences and Science-Based Business. The metabolomics group where she performed her first thesis, under the supervision of Amar Oedit and Peter Lindenburg, was the foundation of her love for metabolomics. During her internship at DSM in Delft, she realised that impact-driven research for sustainable food systems was what she wanted to do. In March 2018, she finished with her double MSc degree, but did not know directly how and where to continue with research. While thinking about it, she started working at Monsanto in Bergschenhoek as technician in the Seed Lab, but she missed the challenge and the mission she wished for. That is when she found the position at Wageningen University as a PhD student in Robert’s Hall team of Plant Metabolomics. She fell in love with the project because of the techniques involved and, mostly, the goal for minimising and upcycling agricultural waste. The distance Rotterdam-Wageningen (~100km) did not hold her back. She applied and got the position. Eirini started with her PhD in November 2018, and since then asparagus has been one of her best friends. Besides gaining experience in metabolomics tools, she also helped with supervision of course tutorials, she organised and got involved in couple of sensory studies and she got very intrigued by the power of advanced data analysis tools. The outcomes of her PhD research are presented in this thesis. At the end of the second year of her PhD, she moved to Wageningen with her hero (and now also her husband) Lennaert and their lovely and smart daughter Nefèli.

Eirini submitted her thesis in January 2023 and in March 2023, she started working at Wageningen Food & Biobased Research as researcher on volatile organic compounds in the Post-Harvest Technology group. Her research contributes in defining chemical markers to predict the quality of crops, to avoid deterioration and unneeded waste.
List of publications


Pegiou, E., Mumm, R., & Hall, R. D. (submitted). Elucidating the flavour of cooked white asparagus by combining metabolomics and taste panel analysis. *LWT.*
## Overview of completed training activities

### Discipline specific activities

#### Courses

<table>
<thead>
<tr>
<th>Course</th>
<th>Institution</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced Food Analysis</td>
<td>Wageningen, The Netherlands</td>
<td>2019</td>
</tr>
<tr>
<td>Big Data Analysis in Life Sciences</td>
<td>Wageningen, The Netherlands</td>
<td>2019</td>
</tr>
<tr>
<td>Chemometrics</td>
<td>Wageningen, The Netherlands</td>
<td>2020</td>
</tr>
<tr>
<td>Sensory Perception &amp; Food Preference</td>
<td>Wageningen, The Netherlands</td>
<td>2021</td>
</tr>
<tr>
<td>Big Data Analysis in Life Sciences</td>
<td>Wageningen, The Netherlands</td>
<td>2019</td>
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<tr>
<td>Sensory Perception &amp; Food Preference</td>
<td>Wageningen, The Netherlands</td>
<td>2021</td>
</tr>
</tbody>
</table>

#### Conferences

<table>
<thead>
<tr>
<th>Conference</th>
<th>Institution</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolomics International Conference</td>
<td>Den Haag, The Netherlands</td>
<td>2019</td>
</tr>
<tr>
<td>Weurman Flavour Research Symposium</td>
<td>Dijon, France (online)</td>
<td>2021</td>
</tr>
<tr>
<td>NMC Benelux Metabolomics days</td>
<td>Utrecht, The Netherlands</td>
<td>2022</td>
</tr>
<tr>
<td>European Federation of Food Science and Technology</td>
<td>Dublin, Ireland</td>
<td>2022</td>
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</tbody>
</table>

1. Poster presentation, 2. Oral presentation

### General courses

<table>
<thead>
<tr>
<th>Course</th>
<th>Institution</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLAG PhD week</td>
<td>Baarlo, The Netherlands</td>
<td>2019</td>
</tr>
<tr>
<td>Scientific Writing</td>
<td>Wageningen, The Netherlands</td>
<td>2020</td>
</tr>
<tr>
<td>Applied Statistics</td>
<td>Wageningen, The Netherlands</td>
<td>2020</td>
</tr>
<tr>
<td>Reviewing a scientific manuscript</td>
<td>Wageningen, The Netherlands</td>
<td>2021</td>
</tr>
<tr>
<td>Career Perspectives</td>
<td>Wageningen, The Netherlands</td>
<td>2021</td>
</tr>
<tr>
<td>Workshop: Hands-on with Artificial Intelligence (online)</td>
<td></td>
<td>2022</td>
</tr>
</tbody>
</table>

### Teaching activities

<table>
<thead>
<tr>
<th>Course</th>
<th>Institution</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPH10806 – lab classes of course Structure and function of Plants</td>
<td></td>
<td>2019</td>
</tr>
<tr>
<td>FC20806 - lab classes of course Food Chemistry</td>
<td></td>
<td>2020 &amp; 2021</td>
</tr>
<tr>
<td>NEM31806 - lab classes of course Plant &amp; Health 2</td>
<td></td>
<td>2022</td>
</tr>
<tr>
<td>Supervision of MSc thesis students</td>
<td></td>
<td>2020 &amp; 2021</td>
</tr>
</tbody>
</table>

### Other activities

<table>
<thead>
<tr>
<th>Activity</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of research proposal</td>
<td>2018</td>
</tr>
<tr>
<td>BU biweekly scientific meetings</td>
<td>2018-2022</td>
</tr>
<tr>
<td>AMS weekly scientific meetings</td>
<td>2018-2022</td>
</tr>
<tr>
<td>PPH monthly scientific meetings</td>
<td>2018-2022</td>
</tr>
<tr>
<td>Literature discussion meetings</td>
<td>2019-2021</td>
</tr>
</tbody>
</table>
The work described in this thesis was carried out in the framework of the Institute of Sustainable Process Technology (ISPT) under the project ‘TKITOEDR-20-11’: Waste2Taste and was co-funded by TKI-E&I with the supplementary grant ‘TKI- Toeslag’ for Topconsortia for Knowledge and Innovation (TKI’s) of the Ministry of Economic Affairs and Climate Policy. Partners in this project are Growers United, ISPT, Teboza BV, Unilever BV, and Wageningen University & Research.

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Metabolomics and sensory studies to uncover asparagus flavour

Eirini Pegiou

2023
Propositions

1. Dimethyl sulphide and 2-methoxy-3-isopropyl pyrazine make ‘asparagus’ flavour.
   (this thesis)

2. The best asparagus quality is found in mid-May.
   (this thesis)

3. The least understandable a data analysis tool appears, the more efficient it is.

4. Plant biodiversity is exceedingly jeopardised by tidy grass fields.

5. It is impossible for AI algorithms to master good social skills.

6. The ultimate strategic sequence to deliver a successful PhD thesis is Work-Write-Chill-Repeat.

7. The best period to become a parent is during the PhD period.

Propositions belong to the thesis entitled:

Metabolomics and sensory studies to uncover asparagus flavour

Eirini Pergiou
Wageningen, 10 May 2023