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36th PhD cycle in Pharmaceutical and Biomolecular Sciences

Development of new technologies for the valorization of brewery by-products.

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List of acronyms

AnV	p-Ansidine
ATP	Adenosine triphosphate
AUC	Area Under the Curve
BSGs	Brewery Spent Grains
BSY	Brewery Spent Yeast
CPT	Citra Pellet
DH	Dry Hopps
DISAFA	Department of Agricultural, Forestry and Food Sciences of Turin
DMS	Dimethyl Sulphide
DMSO	Dimethyl sulphoxide
DMS-P	Dimethyl Sulphide Precursor
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DSTF	Department of Pharmaceutical Science and Technology of Turin
EBC	Eropean Brewery Convection
EFSA	European Food Safety Authority
EO	Essential Oils
FAO	Food and Agriculture Organization of the United Nations
FFA	Free Fatty Acids
FH	Fresh Hops
FL	Full Loading
GC-MS	Gas chromatography/mass spectrometry
HACCP	Hazard Analysis and Critical Control Points
HCT	Heavy Volatiles Citra
HS-SPME	Headspace solid-phase microextraction
HVC	Heavy Volatiles Chinook
IPA	Idia Pale Ale
IS	Internal standards
LCT	Light Volatiles Citra
LL	Low Loading
LVC	Light Volatiles Chinook
MAHD	Microwave-assisted HidroDistillation
MEFCUV	Multiple Effect Fractional Condensation Under Vacuum
MW	Microwave
NAD^+	Nicotinamide adenine dinucleotide oxidized
NADH	Nicotinamide adenine dinucleotide reduced
PH	Pellet Hops
PV	Peroxides

RBSGs	Roasted Brewery Spent Grains
SAM	standard addition method
sc-CO_2	SuperCritical Carbon Dioxide
SD	standard deviation
SIDA	stable isotope dilution analysis
SMM	S-Methyl Methionine
SOP	Standard Operating Procedure
TOTOX	Total Oxidation
VAS	Visual Analogue Scale
WHO	World Health Organization

ABSTRACT

Over the last decade, global attention has increasingly shifted towards sustainable practices, spurred by growing environmental concerns and the recognition of finite resources. The corner stone of these efforts are the principles of the circular economy, which advocate for minimising waste and maximising resource reuse, remanufacture, and recycling. Concurrently, the issue of food waste has gained prominence, highlighting the inefficiencies and environmental impacts of current food production and consumption systems. A growing understanding of the limitations of the linear output and consumption models has propelled the transition towards a circular economy. Traditional linear systems, characterised by 'take-make-dispose' patterns, are inherently unsustainable, leading to resource depletion, environmental degradation, and increased waste generation. In contrast, the circular economy promotes a regenerative approach, where resources are kept in use for as long as possible, with value extracted at every stage and waste minimised through recycling and reuse.

As we reflect on the past decade, it is evident that sustainability, with a focus on circular economy principles and addressing food waste, has become increasingly integral to global agendas and societal priorities. In response, a powerful alliance of diverse stakeholders, including governments, businesses, and civil society organisations, have united their efforts. They have launched initiatives to reduce food waste at various supply chain stages. These initiatives encompass interventions such as improved harvesting and storage practices, streamlined distribution and logistics, consumer education campaigns, and the development of innovative technologies for food preservation and redistribution.

As a proof to this commitment, the Italian Piedmont Region has allocated funds from the European Community to develop a local supply chain with a sustainability approach driven by circular economy principles. The Baladin brewery seized this opportunity and presented a project that aligned with this vision, developing a new production chain that interconnected multiple industries while promoting the valorisation of brewery by-products. This project, falling under the name of “NUTRIBEV,” aimed to create the necessary expertise to establish a new line of functional beverages and baked goods. The project partners have been chosen from different fields of expertise to overcome the difficulties together, as shown in **Table A 1**.

Table A 1. Summary of the main activity of the partners involved in the "NUTRIBEV" project, their main field of interest and their specific activity on the project.

Partners	Main Activity	Project responsability
	Production and development of craft beers and soft drinks	• Creting the recipe for all the possible products • Project management
	Production and development of food supplements, medical devices and cosmetics	• Formulation of integrators and cosmetics • Investigation on the possible introduction of prebiotics from brewery spent yeast
	Physical transformation of biomasses for industrial applications	• Supporting the partners in the industrial scale physical treatments of the biomasses
	Specialised in chemicals and sanitation systems for the food industry	• Provide chemical analysis for all the partners • Design a filter plant for glucose syrup
	Production and development of baked goods	• Formulation of the recipe for the baked goods • Industrial scale production of baked goods
	Investigation of green and innovative technologies for extraction and modifications of valuable compounds derived from different biomasses	• Investigation of the possible innovative technologies to apply, especially for extracting terpenes from hops and fibre for brewery spent grains.
	Microbiological and process intensification research applied to the food industry	• Supporting the recipe formulation • Organization of panel test • Search for the best microorganism to use for an alcohol-free fermentation
	Research on innovative marketing techniques and upcoming growing markets	• Conduct market research to understand consumer interests and the most profitable market segments.

The previous table shows that the "NUTRIBEV" project comprises numerous partners, each with specific tasks designed to exploit their strengths. The investigation conducted during these three years of research can be divided into three main fields: purification of glucose syrup, exploitation of brewery by-products, and extraction of terpenes.

Gem Chimica decided to use sugar as a sugar source for the glucose syrup in the cereal milk. This is a common by-product composed of a complex mix of proteins, fats and sugars with predominantly glucose monomers but with different lengths and linkage points. We decided to operate an enzymatic digestion using a mix of glucosidases, α and β amylases to release all the glucose still trapped in the different polymers. This process was investigated by the DISAFA Department, which gave the digested starch milk to Gem Chimica, who had the task of removing undesired cereal flavour. The purification operation implemented a centrifuge treatment to remove most impurities, such as biomass fragments and colloids. Then, the liquid recovered was furtherly processed using resin filtration; unfortunately, the cost of the whole process was too high compared to the value of the purified syrup, so we decided to focus our resources on the other two fields of research.

The brewery produces a variety of different by-products. Still, we decided to focus on the brewery-spent grains (BSGs) and brewery-spent yeast (BSY) because they comprise 85/95% of the total waste produced. For the BSGs, we immediately understood that this biomass's main problem was its microbiological stability exiting the plant. The BSGs are always recovered with high humidity, making them a perfect substrate for many spoiling microorganisms. So, at first, BSGs was driyed using a press to reduce the humidity out from the brewery, and then Agrindustria partner accomplished the complete drying process. The stabilised BSGs are a very interesting biomass because it is composed mainly of barley malt without 90% of their original starch. Leaving primarily fibre (cellulose and hemicellulose) and proteins.

The produced material was then used for the direct production of BSG flour. It was introduced in various baked goods to reduce the cost of ingredients, reduce the products' total calories without changing the baked goods' organoleptic properties, and reduce food waste. The effective production comprised salty snacks, sweet biscuits and the typical Christmas dessert, panettone. From the performed tests, it was clear that the presence of BSG flour cannot wholly replace regular flour because it does not have the sugar needed for the leavening. For that reason the BGS flour could not be used in massive amount for the panettone without affecting the dessert's fluffy texture; since it is one of its peculiar characteristics, it was prohibitive. The salty snacks, similar to crackers, have a very soft leavening during the baking process, and their main characteristic is crunchiness. The introduction of BSGs can help, thanks to increased protein and fibre in the dough.

We also decided to investigate some non-conventional technologies for extracting and purifying specific fibres of BSGs due to their different nutraceutical properties. In this study,

we focus our attention on β-glucans and arabinoxylans, the most abundant in the BSG biomass. The β-glucans are under investigation for their capacity to regulate the cholesterol blood level if regularly assumed. At the same time, the arabinoxylans are responsible for reducing the glycaemicglycaemic peak after a meal. Both these fibres are pretty valuable and have started to be added as food supplements in many diets, and for this reason, the commercial interest is growing. Aiming to extract these compounds, we investigated several unconventional techniques, starting from hydrodynamic cavitationaunits (rotor-stator) and subcritical water conditions, methods that enable to skip the drying process and reduce the energetic cost. Unfortunately, neither of these approaches could specifically release the desired fibres, so we decided to implement an enzymatic treatment in collaboration with the company Heallo S.r.l.. Thanks to their patent technology, we purified the desired compounds and tried to implement them in various beverages and baked goods. The nature of the product collected made it perfect for implementation in solid food, while the resuspension on the liquid medium was more complicated.

The BSY are the brewery's second most abundant by-product, consisting of residual yeast cells and other by-products remaining after fermentation in the brewing process. It is primarily composed of spent yeast cells, which are the remains of the yeast population that actively fermented the wort during the brewing process. It may also contain other cellular debris, proteins, lipids, carbohydrates, trace amounts of hop residues, and non-fermentable materials from the original wort. BSY includes a variety of nutrients, including amino acids, vitamins, minerals, and nitrogen compounds, derived from the breakdown of malt and other brewing ingredients during fermentation. As a result, spent yeast can serve as a valuable nutritional source for livestock feed, human dietary supplements, or microbial fermentation processes. We try to use the BSY as a source of proteins thanks to their excellent aminoacidic composition of the human diet; however, the flavour brought by the BSY was detrimental to the product.

The project's final focus was improving the extraction of volatile compounds from vegetal biomasses, especially inflorescences and peels, for aromatisation purposes. Since the principal spice used in beer production is hops, we decided to focus on this biomass. Usually, the hop aroma is given to the beer in the brewing industry using the dry-hopping method, a simple maceration operated at low temperatures at the end of the fermentation. However, this methodology is old and far from reaching the best possible extraction yield. Moreover, the dry-hopping procedure is associated with increased contamination risks since, to perform it, you have to contact the beer with a non-sterile biomass. This problem is drastically reduced since beer has a low pH and alcohol to prevent the proliferation of undesired microorganisms. Still, it became much more critical for alcohol-free products, one of the production targets of the “NUTRIBEV” project.

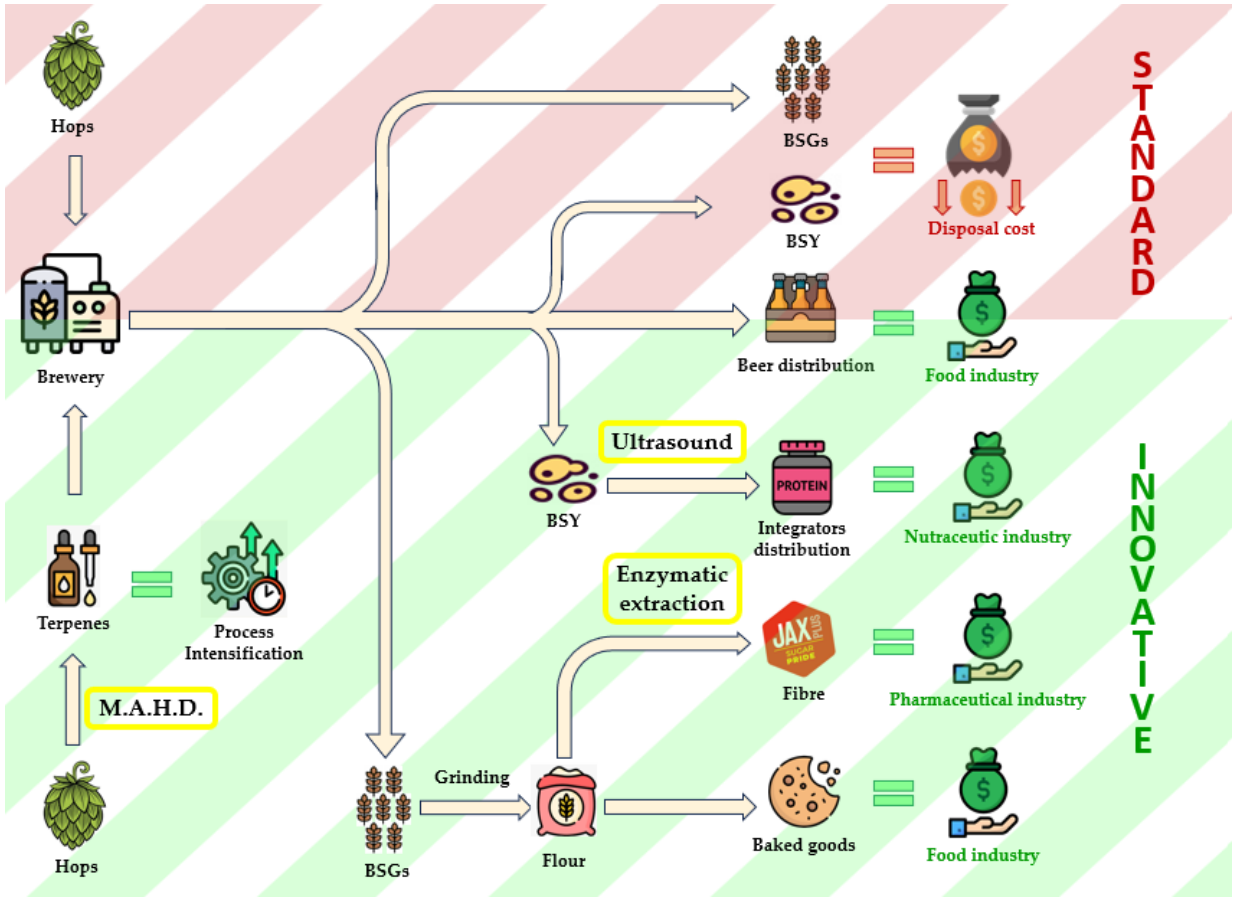
Firstly, water was used as a solvent to reduce the downstream process for applying the produced extract. At first, we tried to use an Aroma Recovery Unit produced by Tropical Food Machinery S.r.l. that works as a refinery tower. The plant can extract the volatiles from the biomass at low temperatures using a robust vacuum system applied in the reactor. Then, all the vapours collected are condensed in different columns depending on their boiling point. With this process, terpenes fraction are recovered from the hops and collected in two solutions: one enriched in the sesquiterpene fraction and one in the monoterpene fraction. This process gave great results initially because the extracted terpenes were already in a aqueous medium, helping the dissolution in the final product. However, the high water content led to high oxidation susceptibility with consequent flavour changes over time, together with possible mould formation.

After this first approach, we decided to extract the terpenes in purity using a microwave-assisted hydrodistillation system (ETHOS X, by Milestone S.r.l.). The relative scale-up was tested as well thanks to a pilot system (ETHOS XL), which started to be commercialised in 2023. The microwave interaction with the vegetal biomass enhances the extraction of the volatile fraction, reducing the formation of hot spots in the reactor that would create the risk of combustion products, reducing the overall quality of the recovered extract. This reactor does not directly heat the biomass because microwaves transfer their energy only to the water molecules, increasing the treatment homogeneity. After optimising the procedure for the recovery the terpenes, the inflorescence of *Cannabis sativa* L., we implemented it in industrial-scale production. The extract recovered from hemp was used for flavouring 150 hectoliters of alcohol-free beers that have been sold both in cans and bottles in local and foreign markets.

The great success achieved in this first industrial-scale application of the extract show the great efficacy of this new technological and processing approach, encouraging its implementation in the traditional beers production, to reduce the production cost. This technology helps reducing the quantity of raw material used thanks to process intensification.

All the research lines followed in these years aimed to lay the foundation of the brewery of the future: all the by-products are exploited and not seen as a waste, and the raw materials are treated with the most suitable technologies to enhance their flavours. In its entirety, the goal is to strongly reduce materials, time and energy consumptions, making the whole brewery more green, efficient and profitable

Graphical Abstract



1 RAW MATERIALS

Four main ingredients are required to produce beer: barley, hops, yeast and water. A careful selection of ingredients affects the product quality and the process standardization.

Barley malt is the cereal most used for brewing because its grains have a high concentration of starch per kg around 65%, and the husk's structure is essential for creating the filtration layer. Also, other cereals are used in brewing, both malted and not, like maize, rice, sorghum, and wheat. These cereals are considered adjuncts and cannot fully replace the barley malt. Often, they are used to give some flavour twist or colour shift, and their application is usually connected to local agricultural practices. The different cultivations of cereals are caused by the country's economic development, which affects the techniques that can be applied to promote growth, the nature of the soil, the periodicity, and the amount of rainfall.

Hops is a crucial component in beer production and is responsible for the bitter taste, which helps in the organoleptic equilibrium of the product. This not only for the flavour but also for their antibacterial and antimycotic activity, which helps a lot for the microbiological stability of the product.

The yeasts are the true beer makers, and their alcoholic fermentation process is crucial for the outcome of the product. They not only produce ethanol and carbon dioxide, which ensure carbonation, but also numerous secondary metabolites that have a significant influence on the flavour of the beer via their metabolic pathways.

Finally, water is quantitatively the most important ingredient in terms of volume and is often underestimated. This preconception stems from the idea that water is merely a solvent for other vital compounds. In reality, the water used for brewing is not pure, we also find various salts and minerals. Salts are also crucial in the preparation of solid foods, as they act as flavour enhancers, and the minerals are important cofactors that are essential for proper propagation and fermentation by the yeast.

1.1 Barley

Barley (*Hordeum Vulgare*) is the source of fermentable sugars released during the mashing phase, thanks to the activity of endogenous enzymes on the stocked starch. Many barley varieties differ in their structure and ripening time and not all can be used for brewing.

Barley is essentially the most commonly used grain for beer production; it is one of the first cultivated grains in Eurasia, having been cultivated 10'000 years ago [1]. Nowadays, it is the fourth most-produced grain after maize (corn), rice, and wheat; its production is around 150 million tonnes per year, and the more prominent producer is Russia with 20.6 million tonnes, followed by Australia and Germany with 13.5 and 10.9 each [2]. This diploid

plant (14 chromosomes) can self-pollinate and can be divided into two categories: the two-row and the six-row. As can be seen in **Figure 1,** these two groups have some morphological differences. The disposition of the kernels is not the same when the head is viewed down its axis. The differences between the two categories continue in their nutritional power; the two have a lower protein content but a higher fermentative capacity with a higher amount of carbohydrates that can released during malting and mashing. Brewers can use both, usually, the choice depends on the brewer's experience or the product availability. The main differences are summarised in **Table 1.**

Table 1. Schematic representation of the differences between using two-row and six-row barley in beer production [3].

Type	PROS	CONS
Two-row barley	✓ Greater Drought tolerance ✓ Thinner husks cause an easier extraction of starch, which is particularly crucial for industrial productions ✓ Give a mellow flavour to the beer	✘ Lower enzymatic activity, though it needs to be malted if used in high doses ✘ More expensive
Six-row barley	✓ High protein levels help in the speed of conversion in fermentable sugars ✓ Used with other grains unmalted can help with the release of also their starches ✓ Cheaper ✓ Thicker husks are more manageable to filter	✘ Produce a higher quantity of dimethyl sulfide during the malting process, which gives a sweet flavour acceptable only in some beers ✘ Higher concentration of polyphenols (tannins), giving an astringent taste ✘ Due to the protein concentration, it is tricky to recreate the same colour of the final product

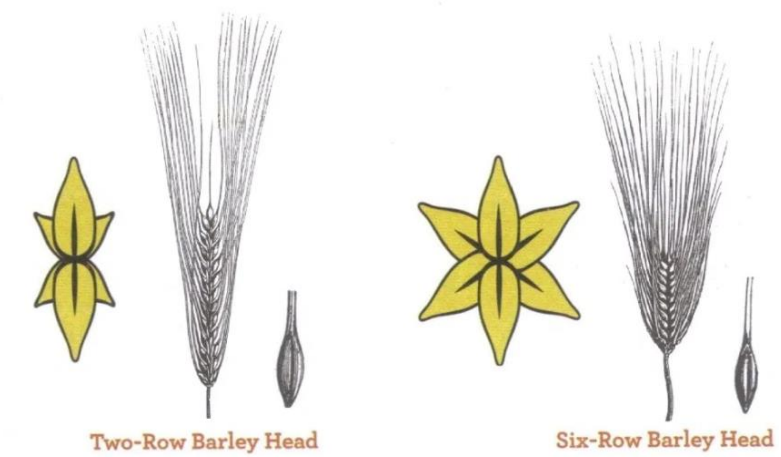


Figure 1. Representation of two and six-row barley. The picture was taken from [3]

1.1.1 Barley structure

The barley kernel's internal structure can be divided into the germ region, the endosperm and the coverings. The germ region is located in the bottom region of the kernel, and it is separated from the endosperm by two tissue layers called the *scutellum* and the *epithelium*. The endosperm is the kernel's most significant part, reaching even 80% of the total volume of the grain as can be appreciated in **Figure 2**. This part is constituted by stocking cells, which contain the starch granules. Depending on their dimensions, these granules can be divided into types A and B. Type A are the biggest, with a diameter between 20 and 30 μm and type B between 3 and 5 μm. The ratio between those two granules can differ significantly from barley to barley, and it correlates both to the genetic properties of the variety of barley and from environmental factors during the development of the grain. The space between each granule is filled by a complex protein matrix that can be dense or loose. The cell walls comprise a central lamella composed of phospholipids and proteins that regulate the internal and external mass transfer. Around this lamella, we find a β-glucan layer covered with different pentosan layers on both sides. The thickness of the starch cell wall depends on the variety of growth conditions. For brewing, barley with thinner cell walls is usually preferred because it is more accessible to break down. The endosperm is surrounded by the aleurone layer composed of protein-rich cells. This region is significant because it stocks the endogenous enzymes produced during malting. The grain coverings are composed of three layers superimposed. The inner layer is called the testa or seed coat and is water-permeable. Above it is the fruit coat, pericarp, and husks, which consist mainly of cellulose.

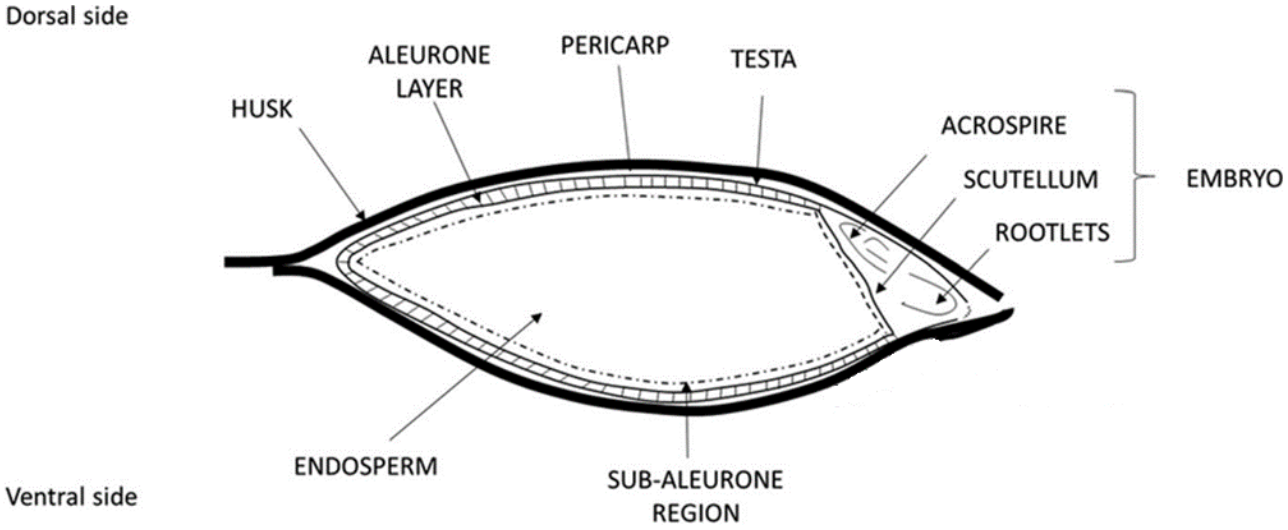


Figure 2. A barley kernel in longitudinal cross section. The picture has been taken from [4].

1.1.2 Barley composition and properties of the components

When the barley is stocked, it must have a moisture level below 15% for long-term storage. The dry matter average chemical composition is shown in **Table 2**.

Carbohydrates are the most abundant class of compoundsin plants (starch and cellulose in particular).

Table 2. Barley average chemical composition.

Compounds	Dry matter %
Total carbohydrates	70-85%
Proteins	10.5-11.5%
Ashes	2.0-4.0%
Fats	1.5-2.0%
Other substances	1.0-2.0%

The simplest sugar is glucose, which is produced from CO_2 and H_2O in the photosynthesis process.

Starch is the barley's most abundant constituent, reaching around 63% of the dry matter. After the glucose is synthesized in the chloroplast, organelles located on the leaves are transported to the specialized stocking cells that polymerise the glucose in the growing starch grains that gain a onion-like structure with sequent layer. Normally the glucose monomers are connected one another thanks to 1→4 glycosidic bonds that creates long helical chains of amylose. The amylose chains also have some branching thanks to occasional 1→6 bonds forming the amylopectin structure (**Figure 3**). Both the 1→4 bonds and the 1→6 bonds utilse α-glucose as building blocks and for that reasons amylose and amylopectin are considered α-glucans that differentiate those polymers to cellulose which is a β-glucan.

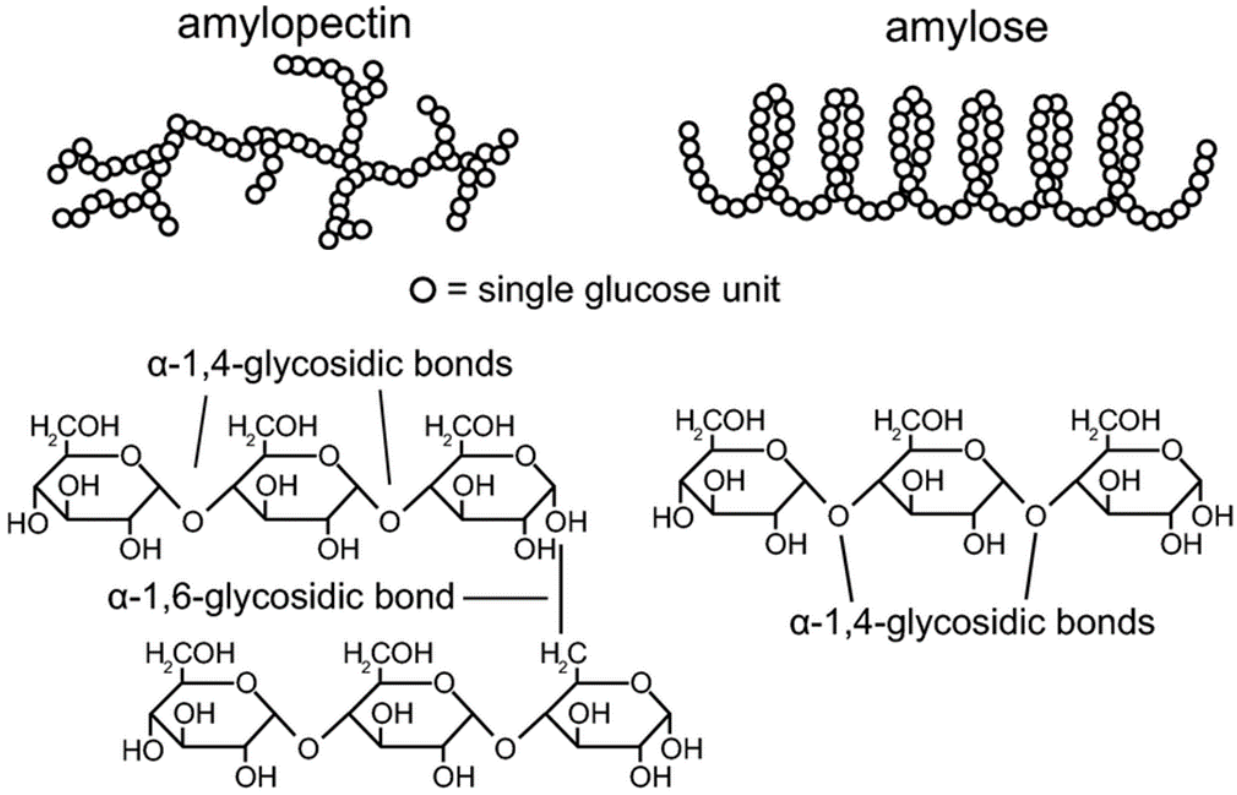


Figure 3. Chemical structure of the corn starch constituting natural polymers amylopectin and amylose. Amylopectin is a branched polymer, whereas amylose is a linear polymer forming a helix [5].

Cellulose is a polymer constituted by long chains of β-glucose linked via 1→4 bonds. This difference in the position of an OH group in the C1 position renders the polymer insoluble in water and not-digestible for human organism. Moreover, it is much more resistant to enzyme activity, and the plant uses this polymer as a structural support for the cells, while the starch is an optimized energy stock. Both these polymers are essential for brewing; starch gives the yeast the desired fermentable sugars to operate the alcoholic fermentation, while the cellulose that remains undamaged during the mashing phase creates the filtering cake layer during lautering.

The other carbohydrates form the hemicellulose, the endosperm's main constituent. They are formed by the aggregation of β-glucans polymers of pentose called pentosans, creating a rigid layer together. The β-glucans consist of long chains of glucose linked via 1→3 and, more often 1→4 bonds. The breakdown of β-glucans starts during the maltation phase and can hurt the finished beer. Pentosans are composed primarily of xylose and arabinose. The main structure comprises long chains of 1→4 xylose residues with some arabinose sugar linked with 1→2 or 1→3 bonds (**Figure 4**). This structure is mainly present in the aleurone layer and the kernel's endosperm. The pentosans can be broken down enzymatically, but since many different enzymes are required at the end of malting, there are still plenty of long chains of pentosans.

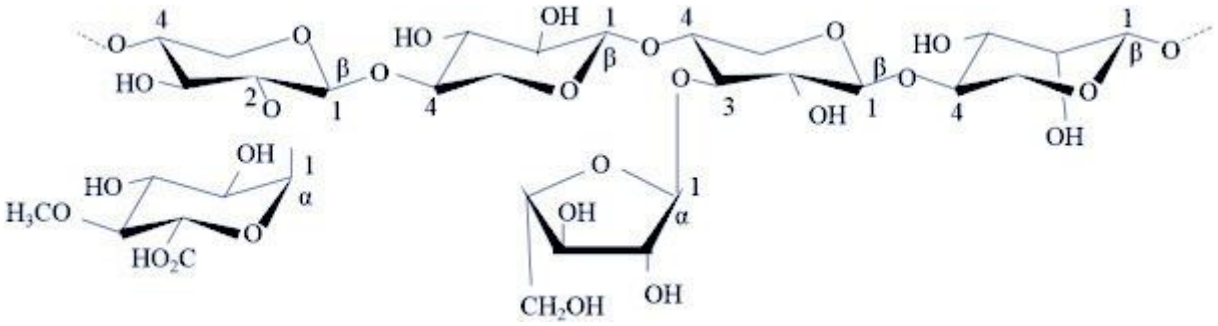


Figure 4: Structure of hemicellulose (xylan) consisting of a xylopyranose backbone, with glucuronic acid (1→2) and arabinofuranose (1→3) side branches [6].

The nitrogen content of barley typically does not exceed 12% of the dry mass. Most of the proteins are stored in the endosperm's cell wall, transport proteins, and they regulate the mass transfer in both directions. Only a tiny amount of the protein in the barley reached the finished beer. Nevertheless, they have an important effect on the quality. They are particularly relevant for the foam formation and stability and for the occurrence of hazes in beer. The essential sources of nitrogen are amino acids and grain proteins. As for the carbohydrates, the yeasts cannot degrade the proteins by themselves. The malting and mashing process helps them create simpler units to work with. Using unmalted cereals like rice, corn, or wheat will not change the nitrogen concentration in the wort because the proteins contained are not soluble, so the yeast will not be able to interact with them. The presence of amino acids is significant in the fermentation and can be classified by the rate of assimilation by yeast in normal fermentation or by the ability of the yeast to produce them (class I), replace them (class II) or necessary because the wort is the only source for the yeast. This classification has been schematized in **Table 3** [7].

Glutelin is around 30% of the total protein of barley, and it is stocked primarily in the aleurone layer. In the final product, we typically found only protein breakdown products, which are soluble in water and do not precipitate during boiling.

Barley contains up to 2% of fat stock, primarily in the aleurone layer and in the seedling in the form of oil droplets. The lipids consist primarily of fatty acids. These molecules comprise a long carbonic chain that ends with a carboxylic group that gives a weak acid behaviour. The fatty acids are categorized by their carbon chain length and by the presence of double bonds. The fatty acids that present at least one carbon hybridized sp^2 are called unsaturated fatty acids. These compounds are essential for beer production since they are necessary for the cell wall of the yeast.

Moreover, they are also responsible for the ageing process of the beer. The fatty acids are quite reactive and can be processed by lipoxygenase enzymes or react independently with free O_2 molecules. Fatty acids with medium and long chains have an adverse effect on foam formation and stability since they interfere with bubble stability thanks to their amphipathic nature.

Table 3. Amino acids classification using the absorption rate parameter, divided in degrees of importance.

	Absorption rate			
	Group A: Rapid	**Group B: Moderate**	**Group C: Slow**	**Group D: Largely unabsorbed**
Class I: Unimportant Amino acid	Glutamic acid Glutamine Aspartic acid Asparagine Serine Threonine			Proline
Class II: Vital amino acid		Valine Isoleucine	Glycine Pheniylalanine Tyrosine Alanine	
Class III: Crucial amino acids	Lysine Arginine	Leucine Histidine	Tryptophan	

The quality of this raw material strongly impacts the final product and can be evaluated by hand or via physical and chemical analysis. When evaluating the quality of the barley, the cereals must have a pleasant, clean smell associated with fresh straw. On the other hand, earthy and mouldy flavours indicate inappropriate storage that can cause a reduced germinative capacity. This evaluation can also be tested by an empiric flow test using our hands. If the barley grains remain stuck to the hand, they have a higher moisture content. The colour of the grain should have a light yellow uniform and bright colour all over the husk. Greenish nuances indicate early harvesting, while grey colours are more associated with rain damage during the growth.

1.1.3 Other cereals

Since the enzyme potential of malt is sufficient to catabolize additional starch, sometimes other unmalted cereals are introduced in the recipe to reduce the total production cost . In this case, the unmalted cereals are called raw grains, and typically, each world region uses the most cultivated one in that region. For example, rice is more used in Asia than in other countries, while sorghum is more used in Africa.

- WHEAT

It has a low protein percentage and high carbohydrates that can be released during the mashing. It creates white beers, giving them the flavour that can remember bread. This cereal has a very high concentration of gluten that gives the typical haziness associated with

Blanche and *Weisser* beers. Since wheat loses its husk during the threshing, it creates a wort with a high viscosity caused by by pentosans instead of β-glucans .

- RICE

It is mainly used in eastern countries like Asia, but it also started to appear in the Western market due to its lower cost of production and malting process than the barley. It is used in lager beer production [28]. Are commonly used grains that have broken during the processing of dehusking and polishing. Since this cereal has no gluten, it is often used as the cereal base for gluten-free beer. In this way, the total gluten to remove is lower than a recipe that does not use rice. This cereal has a very high content of starch that fluctuates between 85 and 90% and is stocked in aggregated granules with a characteristic shape. These morphological characteristics increase the gelatinization temperature for this cereal, around 80-85 °C. This must be taken into consideration when used.

- MAIZE

It is used for its low production price, and because it gives a sweet touch to beer with also high clarity, it is mainly used in the U.S.A. because the corn is produced in large quantities and is related to the local culture [29].

- SORGHUM

This cereal produces beers without barley and is a trendy drink in South Africa. The local population does not have access to the other cereals, which need a more tempered climate and a soil more prosperous of nutrients that this land cannot provide. Many studies have shown some functional properties of sorghum-fermented drinks, but the scientific opinion and how it interacts with the human body is not yet clear [30]. It is also used to make gluten-free beers to reach a segment of users that would have been otherwise cut off; it is not the only way to produce gluten-free beers. Some breweries use enzymatic processing of the beer to degrade the protein into smaller peptides that cannot cause allergies.

A brewer can add some sugar to the wort when needed to reach the desired concentration of fermentable sugars. Typically, saccharose is the one used. It is a disaccharide composed of a molecule of glucose and fructose. When saccharose gets boiled for at least one hour or placed in an acetic environment, the glycolic bond is broken, the monosaccharides are released, and they become easily fermented by the yeast. For this reason, saccharose is usually added during the boiling phase. When desired by the brewmaster, it is also possible to introduce some caramelized sugar with many implications. As with the traditional sugar, when added, the caramelized syrup will increase the amount of fermentable sugars but also change the colour and give some caramel flavour.

1.2 Hops

Hops are the inflorescence produced by the *Humulus lupulus L.* plant. It belongs to the Cannabaceae family native of Europe, southwestern Asia and North America [8]. *H. lupulus* is described as a bine capable of climbing on vertical surfaces like other plants or walls. They can reach six meters, wrapping themselves on other plants. For this reason, it is called Lupulus, which in Latin means a small wolf, making an analogy to the wolf's aggressiveness with their prey. To be cultivated, *H. lupulus* need temperate climates. The average year production fluctuates around 100 thousand tons. The major producers are the United States, Germany, and the Czech Republic, respectively, 44.3, 39.0, and 6.1 tons [9].

Only female plant flowers are of interest to brewers. The inflorescence is attached to the vine thanks to a short stem connected to the central zig-zaging central spring with a floret with a large covering bracts at each bend. The Bracts are the petals of the inflorescence and are characterized by a yellow-green gradient that starts at the base, where the bracts are attached to the strig. These petals have an egg-shaped form and altogether are arranged in a cone shape. Between the strig and the bracts are found the lupulin glands that show themselves as sticky yellow powder composed of many glands stoked in the bracteoles space (**Figure 5**). These small rounded glands contain most of the plant's interesting compounds, both the resins and the EO. The glands are covered by a membrane preventing the inner content from escaping.

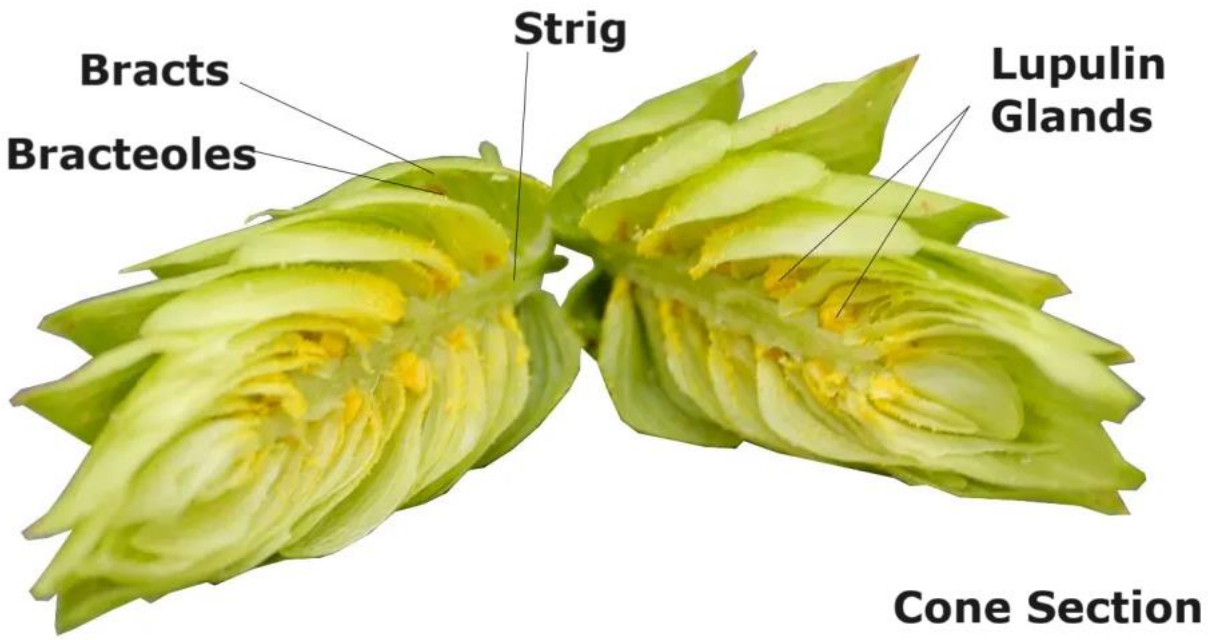


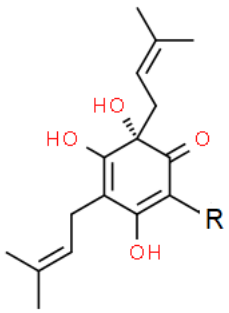
Figure 5. Longitudinal cross section of a hop cone [10].

1.2.1 Hops composition and properties of components

The organic molecules that affect the organoleptic properties are the resins and the EO; the main chemical structures are reported below.

There are two types of resins: hard resins, which are insoluble in hexane and are about 5% of the total resin content, and soft resins, which are soluble in hexane. The latter are divided into α and β acids. The α-acids are primarily responsible for the hop bitterness taste in beer [11]; all share the same partial structure with an acyl side chain that differentiates them, **Table 4.**

Table 4. α-acids structure.



Name	R (= Acyl side chain)
Humulone	$COCH_2CH(CH_2)_2$
Cohumulone	$COCH(CH_3)_2$
Adhumulone	$COCH(CH_3)CH_2CH_3$

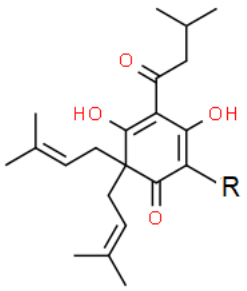
As can be seen, by the structure, the cohumulone has one less atom of carbon than the other α-acids; hence, it is more soluble than the others in water after the isomerization reaction occurs during the hops processing via temperature increase. The cohumulone gives a bitter, harsher taste than the other analogues [12]. The proportion of these molecules changes from hops to hops, giving different characteristic flavours to the beer. Hops cultivation place and soil characteristics and overall temperature significantly impact the proportion of the α-acids.

The other group of soft resins are the β-acids, that like the α-acids have a six-carbon ring with several substituents **Table 5**. They generally do not play a significant role in fresh hops due to their lower solubility compared to α-acids. However, during the ageing process some oxidations increase the solubility, and the beer bitterness [13]. Usually, the ratio between α-acids and β-acids is cited with the percentage of cohumulone as hops indications of quality. The acids ratio greatly changes (0.8 – 3.0) [7]. It depends on the hops species that may characterize different beers.

Hops with poor storage stability can lose a significant amount of their α-acids in just one year. A study on two popular hops, the *Cascade* pellets and the Hersbrucker pellets, have shown a significant reduction in the levels of α-acids after twelve months in a refrigerator. The starting level for the *Cascade* was 7.6%, and for the *Hersbruker*, it was 7.4%. After the time laps, the respective values have decreased to 4.6% and 4.7% [14]. This change is caused

by an oxidation reaction that deteriorates the acids and creates molecules that give the beer some "cheesy" tones.

Table 5. β-acids structure.



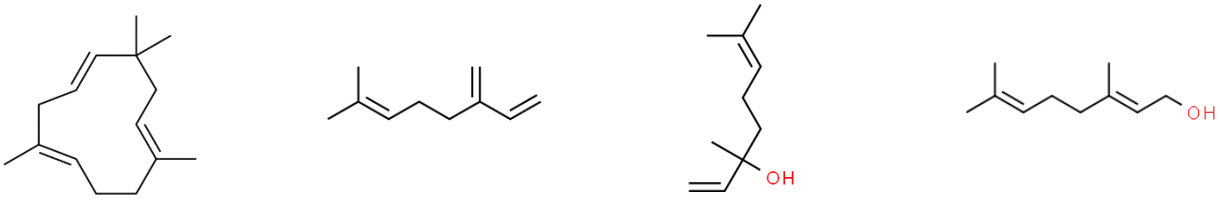
Name	R (= Acyl side chain)
Lupulone	$CH_2CH(CH_3)_2$
Co-lupulone	$CH(CH_3)_2$
Adlupulone	$CH(CH_3)CH_2CH_3$

The EO extracted from hops do not affect the resin's bitterness but are responsible for the aroma. The components of the EO can be divided into three subgroups: hydrocarbons, oxygen-bearing derivatives, and sulfur-containing components.

The hydrocarbons components can represents up to 75% of all the EOs [15]; they can be classified as monoterpene or sesquiterpene. The two main hydrocarbon compounds in beer are humulene and myrcene (**Figure 6**); the first gives an elegant flavour, while the other is described as pungent. The ratio between those two molecules is usually cited on the hops batch. Noble hops will have a humulene/myrcene ratio that exceeds 3.5, but there are variations. It is important to note that as for the soft resins, the EO can deteriorate in time due to oxidation. The palletization process can also alter hop composition; for this reason, there are apparent aroma differences between the leaf or pelletized version of the same hops batch [7]. A study showed that myrcene levels of Cascade hops have decreased from 329 mg/L to 7 mg/L after twelve months of refrigerated storage [16]; also, the oxidized molecules have organoleptic properties, giving a grassy touch to the beer.

Oxygen-bearing components are approximately 25% of the hops' EO [15]. The lesser concentration does not mean that the molecules belonging to this group have a negligible impact on the hop's flavour characteristics. The linalool and the geraniol oil (**Figure 6**) have been studied. They cause the floral and herbal aroma that characterizes many hops like the Cascade and the Continental [14].

Sulfur-containing components are the last subgroups of EO molecules. As already explained, an excess of sulfury tones is generally defective for the beer. It is known that malt and the fermentation process are the significant sources of sulfur flavours, but hops can also contribute to them [7]. High doses of sulfur compounds can be caused by using sulfur-based treatments during the growth to protect the plant from fungus infestations before the harvest.



(a) **(b)** **(c)** **(d)**

Figure 6. Respective structures of (**a**) humulene, (**b**) myrcene, (**c**) linalool, and (**d**) geraniol.

The hops contain 2 to 5% of polyphenols in their dry form. They are entirely located in the spring and the bracts. This family of molecules have essential properties for the beer for their antioxidant effect, astringent taste, and colour mutation of the beer. As a result, they are involved in the formation of hazes and contribute to taste and colour. They are a complex mix of tannins, flavonols, catechins and anthocyanins.

The quality of hops is evaluated by hand assessment and the analytical determination of both resins and EOs. By hand, it is possible to evaluate the crop purity. The presence of leaves and plant stems will reduce the intensity of of the pellet hops since the globulin glands are found just in the inflorescence. It is important to harvest the hops at the proper dryness, which is evaluated thanks to the colour and thickness of the bracts. Of course, the most crucial parameter is the abundance and quality of the lupulin glands. They should present as a shiny golden-lemon yellow powder with a fresh, pungent flavour.

Analytical analysis comprehends a total quantification of the α-acid and β-acid concentration and a total characterization of the volatile profile using gas chromatography techniques.

1.3 Yeast

Yeasts are unicellular eukaryotic organisms belonging to the fungus kingdom. More than 1500 species are currently recognized; they descend from an ancestral progenitor that lived hundreds of millions of years ago [17]. These microorganisms can metabolize sugars in ethanol and carbon dioxide and are used in bakery and alcoholic beverage production. The fact that the metabolism produces carbon dioxide is essential in the bakery industries because it creates the fluffy texture wanted, whereas, in alcoholic production, it gases the beverage, which is usually a wanted characteristic. The ability to produce ethanol instead is not essential during the bakery's levitation process because the ethanol produced is volatile. After all, the dough is brought at a higher temperature than the boiling point [18].

The saccharomyces are the most used family of yeasts for beer production; they can be divided into groups: the top-fermenting and the bottom-fermenting. The top one creates foam during fermentation in the wort's upper region; this group's most crucial yeast is *Saccharomyces cerevisiae*, used in ale production [19]. The bottom-fermenting yeasts are

usually used in lager beer production. They prefer a lower temperature during the fermentation, which is also longer than the top-fermenting yeast. The most important representative of this class is the *Saccharomyces pastorianus* (a.k.a. *S. carlsbergensis*).

The last significant group of yeasts for beer is the *Brettanomyces*; this strain produces Lambic and sour ales beers. Those beers have an iconic acid taste resulting from the different metabolic pathways used by this type of yeast [20].

The main difference between these yeasts is that some can process some sugars that others cannot assimilate. For example, melibiose is a disaccharide composed of a galactose molecule and a glucose molecule liked by an α 1→6 oxygen bridge. Only the organisms with the α-galactosidase enzymes can break the link and release two fermentable monosaccharides; this enzyme is only found in the *Saccharomyces* of the *pastorianus* genus and not in the *cerevisiae*. Another critical factor of the yeast to use in beer production is the secondary metabolic pathways that the yeast we want to have. Those pathways will produce molecules in small concentrations but can provoke an alteration of the beer's organoleptic properties.

Due to their high impact on the final product, typically, the breweries decide to inoculate pure yeast culture to the wort; thus, they have better control over the fermentation process. This cannot be sad for lambic beers; the wort produced for those beers is left on purpose in the air during the cooling process. In this way, more than a hundred types of microorganisms can inoculate during the night overcooling. This process can be made only from October to May because, during the summer, the increased temperature can cause the inoculation of unpleasant yeasts [21]. Climate change reduces the optimal window to produce this kind of beer; the temperature needed during the night can vary from -8 °C to 8 °C. One hundred years ago, the brewers could do this beer for 165 days yearly. Now, this window of opportunity has been reduced to 140 days per year [22].

1.4 Water

Beer is mainly constituted by water, probably the most essential component. Its chemical-physical properties are the key to understanding its impact on the final product. Water is an excellent solvent that is important to dissolve the grain constituents during the mash process and hop flavouring molecules.

The water used for the brewing process is also called brewing liquor, and it is the medium in which all the different preparation steps will occur. In brewing liquor, the content of minerals and salts is vital for enhancing the enzymatic activity during mashing. The essential salts used in the brewery are calcium chloride ($CaCl_2$), calcium carbonate ($CaCO_3$) and magnesium sulfate ($MgSO_4$).

All of these salts tend to dissociate completely in water; the reactions can be represented as follows:

$CaCl_2 \rightarrow Ca^{2+} + 2\ Cl^-$

$CaCO_3 \rightarrow Ca^{2+} + CO_3^{2-}$

$MgSO_4 \rightarrow Mg^{2+} + SO_4^{2-}$

The ions released by the ionic bond's rupture have an essential role; they can alter the pH of the brewing liquor and interact with the enzymes. For example, it is known that calcium tends to afford thermal protection for the mash enzyme [23]. It also inhibits colour formation during the boiling process and facilitates protein coagulation. This activity helps to clarify yeast flocculation and to clarify beer during maturation[24][25]. Magnesium is also an essential cofactor for many enzymes. Malt supplies sufficient magnesium for the catalyst, but higher concentrations will contribute to a harsh bitterness of the final product [26].

The carbonate group (CO_3^{2-}) and the bicarbonate (HCO_3-) are fundamental to wort production.

The carbonate can work as a buffer solution as it happens in blood vessels, and the proportion of the carbonate, bicarbonate and carbonic acid in pH is shown in **Table 6**[7].

Table 6. Proportion rate between all hydrogenated carbonate forms correlated to the solution pH.

pH	CO_3^{2-}%	HCO_3^-%	H_2CO_3%
10	32	68	0
9	5	95	0
8	0	97	3
7	0	81	19
6.5	0	58	42
6	0	30	70
5.5	0	12	88
5	0	4	96

Low pH values of the water used for brewing liquor are advantageous, although most water supplies have a pH range of 7.0 to 10.0. Thus, making the same water treatment or introducing salts is often necessary to achieve the desired pH value.

2 BREWING PROCESS

Industrial breweries follow the same steps as a homebrewer. The main differences are in the production scale, suitable plants and tools. The brewery's procedures are well established, but each producer may create different products to gain market share. In this paragraph, each step will be described in depth to provide a full view of the whole process and the by-products created in the breweries.

2.1 Malting

Malting is a process done on grains where we induce and control the germination process, transforming grains in malt. All types of grains can be malted, but the most used ones are barley, wheat, rice and sorghum.

After harvesting, all grains must be immediately malted or dried at 50 °C with solid ventilation. Grain overheating has to be avoided; the embryos will not germinate. After this operation, the grains can be stored for 18 months at a temperature lower than 18 °C. This way, there will not be a vitality loss and will be monitored to control if the batch has some insects or fungi contamination.

2.1.1 Barley steeping

Steeping is the first step of the malting. In this phase, the barley must be cleaned and hydrated; it is also important to supply it with sufficient O_2. The gas is needed for respiration and the resulting CO_2 must be excreted. The grains are covered with water and the internal moisture content of the grains increases from 12% to 40/45%. The temperature is kept between 10 °C and 16 °C for over a week [27]. The water penetrates incredibly quickly into the embryo region of the barley grain at the base and from there reaches the inner part, the endosperm. Water uptake depends on many factors, e.g. steeping time and temperature, grain size and the genetic and environmental characteristics of the barley. Water absorption is rapid in the first hour of steeping and then gradually slows down. At the end of the steeping phase, it is important to have a homogeneous humidity level on all the barley; otherwise, they will undergo unequal growth and therefore be of poorer quality. For this reason, it is important that the grains have a stable size. Large variations in diameter and length lead to a drastic difference in water content at the end of steeping.

2.1.2 Barley germination

In this period, the germination starts, and the kernel produces a new plant. The enzymes are produced, and the endosperm is transformed with their activity due to the degradation of the cell walls and the protein matrix. In this phase, the amylases are also produced. This protein class can hydrolyse the oxygens bridges on starch, releasing simpler carbohydrate

chains that can be assimilated and metabolized during fermentation [28]. The embryo senses the rise of water content over 30% and starts its morphological and metabolic transformation. At the end of the steeping phase, the rootlets break the base of the corn and become visible at the exterior, and in a few days, they multiply the number of roots and their length. The root formation determines the consumption of starch, so it is essential to reduce as much as possible this process. The two most impactful parameters for the roots' growth are the germination time and the temperature. Long germination periods at warmer temperatures induce a longer root and a more significant loss. A delicate rooting phase will only lose 4% of the total malt dry weight. A few hours after the water intake, the seeding cells start to produce and secrete gibberellic acid and other growth-promoting signals. When those signals reach the aleurone layer, the cells of this structure receive the signal thanks to selective membrane receptors and, after a waterfall signal, start the production of a series of different enzymes. The enzymes produced are primarily α-amylases and β-amylases, glucanases and proteases. These enzymes can cause a release of the sugar stocked in the endosperm in the form of starch. Usually, the grain needs to use all the energy stocked as starch to create all the vegetal structure needed for the sustainability of the plant, such as roots for extracting water and micronutrients from the ground and the leaves to start the photosynthesis and become independent from the starch. During malting, the operator wants to enhance the production of the enzymes but wants to stop the germination as soon as possible. In this way, preserving more sugar for the brewing is possible. The first enzymes to be produced are amylases. Specifically, β-amylases start on the second day of germination while α-amylases the next day. The most important families of enzymes produced during germination are:

- β-Glucanase

  This enzyme can break the glycosylic bonds of the glucans, particularly the β 1→4 links that create the cellulose, the primary structure that gives mechanical resistance at cell plant walls [29]. The damage to the cell wall is crucial because it allows other enzymes to access the starch stored inside the cell that would otherwise be inaccessible.

- Proteinase and peptidase

  Those enzymes are capable of attacking the peptidic bond. The degradation of proteins into peptides and then into free amino acids is crucial because the yeasts must find free amino acids in their growing medium to proliferate.

- α/β-Amilase

  This family of enzymes is the one that breaks down the starch carbohydrates chain, producing the disaccharides and the monosaccharides that yeast can assimilate and ferment. The α-amylase can break the glycosidic α 1→4 bonds of starch in random positions, creating more long chains of polysaccharides. The β-amylase can also

break the α 1→4 glycosidic bonds but only of the last two or three glucose molecules of the polysaccharide chain. It is vital to have both enzymes because, without the presence of α-amylases, the β-amylases would have few ending points to work with. The degradation of starch into simpler molecules would be a prolonged process. The same can be said for the other enzymes. Breaking in random points will not guarantee the release of the desired carbohydrates.

The amount of enzymes produced depends on many factors, such as [30]:

- Genetics of the cultivar chosen and environmental conditions during the development of the plant
- Dimension of the kernel: larger kernels produce higher amounts of proteins
- The total humidity in the green malt is proportional to the amylase content
- Good aeration during the germination promotes the development of α-amylase
- The overall temperature during the steeping and germination phase. Maintaining a cold temperature during germination gives higher amylase values. However, having higher temperatures during steeping and germination will cause an earlier commencement of enzyme production, but the amount will be lower at the end.

The diastic power evaluates the enzymes' overall activity and is expressed in Windish-Kolbach-Units (WK). During the germination, the enzymes already produced start to make some changes and produce some low molecular weight degradation products from high molecular weight products. The degradation products are used to build new cell material for the germination transformation, such as the roots, so they will not be available during the later processing. In this phase, the cell wall of the endosperm starts to be degraded. The middle lamella, made of proteins, is surrounded by a β-glucan layer attached to a porous pentosan layer. The proteases produced start to break down the middle lamella, and in this way, the pentosan layer gets exposed to the xylanase enzyme, which starts the degradation. After the partial degradation of the β-glucan layer opens, the path for the enzyme into the inner endosperm cells where the starch is kept.

2.1.3 Malt kilning

When enough enzymes are produced, we stop the germination process, reduce grain moisture, and start the kilning process [31]. The moisture levels go down to 10% using air ventilation with a temperature varying from 40 °C to 65 °C. We must lower the moisture level again to 6% to guarantee an extended storage capacity. This phase is called kilning. To do so, we can maintain the temperature near 65 °C for 10 h. or bump it to 250 °C for 36 h. and induce the Maillard reaction on the sugar grains [28].

The high temperature enhances this reaction. It causes the interaction between the carbonyl group to reduce sugar with the nucleophilic centre of the amino acids: the amino group. The high molecular diversities that sugars and amino acids can provide cause the

generation of hundreds of possible products of the reaction, counting also the isomers that can be produced by arginine, lysine, asparagine, and glutamine (they have two amine groups). The first interactions between the two active groups cause the formation of a glycosylamine with a substituent that depends on the R chain of the amino acids. This intermediate is unstable and is rapidly transformed in ketosamines by the Amadori rearrangement, an isomerization reaction passing through a Schiff Base intermediate (**Figure 7**).

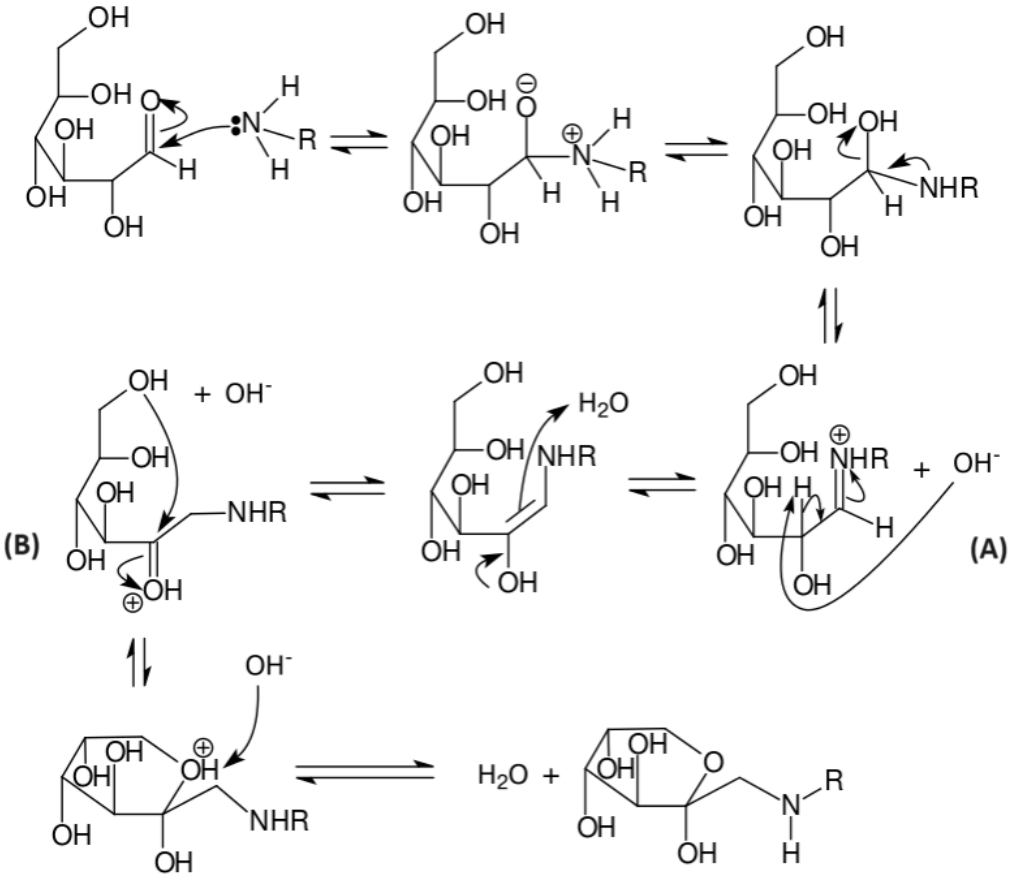


Figure 7. Maillard reaction between a monosaccharides in the aldehydic form and an aminoacids. (a) Schiff base and (b) ketose form. The figure has been taken from [32].

The Maillard products are colour and flavour compounds, and their presence can be either desired or unwanted depending on the type of malt we want to produce. For example, in the case of dark malt, Maillard products are essential for the desired organoleptic properties of the malt. The opposite can be sad for the pale malt used for blonde beers and most other types of malt. To reduce the amount of Maillard products, it is essential to follow these measures:

- Choose a barley that genetically produces a low concentration of protein
- Maintaining a low temperature during the steeping
- Reduce the concentration of oxygen after the third day of germination
- Start the drying procedure between 35-50 °C

Another undesired product that develops an unpleasant flavour in beer that starts its production during the maltation is dimethyl sulphide (DMS). This molecule is associated with vegetable-like or cabbage flavours and is always considered an off-flavour. The DMS is produced for its majority, starting from S-methyl methionine (SMM), which is formed during germination. Since this compound is thermos-labile during germination, it splits into the active precursor DMS-P and free DMS. When heated, the DMS-P frees DMS. Typically, this reaction can happen during kilning and boiling. However, if some DMS-P remains not converted during the fermentation, the yeast can interact with it and release more DMS. The SMM can also be oxidized in dimethyl sulphoxide (DMSO). Some yeast can then reduce this compound in free DMS and release it in the beer. To reduce the production of DMS during the malting and brewing process, it is essential to follow these good practices [33]:

- Choose barley that genetically produces a low concentration of DMS-P
- The micro elements of the cultivar terrain are essential, especially the amount of sulfur compounds
- More significant protein breakdown during germination also are correlated with a higher release of DMS-P
- Increasing the duration of germination brings a significant increase in DMS-P
- An increase in the initial temperature during the drying phase reduces the DMS content

The DMS-P content, on average, is around 4 ppm in dry weight for pale malts.

2.1.4 Special malts

The majority of beers produced worldwide use pale malt to be produced; therefore, most maltings produce only large batches of pale malt. However, more and more beer styles were invented and started to be trending. The differences in taste, colour, aroma and foam primarily depend on the type of malt used. The malts that are not considered pale are named generically special malts and are produced by special maltings that have specialized in producing small batches and have the equipment needed for these unique productions. The most important economically wise special malt are:

- *Dark malt*

  It promotes all the conditions that lead to Maillard products formation as much as possible. Usually, they use barley with high protein content with intense germination temperatures around 20 °C, and after kilning, they rest at 100-105 °C for 4 h. to further enhance the Maillard product formation.
- *Vienna malt*:

  This barley malt is typically used to improve palate fullness and intensify the golden colour of over-pale malts.
- *Caramel malt*:

One of the most used special malts gives the beer an increased depth of colour and aroma, emphasizing the malty flavour of the beer. For its production, it is essential to enhance a tremendous enzymatic breakdown causing the release of low molecular weight nitrogen compounds and sugars. Depending on the intensity of the caramelization process during the roasting phase, we can create different types of caramel malt with more intense colour and flavour.

- *Smoked malt*:

  This unique malt is used to produce smoked beer. Before the kilning, the condensing clouds of smoke are fed to the malt. This process gives intense scratchy flavours that can change significantly depending on the nature of the woo or peat burnt to produce the smoke.

- *Roasted malt*:

  It is generally used in a small percentage, between 0.5 and 2% on the grist, to obtain a dark colour and roasted flavour. The production of this malt follows the classic steps of the pale malt until the kilning. At the end, the malt is transferred in a roasting drum that treats the malt for 1-1.5 h. at a temperature between 180 and 220 °C. This high-temperature treatment induces the formation of caramel substances and roasting-associated compounds such as assamars.

2.2 Wort Production

The crucial phase of beer production is the alcoholic fermentation, which is carried out by the yeast with the sugars contained in the wort. In order to create all the necessary conditions, the insoluble components contained in the malt must be released by the enzymes formed during the maltation. The wort is produced in the brewhouse. The malt is transported from its storage location to the grist mill and milled to a suitable size. After this operation, the milled malt is transported to the mashing tank, where water is added. The enzymatic digestion of the starch takes place in this vessel. When the wort is separated, the insoluble substances are removed and the spent grains are operated in the lautering vessel. The wort is then boiled in the kettle, and the hops and spices are also added at this stage. Boiling takes more than an hour to sterilise the wort. Finally, the trub (precipitated organic particles) is removed from the wort using a whirlpool or centrifuge. When the temperature of the wort drops, the yeast can be inoculated and fermentation can begin.

2.2.1 Malt milling

In order to optimise the activity of the enzymes produced, it is essential to break open the husk and expose the stored starch of the endosperm. This first process is the milling and the malt obtained is called grist. During this mechanical process, the size of the resulting grain fragments must be determined as precisely as possible, as the husk must be treated gently

since it will form the filtration cake during lautering. There are many different types of grinders in the brewing industry. The most common are dry mill, wet mill, hammer mill and dispersion mill.

The malt delivered to the brewery is generally stocked in large, not aerated silos with controlled temperature and humidity inside. Before processing the malt, removing all the impurities present, such as dust, stones or iron objects that could damage the mill if they enter, is essential. Dust is inevitably produced during dry milling and is explosive if it reaches specific concentrations, so it is essential to prevent this accident, removing every possible iron object that can create a spark and ignite the explosion. For this reason, magnets are located before the mill to prevent accidents [34].

It is crucial to have a monitoring plan for the mill since it operates a mechanical treatment, and all the parts can get worn out. However, a proper evaluation can only be done using a dry or conditioner mill. For the others, only an empirical hand evaluation can be made. For accurate analysis, the most used instrument is the *Pfungstadter plansifter*. It is composed of five sieves that separate the grist into six fractions depending on the dimension of the fragments. For statistical reasons, the result of these separations can be associated with a Gaussian. The more the Gaussian peaks in the middle, the better for the brewing process. The desired granulometry of the grist can change depending on many factors. For example, malts with high water content and poorly modified during the malting, if used in a system with intense mashing to balance a poor brewhouse yield, are better if milled more finely than usual.

2.2.2 Mashing

It is the key phase of wort production because the nutrients needed for the fermentation are solubilized thanks to the activity of the endogenous enzymes of the malt. Only the soluble substances can be carried out to the beer bottle. It is, therefore, necessary to convert all the insoluble material of the grist. The soluble substances we want to recover are sugars, dextrins and specific proteins or free amino compounds. In the insoluble fraction remains cellulose and high molecular weight proteins. From an economic perspective, converting as much insoluble material as possible reduces the raw material used. However, reaching a good quality of the extract with the desired free compounds is crucial. This result depends on the enzyme activity that breaks down the chemical bonds of the macrostructures described in sections 1.1.1 and 1.1.2. The activity of the enzymes depends primarily on the temperature. Each enzyme has a specific optimal temperature for catalysis. The enzyme's activity increases slowly, reaching its optimal temperature. When this temperature passes, the enzyme's inactivation is relatively rapid due to the unfolding of the protein as described in **Figure 8**. Since the enzyme's three-dimensional structure is also determined by the ionic force of the solvent in which the enzyme is also, the pH affects the enzyme activity[35]. The

optimal would be to start the mash at a 5.2-5.4 pH and to produce a wort of 5.0-5.2 pH [36]. These pH ranges favour enzymatic activity, although each has slightly different optimum pH levels. Studies have reported that the enzyme activity is not affected too much at lower pH but can decrease rapidly is the pH becomes more basic (**Table 7**). Working at a pH higher than 5.6 leads to dull malt flavour and lacks definition because it negatively affects hops.

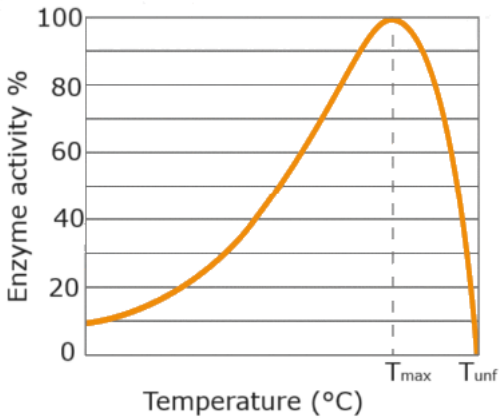


Figure 8. Correlation between enzyme activity and temperature. Tmax= optimal temperature Tunf= Unfolding temperature. This graph has been taken from [15].

Table 7. α-amylase activity at different pH values [37].

pH	Activity (%)
4.8	98
5.0	99
5.2	100
5.4	95
5.8	85
6.2	65

During the mashing phase, many enzymes are activated and start the hydrolytic digestion. The most important are starch, β-glucan, proteins and converting triglycerides into fatty acids. Alcohol is the most essential component of beer and forms thanks to sugars' fermentation. Therefore, deleting the starch as much as possible in fermentable sugars, primarily maltose, is essential. For economic reasons, it is vital to free as much maltose as possible and not to leave in the wort any dextrins; however, those compounds can be desired for the aspect and mouthfeel of the beer because they give haziness and viscosity.

The starch degradation can be divided into three stages: gelatinization, liquefaction and saccharification.

When the hot water is introduced into the mashing tank, it is incorporated into the starch. This rapid intake, helped by the fact that the grains are in the grist form, causes a volume expansion of the starch. When the inner pressure of the starch became higher than the elastic resistance of the starch granules membrane, the starch burst out. The starch is losing its

crystalline ordered state and become amorphous. This change in the disposition of the starch drastically increases the viscosity of the solution. **Figure 9** showcase the change of viscosity during the mashing. The temperature required for gelatinisation is between 59 and 65 °C, depending on the type of barley. Other cereals have different gelatinisation temperatures. Rice, for example, gelatinises at higher temperatures, between 75 and 85 °C. This difference must be considered when using different cereals since gelatinization is an essential precondition for a complete breakdown of the starch. If not operated correctly at the end of the mashing phase, this can result in low extraction yields, lower final fermentation, difficulties with filtration and increased turbidity. No enzymes are involved in gelatinisation, so no metabolic degradation takes place. Nevertheless, only the gelatinised starch can be easily attacked by the enzymes. If some of the starch remains ungelatinised, it takes days for it to be completely hydrolysed, as in normal germination.

At this point, the liquefaction. The amylose and amylopectin chains start to be broken down into smaller chains by α-amylase. This enzyme can break the 1→4 glycosidic bond randomly, creating multiple dextrins. This activity causes a rapid reduction of the viscosity of the liquid. β-amylases work slowly in this phase because they can only attack the non-reducing ends. The possible substrate for β-amylases increases each time that α-amylase breaks a random bond. During this event, a single amylose chain is broken in two, doubling the possible interaction sites for β-amylases.

Progressively, α-amylase creates more dextrin chains attacked by β-amylases, releasing maltose molecules from the non-reducing end starting the saccharification phase. Since not all the dextrin chains produced have an odd number of glucose molecules, β-amylases can release some glucose and maltotriose sugar. In all cases, this enzyme class cannot break down the 1→6 glycosidic bonds, so there are always 2 to 3 glucose residues left attached to a glucose residue linked with a 1→6 bond. During the germination phase, the aleurone layer produces an enzyme called dextrinase, capable of breaking both the 1→4 and the 1→6 glycosidic bond. However, this enzyme reaches its activity peak an 50/60 °C, so it is already inactivated after gelatinization.

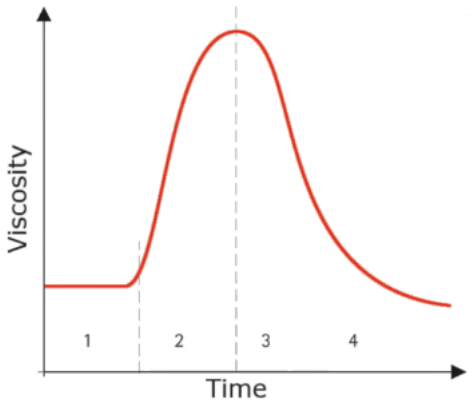


Figure 9. Change of liquid viscosity during mashing. (1) ungelatinised starch; (2) gelatinisation; (3) liquefaction; (4) saccharification.

To sum up, we can say that α-amylase breaks down the long starch chain, releasing smaller dextrin and freeing more non-reducing ends for the β-amylases. This enzyme reaches is peak activity between 70 and 74 °C and denaturates at 80 °C, while the optimum pH is between 5.6 and 5.8. The other enzyme of the family, the β-amylase, releases glucose and maltose molecules from the non-reducing ends of glucose 1→4 chains. It acts optimally at 5.4 pH and 62 °C but is very thermosensitive. At 65 °C is already inactivated. The breakdown of the starch polymer must be monitored as closely as possible because undegraded starch and larger dextrin increase the final haziness.

The iodine test is the most common test used in brewery to evaluate starch degradation. To perform it, only a 0.2N tincture of iodine solution is needed (iodine and potassium iodide in an ethanol solution). The molecular Iodine I_2 has a low solubility, so it is used as potassium salt. When it dissociates, it frees triiodide ions (I_3^-), which can create a complex with the amylose chains that fold on the ions, creating an helix form that traps the triiodide ions inside. In this form, the iodide changes its absorption spectrum from the free form, creating a perceivable colour shift in the visible range (**Figure 10**). So, the free iodide is brown, while the complex form is perceived as violet-blue [38]. So it is possible to use this phenomenon to evaluate the degradation of the starch because if the colour of the solution remains brown it means that there are no long chain of amylose capable of creating the complex and shifting the colour to violet-blue. In brewing, it is suggested to make this test on each production at the end of the mashing phase to validate the step's success. It is essential to use a wort at room temperature. Otherwise, the thermal energy can cause the dissociation of the amylose-iodide ion complex and a false evaluation of the test. If the Iodine solution is added when the wort is still hot, it is sufficient to wait and see if decreasing the temperature leads to some complex formation[39].

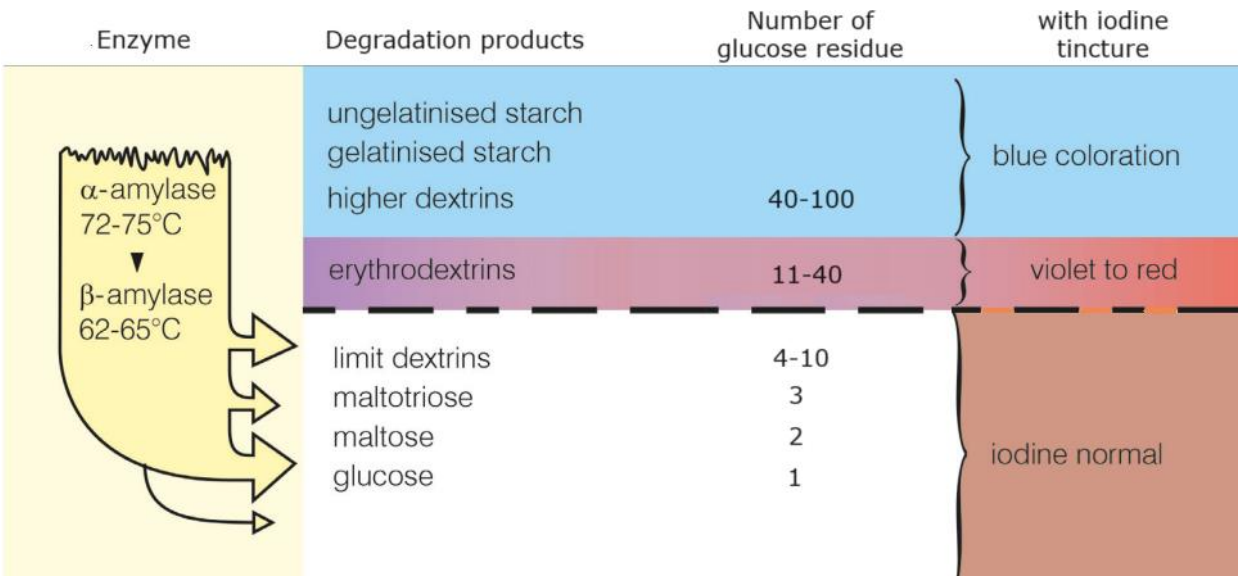


Figure 10. Scheme of iodine interaction with amylose chains depending on their length.

Generally, at the end of mashing, the sugars remaining in the solution are maltose (65.5%), maltotriose (17.5%) and the remaining 17% is divided equally between sucrose, glucose and fructose [40]. These sugars can all be fermented by top-fermenting yeast, starting from the monosaccharides and proceeding with the more complex carbohydrates. The percentage of fermentable sugars in the wort extract determines the attenuation limit of the wort produced. This value establishes the maximum alcohol content the beer can have at the end of the wort fermentation. The respective proportion between all the different categories of fermentable and unfermentable sugars is determined by the enzyme activity during the mashing. So, the final composition of sugar depends on how the mashing procedure is operated. The most critical parameters to control are the mash's temperature, pH, time and concentration.

The enzymes exhibit unstable behaviour even if a stable temperature is maintained during the mashing phase. In the first 10 to 20 min., the enzyme reaches its activity peak, remaining for approximately 10 min.. After that, the activity of the enzyme decreases rapidly, slows down after 40 min., but never stops as shown in **Figure 11**. In general, it can therefore be said that the concentration of the extract solution increases with increasing mashing time, although the rate of increase is always slower. Moreover, the percentage of the different sugars will shift towards smaller carbohydrates, mainly maltose. The mash concentration also influences the enzymes' thermal stability. In a thinner mash, more extract goes into the solution, but the enzymes are more susceptible to thermal inactivation. In comparison, thicker mashes tend to protect the enzyme thanks to its colloid form.

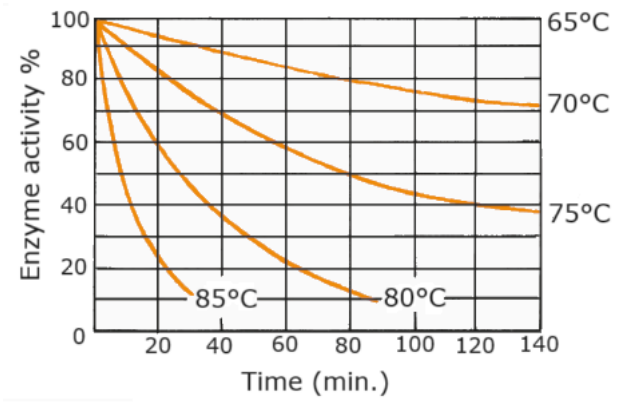


Figure 11. Dependence of α-amylases enzyme activity over time at different temperatures. The graph has been taken from [15].

During mashing, starch is not the only polymer that undergoes some structural changes; β-glucans follow the same path. Their degradation, as mentioned in section 2.1.2 (malt germination), starts during the cytolysis of the maltation. Good quality malt has already degraded the high molecular weight β-glucans to prevent lautering difficulties and increase viscosity. The remaining β-glucans chains are crosslinked one another by hydrogen bonds creating a structure called soluble fringed micelles. Some micelles may interact with one other, with some of the chains that form them while remaining linked to the protein cell walls. During gelatinization, the starch exits from the granules and the β-glucan chains start to bind with the protein released. At this moment, the endo-β-1→4-glucanase starts its degradation on the interlinked chains. Its optimum temperature is between 40 and 45 °C. So, if a higher degradation of β-glucans is desired, it is essential to rest a long time at this temperature, leaving only soluble low molecular weight chains of β-glucan. This stop is done especially when the malt quality is not high because it has more β-glucans than expected or if the maltation has been suboptimal. It is essential to do this degradation step as soon as the mash starts because when the gelatinization temperature is reached, the activity of endo-β-1→4-glucanase is already too low to make some difference.

Also, proteins undergo degradation during mashing, but since these polymers are far more complex than starch and β-glucan, different proteinases must work together to complete a total degradation. Some protease is active at 45-50 °C, creating low molecular weight proteins like peptides and free amino acids (FAN). The active proteases at 60-70 °C release high molecular weight degradation products, which are important for the foam stability (10-40kDa) and the body of the beer and tend to increase haze. A suitable concentration (20-22 mg/100mL) of amino acids is essential for the growth and correct fermentation of the yeasts. If lower concentrations are reached, a reduction in the propagation and a consequent delay in the fermentation and maturation and, finally, some green bouquet undesired flavour is expected. If a high-quality malt is used, it has already been modified, and the desired concentration of FAN is already reached, so it is possible to

skip the 45-50 °C step nearly altogether. Because we already have the FAN needed for the fermentation, we want to produce high molecular weight protein to increase the beer's foam stability and head quality. If this is not the case and the malt used has been poorly modified, releasing the right amount of FAN is essential, even if it means reducing the foam stability.

In the end, the mashing phase consists of activating the desired enzymes that degrade the substrates and release the desired components in the wort. **Table 8** and **Figure 12** summarise the enzyme range and respective activity.

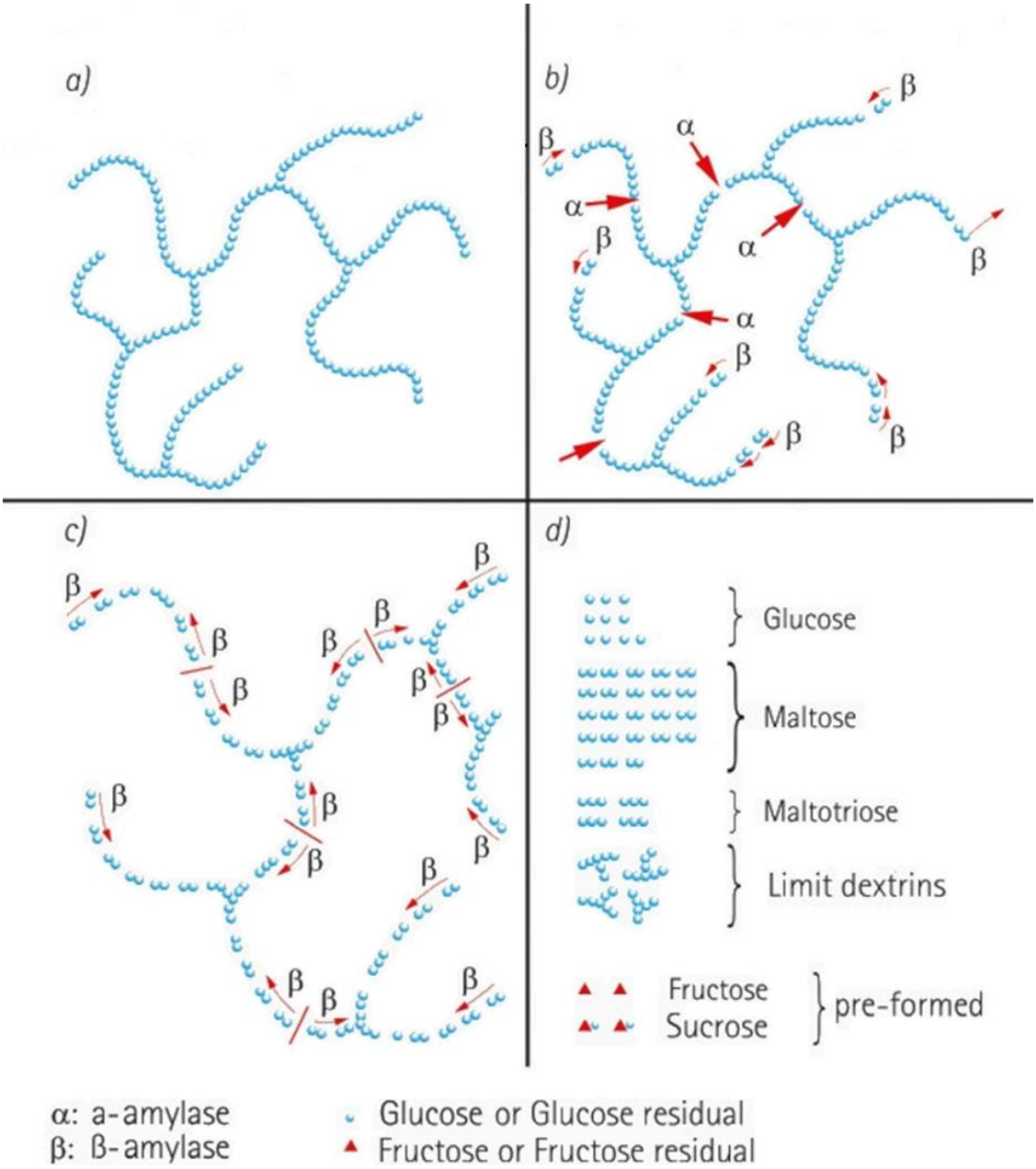


Figure 12. Starch degradation during mashing. a) amylopectin structure prior digestion; b) liquefaction step; c) saccharification step; d) free carbohydrates found in the final wort. This scheme has been taken from [15].

Table 8. Different enzyme activity promoted at the various temperature ranges.

Temperature (°C)	Effects promoted
45-55	β-glucanase activity
45-55	Proteinase and peptidase start to degrade proteins, releasing FANs.
55-60	The activity of β-glucanase ceases, but the proteinase still works, and the gelatinization of barley starts.
60-65	α-amylase attacks the starch, starting the dextrin release.
65-70	β-amylase activity leads to the breakdown of dextrins into maltose.
70-72	The formation of glycoproteins leads to the stability and texture qualities of beer foam.

One of the biggest fears for a brewmaster is oxidation reaction since they create unpleasant flavours associated with old beer. Since all the components of atmospheric air are soluble, the O_2 molecules found in the atmosphere can solubilize in the wort and then oxidate some compounds. This phenomenon must be blocked as much as possible in all the production steps, and the mashing is no exception. Since the solubility of a gas in a liquid solvent depends on the temperature, pressure and exchange surface. The result of oxidation is a darker colour on the wort and the beer and some unpleasant flavour like wet-dog and reduced stability and intensity of the desired flavours. Oxidation in this phase is typically caused by not de-gassed water, the introduction of the wort from above or a vortex formation caused by a too vigorous agitation or over pumping.

2.2.3 Lautering

At the end of the mashing phase, the mash consists of a liquid part, the wort, and some undissolved substances known as brewery spent grains (BSGs). The remaining solids consist mainly of the husk of the grain and all substances that do not dissolve and have precipitated. As only the wort is used for fermentation, it is important to separate the solution from the spent grains as much as possible. This filtration process is called lautering and is operated in a special lautering tun with a perforated false bottom. The modern lautering tun also has a rotational system called the ranke unit. This unit comprises an even number of cutting units that penetrate the BSGs cake during the rotation of the ranke unit. The knives are explicitly shaped, allowing the spent grains to move fastest without disrupting the layering. Is it possible to change the ranke unit's height modifying how close to the false bottom the knives can reach.

The height is generally controlled by software that changes the ranke unit's position depending on the wort-filtered turbidity. If the turbidity of the wort increases, the ranke is

raised, creating a more complex pathway for the wort, making the filtered solution cleaner. It is essential to lose as little weight as possible to reduce food waste. During the wort filtration, the BSGs operate as the filter material, creating a filtering cake on the bottom of the lauter tun. This process is divided into two phases; in the first one, we let the wort be filtered for gravimetry and simply drain off the BSGs. The liquid collected in the first step is called the first wort. At the end of this phase, the latter still contains extract, so it is essential to recover it. The second phase, sparging, in which fresh hot water is poured from the top of the lautering tank to wash out the extract, remained imbued in the BSGs. This operation gradually dilutes the wort; the liquid recovered in this phase is called the second wort. To reach the desired concentration of sugars at the end of the lautering procedures, the first wort must contain at least 4-6% more extract than the beer we want to produce. Since the extract retained is washed out by hot water, the extract content decreases rapidly at first, and then the slope decreases.

The amount of sparging water used depends significantly on the concentration of the first wort. As described in **Table 9**, the thicker the mash produced, the more sparging water must be used to wash the BSGs correctly.

Table 9. Differences in water ratio between first wort and sparging water depending on the extract concentration.

Extract% (w/w) in the first wort	**The ratio between first wort and sparging water**
14	1 : 0.7
16	1 : 1
18	1 : 1.2
20	1 : 1.5
22	1 : 1.9

The higher the amount of sparging water passes through the grains, the higher the extraction yield of the mashing will be. Although more water is used, more will need to be evaporated during boiling to reach the desired extraction concentration. So, choosing the right amount of sparging has to consider both the extraction time and yield and the energy cost of the evaporation of the water in access. Since the filtration speed is opposite to the viscosity of the filtered liquid, it would be faster using water at 100 °C. Unfortunately, the late saccharification would be blocked at this temperature since the β-amylases become deactivated above 80 °C.

The turbidity of the filtered solution can change significantly depending on the quality of the cereal used and the specifics of the lautering tun. The haziness in this production phase has to be associated with the presence of fatty acids and protein breakdown. The yeast requires those substances during cellular growth to synthesize the cell membrane. For this

reason, worts with higher turbidity have shorter fermentation periods, leading to a more fresh and fulfilling aroma in the final product. The sparging phase can be done continuously or divided into separated sparges. Of course, sparging becomes faster if done continuously, but the extraction yield is higher if it is divided into two or three steps. It happens because, with a time rest, the sparging water has more time to penetrate inside the BSGs and extract the desired contents.

The sparging is performed until the desired concentration is reached in the boiling kettle, and usually, the extract concentration of the final drops of the second wort is around 4%. Some breweries continue the sparging, send the remaining water in the mashing tank, and use the recovered water with some sugars from the previous batch for the new one. When the lautering ends, the BSGs must be removed by the bottom, the knives are raised, and a large valve opens to let the BSGs exit the lauter tun. All this procedure, from the charging of the tun to the emptying, can take up to 2 h. if the mash needs a long sparging. In **Figure 13** is represented the whole lautering process enlightening the how the characteristics of the wort exiting the lauter tun changes during the process and how the ranke unit should be operated to reach high extraction yields.

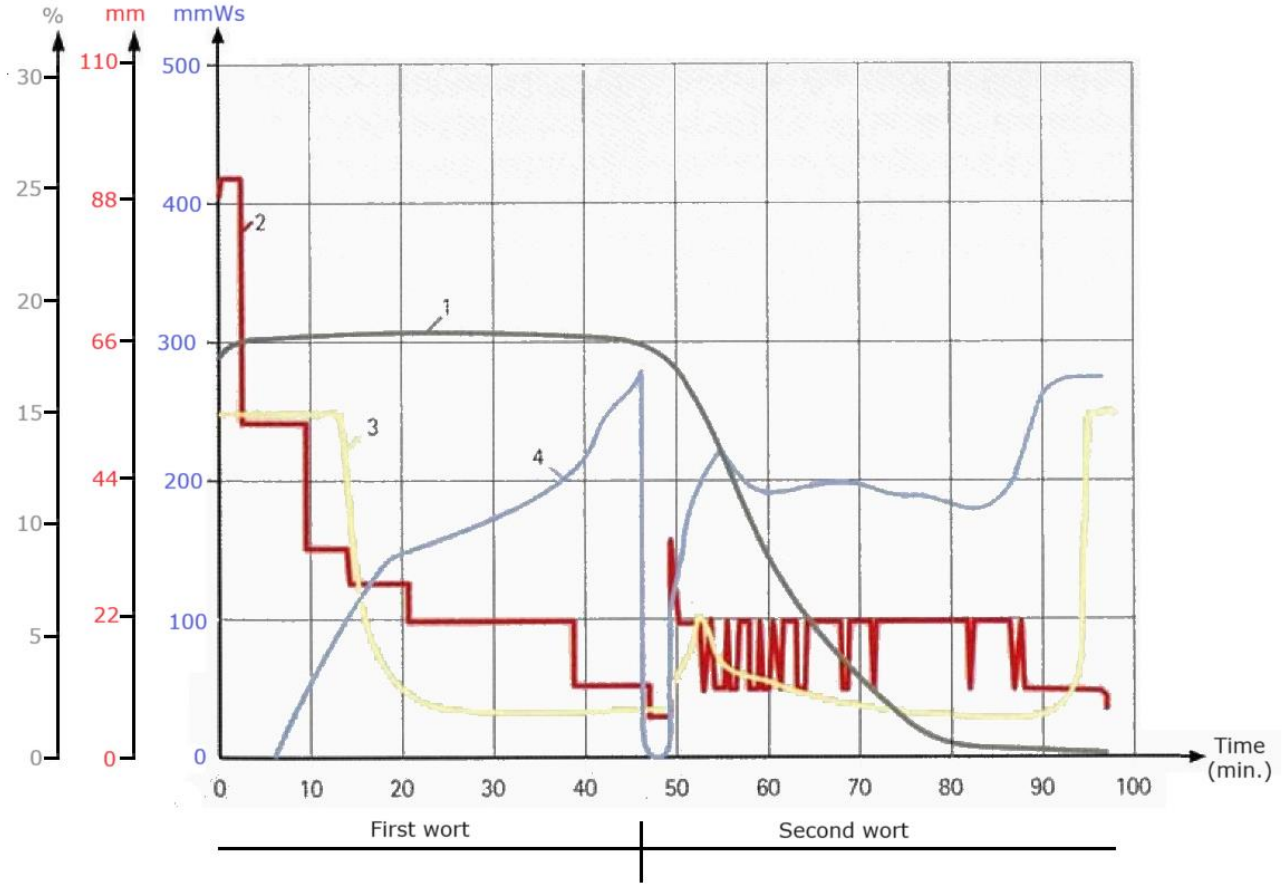


Figure 13. Variation of the most important parameters during lautering. 1) extract concentration in discharge from the lautering tun; 2) height of the knives; 3) turbidity of in EBC; 4) pressure in the water column. The graph has been taken from [15].

2.2.4 Wort boiling

After the wort lautering, it gets boiled for 60 min.. The primary purpose is to sterilize the wort; in this way, when the yeasts are added, no other microorganism competes in the environment. However, heating the kettle until the boiling point will also cause some reaction. This operation is done inside a specific boiling kettle equipped with all the sensors and features needed for the operation. In this production step, the hops are introduced for the first time in combination with the desired spices we want to use to flavour the beer. The spices are recommended in this phase because the thermal energy introduced in the boiling kettle will increase the mass transfer from the vegetal matrix (spices used) to the solvent (the wort). Moreover, it is impossible to use sterile spices, so introducing such ingredients after the boiling phase could cause significant contamination problems.

During this process, several critical processes occur:

- Extraction and isomerization of hops α and β acids.
- Precipitation of proteins and formation of polyphenols-proteins complexes
- Wort concentration due to water evaporation
- Wort sterilization
- Inactivation of all the remaining enzymes
- Thermal exposure of the wort causes the Maillard reaction
- Lowering the pH of the wort
- Evaporation of undesired aromas

As described in Chapter 1.2, hop's important components are the resins, the EOs and the polyphenols. The α-acids are the most crucial compound family for their bittering capacity. These compounds are completely insoluble in water. However, those compounds can undergo a spontaneous isomerization reaction that changes their chemical structure from α-acids into iso-α-acids. In this form, the compounds are soluble in water; unfortunately, this reaction will occur very slowly at room temperature. However, the isomerisation becomes more frequent when more energy is introduced into the system. So, during the boiling phase, the hops are introduced to extract and solubilize the desired bittering compounds. The rate of conversion of iso compounds depends on many factors. The most relevant is the duration of the hops boiling. Most α-acids are isomerized in the first 15 min. of boiling before the isomerisation rate decreases.

To isomerize all the α-acids, one hour of boiling is suggested. Also, the pH plays a crucial role in the isomerization rate. The higher the pH, the higher the isomerization rate (above a pH of 5.4). However, it has been shown that the bitterness obtained results more balanced and finer at lower pH. Also, the dimension of the hop fragments is essential to evaluate the extraction rate. Hops milled finer will have a higher extraction yield. The concentration of isomerized resins is used to evaluate the beer's bitterness. IBU (International Bitterness Unit)

is the reference scale equal to the beer's part per million iso-α-acids [41]. The EOs of the hops are mostly lost during the boiling phase since they are volatile compounds even at room temperature. Only the hops introduced in the last minutes of boiling or during the whirlpool will give some aroma to the beer.

All the proteins that remained soluble during the previous steps of wort production start the denaturation. Due to the breaks of the non-covalent interactions that create the secondary, tertiary, and quaternary structures, the proteins generally precipitate, lowering the concentration of high molecular nitrogen compounds in the wort. During the coagulation, the amino acids that form the proteins can bond with the hops polyphenols, accelerating the precipitation of both compounds. This phenomenon also includes all the free enzymes that have remained active at the end of mashing and lautering. So, at the end of the boiling, the saccharification must be completed because the remaining starch will not be digested.

The most direct consequence of prolonged boiling is the water evaporation that causes an enrichment of the wort. Modern breweries want to evaporate around 4% of the total water transferred in the boiling kettle, which is very low compared to the 10-15% of the old days. This change has been possible thanks to the increase in technical skills and the quality of analytical measurement. Of course, water evaporating is expensive from an energy perspective, so it is vital to reduce as much as possible this waste.

The thermal treatment induces the formation of many Maillard products (described in subsection 2.1.3) and Strecker aldehydes. These reactions altered the wort colour; this change is caused by many factors that occur simultaneously. The most prominent one is the Maillard reaction described in the malting process; the wort contains a high concentration of free monosaccharides and amino acids after the mashing degradation done by the enzymes. The high temperature also induces the oxidation of the polyphenols, also known as tannins, extracted from the hops and the grain's husks. Finally, also the caramelization reaction can occur. The reactions produce hundreds of chemical compounds like the furans family, responsible for the classic nuts flavour associated with caramel. The formation of caramelan and caramelen via dehydration of respectively 2 and 3 molecules of maltose generate the brownish compounds that characterize the caramel texture [42].

During the boiling, the wort pH tends to decrease due to water evaporation and the introduction of the hops that contribute with some acid compounds. At the start of the boiling phase, we have a pH around 5.6-5.5 and finish around 5.4. Many processes work better and faster at lower pH. For example, at lower pH, the colour of the wort changes less, and a more refined and clean taste of the hops is maintained; finally, spoiling microorganisms tend to prefer higher pH. It is possible to artificially modify the pH by introducing some weak acids, such as lactic acid, to reach the desired pH at the end of the boiling phase.

The wort contains many volatile aromatic compounds. The boiling phase causes a net loss of these compounds. However, this is a desired loss since most of them negatively affect the overall organoleptic properties of the beer. The compounds we are talking about are primarily degradation products such as pentanal, hexanal and heptanal (all aldehydes derived from the degradation of fatty acids), Maillard products, and the remaining DMS. The boiling point of these substances is lower than 100 °C, so their concentration is halved in less than 15 min. of boiling time. The most significant component we want to remove flavour-wise is the DMS, which can leave a vegetable-like aroma in the beer. During the boiling, the desire is to direct towards thermal splitting of the DMS precursor and then to remove the DMS formed. As mentioned in the kilning subsection 2.1.3, most of the DMS-P and DMS have to be removed thanks to a correct maltation of barley. The longer the boiling goes on, the less DMS will remain in wort as shown in **Table 10**.

Table 10. Variation of DMS and DMS-P during the different steps of production.

Time point	No. of samples	DMS (µg/l)	DMS-P (µg/l)
Unboiled wort	12	293	167
Start of boiling	8	91	168
Cast wort	104	25	57
Pitched wort	28	49	23
Beer	90	40	16

At the end of the boiling process which can last between 45 min. and 90 min. the wort is sterilised. However, it is necessary to extract the hops matrix that has been introduced; the liquid is brought into the whirlpool vessel, which has a cylindrical form with no internal fittings. A vortex is created to separate the liquid from the vegetal matrix, pumping the wort tangentially. Creating a centripetal force on the debris that pushes them into the bottom cone where they can be easily removed [43].

2.3 Beer production

The discovery of microorganisms as living beings was the first step to understanding the fermentation process that has been so used by humans but never really understood. The works of Louis Pasteur [44] have given the knowledge necessary to understand the process and what could go wrong.

As mentioned in section 1.2.4, dedicated to yeasts, many strains are used in brewery, and each one brings different flavours to the beer. The reason for the diversity created can be found in the metabolic pathways used by each group of yeast that led to different products at the end of the chain.

2.3.1 Fermentation

The fermentation process can be divided into two stages: the initial period in which the yeasts inoculated colonized the new environment and prepared themselves for the nutritional uptake. The second stage is when the sugars are catabolized to ethanol and carbon dioxide.

In the first phase, the yeast cells' activity focuses on preparing the cell wall and absorbing oxygen and nitrogen from the medium. The cell wall must be permeable to the nutrients, otherwise uptake is impossible. Sterol synthesis is the most important factor in this process; like all anabolic activities, it requires energy to be fuelled. The energy required for synthesis comes from the catabolic breakdown of glycogen, the yeast's food reserve. The inoculated population must be in a prosperous state, otherwise it will not have the glycogen reserves necessary to survive the first phase and utilise the nutrients of the medium.

In this phase, it is vital that the wort has been oxygenated, operation done during the whirlpooling, because the synthesis of the sterols needs ATP and oxygen. A wort that is not oxygenated enough will block the sterols production, and yeast will never reach phase two, where there is ethanol production. The synthesis of sterols starts from the pyruvic acid that is transformed by enzymes into mevalonic acid; this acid is condensed, consuming ATP to produce Squalene. This sterol undergoes a sequence of oxidations and other reactions leading to the production of zymosterol, ergosterol, and lanosterol, which are the ones that will change the cell wall permeability. If the cell has low levels of mevalonic acid, it can use the fatty acids derived from the wort, especially the oleic and linoleic acids [37].

The glycogen is rapidly depleted during the sterol synthesis. However, when the wort constituents start to enter the cells, the level begins to increase and remains high until the medium starts to lack nutrients, and the cell uses its reserves to survive.

When the cell wall is ready to start the sugar intake, it prioritises glucose and fructose because they are the most accessible carbohydrates to assimilate. The membrane enzymes responsible for the uptake of bigger sugars like sucrose, maltose or maltotriose have a high sensitivity to the glucose concentration in the wort, and until it does not reach a certain threshold, the permeases are blocked. This process is called the glucose effect [45].

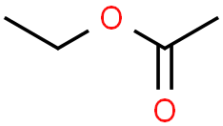
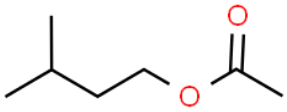
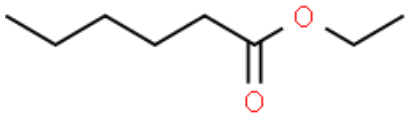
At this point, the cell starts the fermentation process. The sugars are decomposed via the glycolysis in pyruvic acid, which, in the absence of oxygen, undergoes the fermentation and not the respiratory chain to recharge the cell of the NAD^+ consumed during the glycolysis [45]. The pyruvic acid undergoes a decarboxylation done by the pyruvate decarboxylase enzyme, which produces the acetaldehyde, which is reduced to ethanol using NADH for the reducing power, bringing the regeneration of NAD^+.

Not all the carbohydrate molecules follow this pathway. Some can diverge to create some valuable compound for the cell sustain. The final products will be less representative than

the ethanol, reaching 1mg/L in the beer. Those molecules are not less relevant because they can have low flavour thresholds and impact the beer's organoleptic properties [46].

The most important products of these secondary pathways lead to the production of esters, ketones, phenol, and glycerol. The esters are produced via the condensation of an alcohol and a carboxylic acid with the release of a water molecule. It has been estimated that there are more than 90 esters in the beer; the relevant ones are ethyl acetate, isoamyl acetate, and ethyl hexanoate (**Table 11**).

Table 11. Ester summary of structure flavours and threshold.

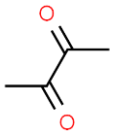
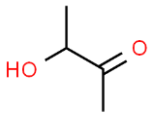
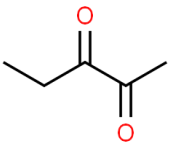
Compound	Structure	Threshold (mg/L)	Flavour
Ethyl acetate		33	Fruity with solvent undertone
Isoamyl acetate		3	Bananas
Ethyl hexanoate		123	Apples

The esters production is not a spontaneous reaction, and it is catalysed by some enzymes produced by the yeasts in the beer. This type of products of the metabolism are a key signature of the yeast strain used; different strains will display an entirely different range of esters. If the yeast has a high growth factor, the production of esters will increase. It is odd to see a boost in esters production when the wort is poorly oxygenated because it is correlated with a slow growth by the yeasts [37].

The other relevant group of compounds are the ketones, which have raised interest in the last decades. The most important representative of this group is diacetyl, but others are also characterised by a very low flavour threshold concentration (**Table 12**). The synthesis of diacetyl starts from the pyruvate, the final product of glycolysis. Two pyruvate molecules are condensed into α-acetolactate, releasing carbon dioxide and the acetolactate synthase. This five-carbon molecule undergoes a decarboxylation catalysed by the acetolactate decarboxylase, which produces the diacetyl.

In the first decades of the 1900s, diacetyl levels in U.S. beers were typically found around the 0.2/0.3 mg/L range, while our days are maintained above 0.05 mg/L [35]. The vanilla tones brought by the diacetyl were considered attractive. However, this molecule is a bit unstable and, at that high level, could turn into unpleasant flavours, so those beers had a short shelf life and do not travel well, essential qualities needed in a globalized world.

Table 12. Ketones summary of structure flavours and threshold.

Compound	Structure	Threshold (mg/L)	Flavour
Diacetyl		0.1-0.15	Buttery and vanilla
Acetoin		1.0	Fruity musty
2,3-Pentanedione		1.0	Honey

2.3.2 Maturation

When the alcoholic fermentation reaches the desired point, and the yeasts have converted the right amount of wort's sugars in ethanol, the fermentation is stopped by lowering the temperature to 2.0/2.5 °C. It is essential to leave the beer for some time to maturate; secondary fermentation can occur in this period, changing the final product's organoleptic properties. For example, white beers must undergo a lactic fermentation that can be done by two bacteria genera: the *Lactobacillus* and the *Pediococcus*. This fermentation produces different molecules that significantly impact the beer texture and flavours like the lactate, glycerol, and 2,3-butanediol [46].

During the fermentation, the yeast that have fermented the wort start to flocculate on the tank's conical bottom, creating a dense sludge. The deposition of the yeast cause a reduction of the haziness of the beer that became clearer and easier to filter. The yeast that have reached the bottom of the fermentor drastically reduce their metabolic activity since the free sugar remaining are very limited and the thickness of the yeast sludge reduces the mass exchange between the liquid wort and the yeast deposit. For this reason, most of the yeast, after a quiescent period, start to die and to lysate, releasing the inner cytoplasm. This release causes a slight increase in the beer's pH thanks to all the protein and amidic compounds released. While the cellular membrane after the lysis remains on the bottom with the undead yeast cells.

At the end of the fermentation, the brewmaster can decide to introduce some hops inside the beer, leaving them for the whole maturation phase. This process is called dry-hopping, and it is used to imbue the aroma and scent flavour of the hops into the beer. The temperature and timing of the introduction of the hops can change from brewery to brewery, depending on the own tradition. Usually, the hops are introduced or at the end of the fermentation when the beer starts to cool down from 22-28 °C to 2-5 °C or when the beer

has reached the final maturation temperature. The hops introduced during the dry-hopping do not increase the bitterness of the final product as the energy of the system is too low for the isomerisation reaction of the resins. However, a prolonged maceration of three to four weeks creates the perfect environment for the extraction and suspension of the EOs in the lupulin glands.

2.3.3 Packaging

Before bottling, the beer or the canning has to be filtered to remove all the trub residues and yeast cells. This process stabilises the taste and gives the product its typical bright and shiny texture, making the product more attractive.

The final adjustment that has to be done to finally produce the product that can be put on the market shelves is the gassing procedure. Each beer wants to reach a precise amount of carbon dioxide dissolved in the beer. The measure used is typically the volume of the gas dissolved in each volume of beer at 1 bar and 25 °C. Two methods can be used to achieve the target: the first one is the beer carbonation using a carbon dioxide gas bottle to introduce the CO_2 quantity desired in the beer. This process is the easiest and gives high control of the result. The other method is the classical one that has been used since the Middle Ages; before introducing the beer in the bottle or the keg, a new batch of fresh yeasts is inoculated in the beer and some sugar. During this process, the beer also gets oxygenated, and the fresh yeasts introduced will be able to metabolize the new sugar down to carbon dioxide. This method is far more viable for homebrewers but is also used in other industries. When using this process, the beer will need some weeks before the yeasts will finish the degradation of the sugar, and the carbon dioxide levels will reach the desired parameter[47].

During the packaging process, it is vital to introduce the least quantitative possible air inside the beer. Otherwise, the oxygen will be transferred from the gas form of the upper bottle to the beer, and when it reaches the liquid, it will be able to oxidize the flavour molecules, altering the organoleptic properties. The fact that the oxygen in the liquid solution is consumed causes an ongoing gas transfer to the liquid. For this reason, the overall taste, flavours, and textures can change when the product leaves the brewery and reaches the consumer [48].

3 BREWERY BY-PRODUCTS

Beer is the most consumed alcoholic beverage worldwide, and its consumption is widespread on all continents. The brewing process described in chapter 2 inevitably produces some by-products, primarily BSGs and spent yeast. In the last decades, the sensibility of recycling and giving new life to waste has grown significantly. This green approach reduces food waste and raw materials if translated into the food industry. The extraction processes that have been described aim always to reach a higher yield to reduce the quantity of raw materials needed.

Moreover, the by-products produced must be disposed, and this operation can be another cost for the brewery. Raising awareness of the overpopulation problem and its correlation to the increase in food requirement and cost cannot be solved by eliminating food processing wastes. However, all the parts of the raw material used have their qualities and possible applications to exploit.

Modern brewing involves several stages, each contributing to the generation of specific by-products, many of which can be repurposed or recycled in an environmentally sustainable manner. Here is a technical description of some essential brewery by-products:

- **Spent Grains**: After mashing, the spent grains are the solid remnants of malted barley and other grains. These grains have had their sugars extracted during mashing. Spent grains are commonly used as animal feed, particularly for livestock such as cattle and pigs. Additionally, they can be processed into products like granola bars or used in the production of biofuels. It comprehends 85-90% of the total by-products generated.
- **Wort Trub**: consists of the solid particles that settle at the bottom of the brewing kettle after boiling and consists of coagulated proteins, hop residue, and other solids. Trub contains proteins, hop compounds, and other organic matter. While excess trub is typically separated from the wort during brewing, it can be repurposed for fertilizers or even as a nutrient source for yeast production. It comprehends 5-10% of brewery by-products.
- **Yeast Slurry**: Yeast slurry is a mixture of yeast and other solids that accumulate at the bottom of fermentation vessels after the completion of the fermentation process. Yeast slurry is a mixture of yeast and other solids that accumulate at the bottom of fermentation vessels after the completion of the fermentation process. It contains live and dead yeast cells, proteins, and other fermentation by-products. This by-product can be repatched into subsequent batches for fermentation. However, the repatched yeast's vitality and metabolic activity will be lower than the previous batch.

Additionally, it is used to produce nutritional supplements and animal feed. It comprehends 5-10% of the total by-products.

- **Brewery Effluent**: The liquid waste generated during all brewing processes. It is composed mainly of the water used for cleaning equipment and cooling. It contains organic matter, cleaning agents, and sometimes residual chemicals. To minimize environmental impact, brewery effluent undergoes treatment processes such as sedimentation, aeration, and microbial digestion before being discharged or reused. Regarding volume, brewery effluent can be significant, depending on the efficiency of water use and wastewater management practices. However, the chemicals' effective concentration is shallow and treatable with depuration systems.
- **Carbon Dioxide**: CO_2 is a by-product of fermentation and is released during brewing. Captured CO_2 can be repurposed for carbonating beer, extending the lifespan of packaged products, or sold for use in other industries. CO_2, while not a solid by-product, is significant and can be captured for various uses. Its proportion is generally smaller in volume but highly relevant regarding value and applications.

Efforts to manage and repurpose brewery by-products align with sustainability goals, promoting resource efficiency and minimizing environmental impact within the brewing industry. Advanced technologies and innovative processes continue to emerge to optimize the utilization of these by-products further.

The by-products created by breweries usually have few disposal issues since they are biodegradable and not environmentally risky. Only the brewing effluents are toxic and must be processed specifically before their release in the environment. The effluent typically contains traces of strong bases like sodium hydroxide or strong acids like nitric acid and also some oxygenating compounds such as peracetic acid. This research decided to focus its attention primarily on abundant by-products that can find applications in the food or pharmaceutical industry since all the partners specialise in those market sectors. For this reason, most of the work has been operated on the BSGs and yeast slurry, which combined comprise 95% of the by-products produced while brewing.

3.1 Brewery spent grains (BSGs)

Brewery spent grains represent the most abundant by-product of the brewing process, roughly 85%. These spent grains, primarily derived from malted barley, undergo a transformative journey from integral brewing ingredients to a rich, fibrous residue with multifaceted applications across various industries. Beer production in 2023 is expected to exceed 1.9 billion hectolitres worldwide, generating 38 million tons of BSGs, with more than 3.5 in Europe alone [49].

The genesis of brewery spent grains lies in the mashing process, a fundamental step in brewing where malted grains, usually barley, release their sugars to form the wort. Once

this crucial extraction of fermentable sugars is complete, the BSGs follow the filtration, and after the sparging, they remain as the solid residue in the lautering tun. These spent grains retain a remarkable composition, showcasing a rich tapestry of fibres, proteins, and residual sugars.

In their post-brewing state, spent grains exhibit distinctive physical and sensorial characteristics, forming a granular and fibrous matrix. The texture is coarse, and the colour, influenced by the type of malt used, ranges from light tan to dark brown. The aroma carries a subtle sweetness, a lingering reminder of the sugars that once permeated these grains during the mashing process. Spent grains possess a grainy texture, a testament to their origins as integral components of the brewing process.: The colour spectrum of spent grains mirrors the diversity of malts employed in brewing, contributing to the visual appeal of this by-product. The slight sweetness in the aroma encapsulates the inherent connection between BSGs and the sugars that defined their initial purpose. However, the sweetness has a light but persistent flavour of flour, cereals, and hearty bread. The journey of brewery spent grains extends far beyond the brewhouse, finding purpose in various industries and applications that champion sustainability and resource efficiency. The most common uses are:

- Animal Feed: Perhaps the most traditional application, spent grains find a second life as a nutritious component in animal feed formulations. Cattle, poultry, and swine benefit from the fibrous content and protein residues within spent grains.
- Biogas Production: Through anaerobic digestion, spent grains contribute to the generation of biogas, primarily methane, supporting on-site energy production and aligning with sustainable brewery practices.
- Composting and Soil Enrichment: Spent grains, when composted, enhance soil fertility by introducing organic matter and essential nutrients. This application aligns with circular economy principles, promoting the cyclical use of resources.

The most common use at the moment is animal feed. Unfortunately, the exploitation also increases the value of the by-product. On average, a tonne of BSGs is sold to the farmers between 25 and 40 €. The low evaluation of this biomass is caused primarily by its logistic problems. When they exit the brewing production, the BSGs are soaked wet, with a humidity between 75% and 90%. This status makes handling for weight reasons and stocking more complicated. The BSGs are recovered after a long thermal treatment, so they have a low microbic activity. However, their rich composition of polysaccharides and protein combined with the high-water content make the BSGs very susceptible to microbial growth and spoilage. For these logistic problems, the BSGs are undersold to local farmers to reduce the complications of product stabilization.

3.1.1 BSGs composition

The biomass remaining at the end of the mashing and lautering comprises the seed coat, the pericarp and the husk layers that protected the endosperm where the starch was stocked. Some traces of un-digested starch can remain on the endosperm or the aleurone layer, but the quantities are irrelevant if the mashing was correctly operated. The BSGs are a heterogeneous matrix when comparing the by-products of different breweries. This lack of consistency is caused by different factors, mainly the wort-producing plant that can have different extraction yields and the cereals used. Using different recipes during mashing will change the composition of the collected by-product. As explained in Chapter 2.1, how the cereals get malted significantly influences the beer produced and the quality of the BSGs produced. If the cereals have not reached the desired diastatic power, the enzymatic digestion during the mashing phase will be frustrated. In this case, the hydrolyzation of the carbohydrates stoked in the starch polymer will not be completed, and more sugars will remain in the BSGs.

These differences are very significant when comparing BSGs from different breweries because they usually will have different operating machines and raw material providers. However, within brewery sampling, BSGs are relatively homogenous since the machines used and the malted cereals are unchanged. **Table 13** presents the variation in the chemical composition of BSGs from different breweries and analysedanalysed by different laboratories.

Table 13. Chemical composition of BSGs evaluated by different research groups expressed in g per 100g of dry material; n.d. not determined.

Component	Kanauchi et al. [50]	Carvalheiro et al. [51]	Mussatto and Roberto [52]	Xiros et al. [53]	Waters et al. [54]	Meneses et al. [55]	Avg ± St. Dev.
Hemicellulose	21.8	29.6	28.4	40	22.2	19.2	26.9 ± 7.6
Cellulose	25.4	21.9	16.8	12	26.0	21.7	20.6 ± 5.4
Starch	n.d.	n.d.	n.d.	2.7	n.d.	n.d.	2.7 ± n.d.
Protein	24	24.6	15.2	14.2	22.1	24.7	20.0 ± 5.0
Lignin	11.9	21.7	27.8	11.5	n.d.	19.4	18.5 ± 6.9
Lipids	10.6	n.d.	n.d.	13	n.d.	n.d.	13.0± n.d.
Ash	2.4	1.2	4.6	3.3	1.1	4.2	2.8 ± 1.5

As seen from this table, the BSG is a lignocellulosic matrix, the more representative components being fibre, cellulose and hemicellulose, protein, and lignin. Lignocellulose is a complex structural material found in the cell walls of plants, providing strength and rigidity. The primary components of lignocellulosic matrices are cellulose, hemicellulose, and lignin.

- **Cellulose**: is a linear polymer of glucose molecules linked together by β-1,4-glycosidic bonds. These long chains of cellulose molecules are organized into bundles called microfibrils. Hydrogen bonds hold together the microfibrils and form a crystalline structure, contributing to the strength and rigidity of the cell wall.
- **Hemicellulose**: Hemicellulose is a branched polymer of various sugar molecules, including glucose, xylose, mannose, and others. Unlike cellulose, hemicellulose is amorphous and forms a matrix around the cellulose microfibrils. It helps bind the cellulose fibres together and provides flexibility to the cell wall.
- **Lignin**: Lignin is a complex polymer composed of phenolic compounds. It fills the spaces between cellulose microfibrils and hemicellulose, acting as a binder and providing rigidity to the plant cell wall. Lignin also makes the cell wall resistant to microbial degradation.

This high content of both fibre and proteins makes the BSG an exciting by-product for reintegration in the food industry and pharmaceutics integrators [56]. The predominant structure is hemicellulose. It is composed primarily of arabinoxylan and can reach up to 40% of the dried mass. Arabinoxylan structure is common in all cereals and grass-type plants. It is composed of a backbone of xylose sugars linked by a β-(1,4) glycosidic bond. However, the residues can be substituted by arabinose residues that can be esterified by ferulic acid afterwards [57]. The arabinoxylan chains can create hydrogen bonds with cellulose and other arabinoxylan chains, but this is not the only interaction they can create. When the ferulic acid is esterified to an arabinose sugar, creating a diferulic acid bridge with another acid attached to another arabinoxylan creates a much stronger interaction and increases the biomass's mechanical resistance [58]. The monosaccharides present in this by-product are primarily glucose, xylose, and arabinose, and there are only traces of rhamnose and galactose. The majority of the glucose is found in the form of cellulose, with only small traces of starch, which is challenging to exploit. The mechanical resistance of the matrix is also caused by a significant presence of lignin, around 10-28% of total dry weight. This component is formed by three monomers: *p*-cumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These three building blocks are linked together creating a branching network structure. It is considered a poly-phenolic macromolecule structure that gives rigidity and integrity to the structure. Nutritionally wise another important component of the BSGs are the proteins. Depending on the characterization made on the BSGs we found an average concentration of 20% of dried mass. The most rappresentative proteins are hordeins, glutelins, globulins and albumins [59].This concentration is quite high compared to other cereal-based food; moreover the quality the BSGs proteins is very high thanks to a high concentration of essential amino acids (around 30%) as can be appreciated in **Table 14**. The BSGs present also a variety of minerals like phosphorous, magnesium calcium and traces of silicon.

Table 14. Amino acid content of BSGs derived from 100% barley malt [54].

Non-essential amino acids	% in BSGs
Histidine	26.27
Glutamic acid	16.59
Aspartic acid	4.81
Valine	4.61
Arginine	4.51
Alaine	4.12
Serine	3.77
Tyrosine	2.57
Glycine	1.74
Asparagine	1.47
Glutamine	0.07

Essential amino acids	% in BSGs
Lysine	14.31
Leucine	6.12
Phenylalanine	4.64
Isoleucine	3.31
Threonine	0.71
Tryptophan	0.14
Methionine	n.d

The BSG is an abundant and valuable by-product of the food industry. The only drawback it faces for a proper exploitationis the microbiological stability. The BSGs come out with a high moisture level, making handling and stocking expensive. For this reason, the animal feed of local farmers has been the primary outlet for the BSGs. Many studies have been conducted on the microorganisms responsible for the spoilage of BSGs. After 30 days at room temperature, many fungal species colonized the by-product. The ones identified are *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, and *Rhizopus* [60].

When collected, the BSGs have been cooked for over 1 hour during the mashing phase, and they can be considered microbiologically stable and within the limits for food use. Therefore, stabilising the by-product as soon as possible is vital to maintain its nutritional characteristics and applications intact. To make the BSG stable, the water concentration must reach 15% or be frozen.

3.1.2 Materials and methods

The BSGs used in this work came from the Baladin Brewery and were used to produce "Nazionale®", a blonde ale that uses only barley malt in the mash. We decided to use this type of BSG because it is one of the most popular beer produced and uses only barley malt in the mash recipe. At the end of the filtration, the BSG separated from the wort is removed from the lauter tun and exits the brewery. The BSGs collected had a moisture level around 90%, and in this state, the microbiological stability was a severe problem, so we decided to treat it immediately with a helicoidal press to reduce the humidity level. We used the Vaslin Veritas 15 (**Figure 14**) press that usually treats grape pomace for the maximum extraction of the grape juice for wine fermentation. The press is composed of a charging tank capable of treating at least 1.5 tonnes of grapes. The charging tank has a cylindrical shape with a diameter of 900 mm and a length of 2300 mm. In both heads, a cap can move towards the middle section of the charging tank, pressing the matrix charged inside. The press follows

a specific protocol divided into alternated pressing and decharging moments. As can be seen by the picture, the charging tank is not completely sealed, and liquids can quickly exit from the numerous splits on the tank. When exiting from the biomass, the water leaves more space for further compression operated by the machine.

For this reason, it is essential to have a proper flow-out pathway. Rotating the charging tank to ensure higher water extraction and the machine automating all these operations are also important. Its pressing cycle can vary from 20 to 40 min., depending on our chosen protocol. At the end of the processing, the moisture level of the BSGs collected was around 70%, and just after this operation, they were shipped to the Agrindustria srl facility for the final processing.

Figure 14. Vaslin Veritas 15 (Vaslin S.r.l.) grape pomace press.

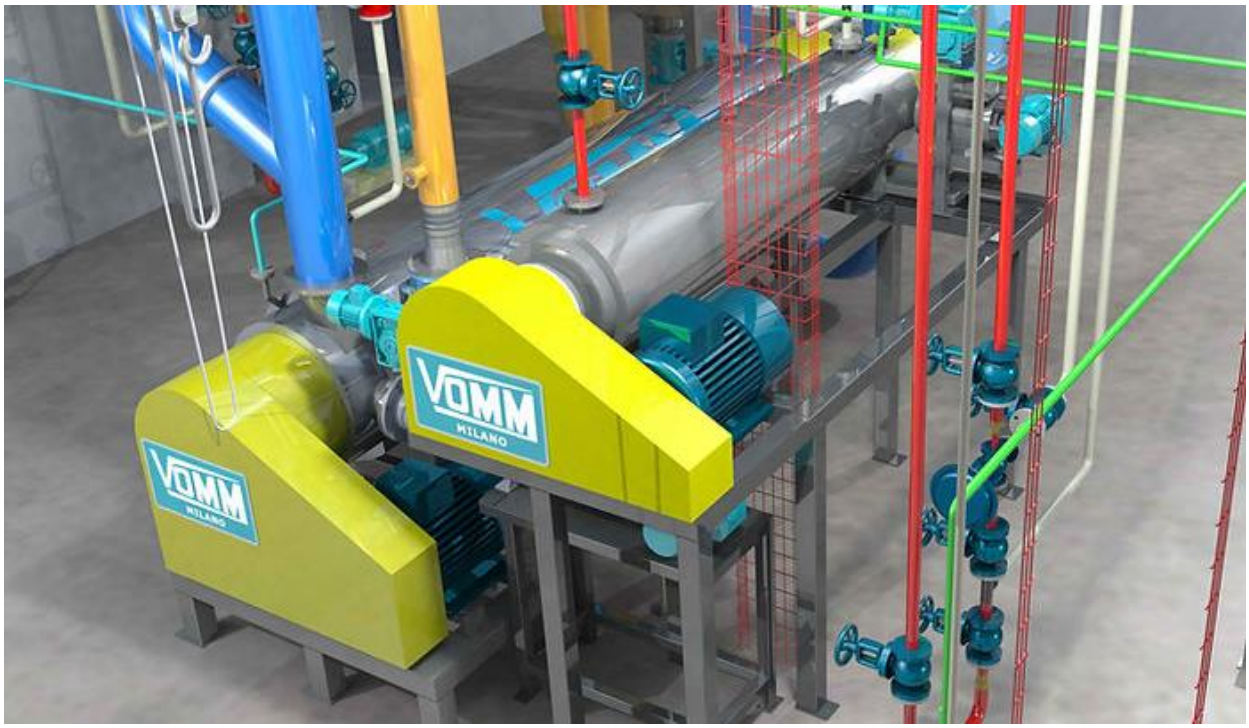
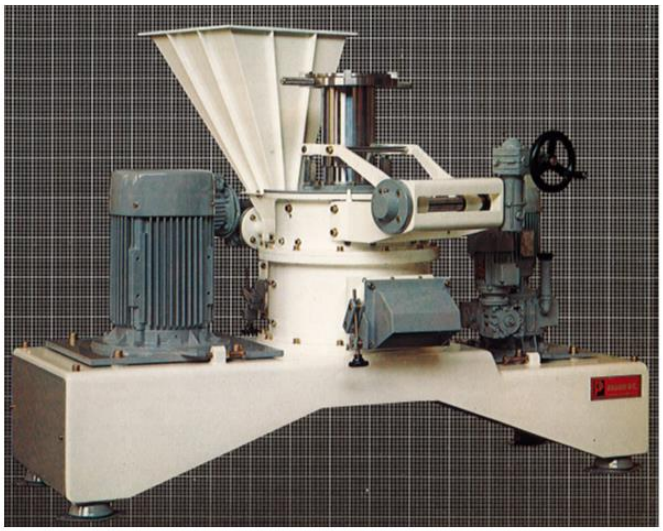


Figure 15. 450 turbodryer (VOMM S.p.a.) used on the BSGs.



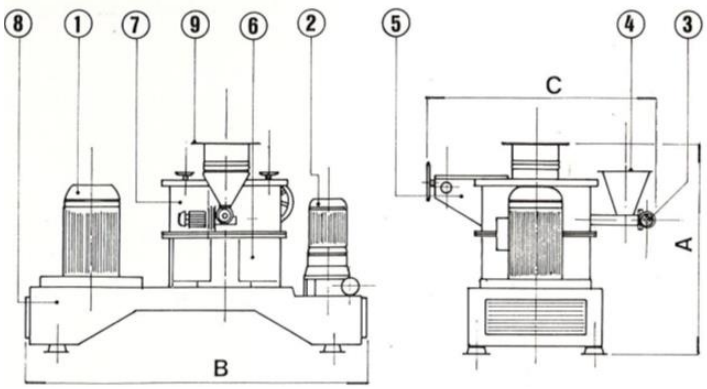


Figure 16. Mach F2 (Flavini S.r.l.) 3D picture and its prospective drowing. 1) mill engine, 2) selector control variator, 3) gearbox screw, 4) product feed screw, 5) lifting lid, 6) mill chamber, 7) screening chamber, 8)mill supporting structure, 9) exiting ground product pipe.

When the BSGs reach Agridustria, they are dried using the VOMM 450 Turbodryer (**Figure 15**). VOMM This technology is based on creating a thin film of material maintained in high turbulence, which allows the optimization of heat exchange due to the transmission of heat by conduction and convection. The chamber inside which the material to be dried is placed is jacketed, and a heat transfer fluid such as diathermic oil, steam, or hot or cold water circulates to maintain a constant temperature inside the machine according to the process needs. The drying was carried out with a usage power of 30Kw. The system, set with this power, leads to an electrical consumption of 0.6 kWh/kg produced and a thermal energy consumption of 1Kwt/litre of evaporated water. The drying process allowed the humidity of the product to be reduced to a level of 12%.

A Favini Mach F2 micronization mill was used for the micronization process (**Figure 16**). The mill takes the material to be subjected to the process via a hopper and is sent to the grinding chamber via a variable speed dosing screw. In the grinding chamber, the product impacts against the pegs present, leading to the product's disintegration. The flow of air, which rises along the internal walls of the grinding chamber, determines the transport of the particles of disintegrated material through the diffuser ring of the selector. In this area, each of the ground particles is subjected to opposing forces between them (drag force exerted by the air and centrifugal force exerted by the rotation of the selector), which leads to a further reduction in the size of the particles up to the set values. The micronization was carried out with a usage power of 40Kw. The system, set with this power, produces an electricity consumption of 1 kWh/kg.

The micronization process allowed the humidity of the product to be reduced to a level of 9%, and the yield of the process was equal to 95% (20 kg). Once micronized, the grains were delivered to DISAFA for oxidative and microbiological stability evaluations.

For the evaluation of oxidative stability, the micronized grains were divided into 3 aliquots in order to evaluate their conservation at three different temperatures: freezing (-18 °C), refrigeration (4 °C) and environmental (20 °C). The samples were stored for nine months, and the analyses were carried out at time 0, after 3 and 9 months.

To evaluate the oxidative stability, the fatty substance present in the thresher matrix was extracted through a solid-liquid extraction. 10 g of sample were placed inside a Falcon, added with 20 mL of a 3:2 hexane and isopropanol solution, and stirred for 30 seconds with a vortex. Subsequently, centrifugation was carried out at 8000 rpm for 10 min at 10 °C, to facilitate the separation of the matrix from the solvent. Once centrifuged, the supernatant was separated from the precipitate and transferred into a 50 ml glass flask. A second extraction was then carried out using 20 mL of solvent. Subsequently, the solvent was evaporated via Rotavapor and the pure fat fraction was transferred into 2 ml amber vials and stored in the dark at -18 °C until analysis.

To monitor the oxidative state of the grains, the free fatty acids (FFA), the number of peroxides (PV) and the value of *p*-Anisidine (AnV) were determined on the extracted oils. The first index represents the most straightforward method for monitoring the hydrolytic rancidity of lipid-containing foods. Peroxides, as primary oxidation compounds, are formed in the early stages of lipid oxidation and can be subjected to subsequent oxidation reactions to generate various non-volatile and volatile secondary products. In general, the lower the PV, the better the oil quality. However, PV levels decrease as the oil's oxidation level advances and as secondary oxidation products appear. The *p*-Anisidine value measures the secondary oxidation compounds generated by the degradation of hydroperoxides, low molecular weight products characterized by a typical and unpleasant rancid odour and acrid taste. As with PV, the lower the AnV, the better the oil quality. Using the primary (PV) and secondary (AnV) oxidation parameters to evaluate the oxidative stability and shelf-life of the samples comprehensively, the TOTOX value was calculated as follows:

The TOTOX value shows the entire history of the primary and secondary breakdown of products. Processors use the TOTOX value to determine the overall oxidation state of an oil. The lower the TOTOX value, the better the quality of the oil. High-quality oils should have a TOTOX value < 10.

$$TOTOX = 2PV + AnV$$

To determine the free acidity, the number of peroxides and the p-Anisidine value of the oils, the FoodLabFat methods and instrument (CRD S.r.l., Italy) were used. The instrument detects the absorbance of the mixture of reagents and sample at 630, 505, and 366 nm, respectively.

The free fatty acids present in the sample, in conditions of pH < 7.0, react with a chromogen, developing a colour whose optical density, measured at 630 nm, is proportional to the concentration of the acidity of the fat, expressed as % of oleic acid.

Peroxides are capable of oxidising Fe^{2+} ions to Fe^{3+}. The Fe^{3+} ions are complex, forming a red chromophore whose absorbance, measured at 505 nm, is directly proportional to the concentration of peroxides in the sample, expressed as meq O_2/Kg.

The aldehydes derived from the secondary oxidation of fatty substances react with P-anisidine, determining an absorption measurable at 366 nm. The p-Anisidine value is expressed as AnV (Anisidine value) according to the AOCS reference method (Cd 18-90).

To complete the analysis of the by-product collected, we conducted some microbiological tests to evaluate the presence of spoiling microorganisms. The evaluation uses traditional microbiology techniques such as selective cultivar terrain and selective molecular biology tests like qPCR to verify and double-check the result. We evaluated the presence of *Lysteria monocytogenes*, *Bacillus cereus*, *Salmonella spp.*, *Staphylococcus aureus*, and *Clostridium spp*.

Also, a total aerobic and anaerobic count has been operated using Tryptic soy agar as terrain. For each analysis, 10g of sample were weighed and introduced in a solution of Buffered Peptone Water and Listeria Enrichment Broth for 24 h. at 37 °C. After this time, 1 ml of the broth was used to plate the petri dish, and then the Petri was stocked for 24 h. at 37 °C. After one day from plating, the cell growth was evaluated, and the results were checked using the TaqMan® methodology for the selective species we wanted to screen. The following **Table 15** summarises a scheme of the microbiological analysis made.

Table 15. Tests operated on the BSG flour to evaluate the microbiological stability.

Microorganism species	Selective terrain	qPCR double check
Total aerobic count	TSA (tryptic Soy Agar)	No
Total anaerobic count	TSA (tryptic Soy Agar)	No
Lysteria monocytogenes	ALOA	Yes
Bacillus cereus	PEMBA	Yes
Salmonella spp.	RAPID S	Yes
Staphylococcus aureus	BPA	Yes
Clostridium spp.	SFP	Yes

The flour produced can already find some applications in baked goods such as biscuits or salty snacks, replacing part of the traditional flour. Switching the recipe completely using only BSG flour is impossible since this matrix has a deficient concentration of starch and free sugars, making the traditional baking process impossible. However, it has been shown many times that an introduction in variable concentrations between 5% and 25% can ensure baked goods' desired flavour and texture characteristics. A biscuit that presents some BSG will be much more similar to biscuits produced with integral flour. The BSG will shift the final product's colour towards a dark, beige and brown colour, reducing the fluffiness and increasing the crunchiness of the good. These modifications are caused by the reduction of the starch and sugar concentration in the dough, which directly affects the yeast's leavening capacity. Less fermentable sugar for the yeast will cause a reduction in the CO_2 produced during the cooking process, leading to a less fluffy and soft dough. Moreover, the increased concentration of vegetal fibre will affect the product's texture, increasing its crunchiness and hardness.

The BSG flour produced has been sent to Albertengo facility, where they tested an industrial-scale production of 4 different batches with increasing concentrations of BSG flour inside a recipe already used for commercial purposes. The sweet biscuits were produced according to the recipe shown in **Table 16**. In particular, the BSG was used to replace whole wheat flour at increasing percentages from 25% to 100%. A Kitchen Aid© planetary mixer with a leaf arm was used to form the dough. Once the dough was formed, it was left to rest for 30 min. in the refrigerator to allow the gluten network to gain strength.

Subsequently, biscuits were formed, characterized by a thickness and diameter of 3 and 6 cm, respectively, and their surface was pricked to create escape routes for moisture during cooking. The cooking process took place at 180 °C for 12 min.

Table 16. Recipe for the BSG biscuit industrial production.

Ingredients (g)	Standard	100% BSG	75% BSG	50% BSG	25% BSG
Wheat flour	300	300	300	300	300
Wholegrain flour	120	-	30	60	90
BSGs	-	120	90	60	30
Cane sugar	120	120	120	120	120
Margarine	210	210	210	210	210
Oat milk	35	35	35	35	35
Baking powder	68	68	68	68	68
Salt	0.5	0.5	0.5	0.5	0.5

The biscuits thus obtained were subjected to physical analysis, analysis of colour, texture and tasting by a trained panel, and analysis of the total polyphenol content and antioxidant activity. The physical analyses took the dimensions of the biscuit, width (W), thickness (T) and spread, a value that describes the "melting" of the biscuit due to the melting of fats and sugars during cooking, as well as the humidity. The analyses relating to the biscuit size were carried out following the official AACC 10-50.05 method. Humidity was measured with a Radwag mac 210NH thermobalance using 3 grams of ground sample and operating at 120 °C, and three replicates were carried out for each sample. The colour analysis of the biscuit surface was carried out using the Konica-Minolta CM-5 spectrocolourimeter (CIE L*a*b* system) with a measurement area of 8 mm, an observation angle of 10°, an illuminant D65 and a wavelength spectrum between 360 and 740 nm. The colour measurement was carried out excluding the specular component, and nine replicates were carried out for each sample. The textural analysis (biscuit hardness – Newton and crunchiness – number of peaks) was conducted using a TA-TX Plus Texture Analyzer (SMS-Stable Micro System, Surrey, UK) equipped with a 25 kg load cell. An HDP-BS cutting blade at a speed of 1 mm/s and a measuring distance of 99% was used for the analysis. For each sample, 12 replicates were carried out. To evaluate the organoleptic properties of the biscuits, a satisfaction test was used with a 9-point hedonic scale where 1 indicates extremely unpleasant, 5 indicates neither pleasant nor unpleasant and 9 indicates extremely pleasant. The consumer was asked to evaluate the following parameters: appearance, smell, taste, aroma, structure and compressive satisfaction of the sample. The propensity to purchase was also assessed on a 7-point hedonic scale where 1 corresponds to definitely no, 4 I do not know and 7 definitely yes. As regards the analyses relating to the total polyphenol content and antioxidant activity, the methods reported [61].

The BSG flour can also be applied in some salty snacks using the same approach as the biscuits. We decided to roast the BSG for this application since it can be an exciting twist on the flavour, enhancing the taste and saltiness of the final product. The roasting process was operated by leaving the BSGs at 180 °C for 12 min.. The roasted BSGs will be named RBSGs

The recipe shown in **Table 17** was used to produce the salty snacks. The BSG flour was used to replace the flour in percentages of 5%, 7.5% and 10%. A Kitchen Aid© planetary mixer with a leaf arm was used to form the dough. Once the dough was formed, it was left to rest for 30 min. in the refrigerator to allow the gluten network to gain strength. Subsequently, biscuits were formed, characterized by a thickness and diameter of 3 and 4 cm, respectively, and their surface was pricked to create escape routes for moisture during cooking. The cooking process took place at 180 °C for 30 min.

Table 17. Recipe for the BSG salty snacks industrial production.

Ingredients	Standard	5% Dry BSGs (5%BSG)	7.5% Dry BSGs (7.5% BSG)	10% Dry BSGs (10%BSG)	5% Rosted BSGs (5%RBSG)	7.5% Rosted BSGs (7.5% RBSG)	10% Rosted BSGs (10%RBSG)
Wheat flour	150	143	138,75	135	128	123,75	120
BSGs	-	7	11,25	15	7	11,25	15
Yeast	8	8	8	8	8	8	8
Sugar	10	10	10	10	10	10	10
Salt	2	2	2	2	2	2	2
Butter	45	45	45	45	45	45	45
Olive oil	15	15	15	15	15	15	15
Water	35	35	35	35	35	35	35

The snacks obtained were subjected to structural, colour, tasting, and physical analyses, analysis of colour and texture by a trained panel, and analysis of the total polyphenol content and antioxidant activity. The physical analyses took the dimensions of the biscuit, width (W), thickness (T), and spread, as well as the humidity. The analyses relating to the biscuit size were carried out following the official AACC 10-50.05 method. Humidity was measured with a Radwag mac 210NH thermobalance using 3 grams of ground sample and operating at 120 °C, and three replicates were carried out for each sample. The colour analysis of the biscuit surface was carried out using the Konica-Minolta CM-5 spectrocolourimeter (CIE L*a*b* system) with a measurement area of 8 mm, an observation angle of 10°, an illuminant D65 and a wavelength spectrum between 360 and 740 nm. The colour measurement was carried out excluding the specular component, and nine replicates were carried out for each sample. The textural analysis (biscuit hardness – Newton and crunchiness – number of peaks) was conducted using a TA-TX Plus Texture Analyzer (SMS-Stable Micro System, Surrey, UK) equipped with a 25 kg load cell. An HDP-BS cutting blade

at a speed of 1 mm/s and a measuring distance of 99% was used for the analysis. For each sample, 12 replicates were carried out. A satisfaction test was used to taste the biscuits with a 9-point hedonic scale where 1 indicates extremely unpleasant, 5 indicates neither pleasant nor unpleasant and 9 indicates extremely pleasant. The consumer was asked to evaluate the following parameters: appearance, smell, taste, aroma, structure and compressive satisfaction of the sample. The propensity to purchase was also assessed on a 7-point hedonic scale where 1 corresponds to definitely no, 4 I do not know and 7 definitely yes.

The BSG flour has been treated with many technologies for extracting and exploiting its valuable compounds. Since the main application can be found in the food industry and to use it as a supplement for integrators and functional food, we had to avoid organic solvents. We focused our attention on the exploitation of water and green and innovative extraction technologies. At first, we wanted to investigate the possible applications of BSG as a source of soluble vegetal fibre for direct applications in the beverage industry. The BSGs have been processed with ultrasounds to evaluate the release of soluble fibres in water and in a solution of 70% ethanol. The equipment used was a Hainertec Ultrasonic generator. The extraction protocol consisted of 15 min. of treatment at 500W derogated. To ensure that the temperature during the extraction would not increase above 35 °C, the sample was cooled using a bath of water/acetone ice. We took 3 gr of the BSG to prepare the ultrasound solubilization process. At this point, we added 60 mL of the solvent (water or 70% ethanol) and sonicated the mixture as described above. At the end of this process, the sample has been filtrated and washed with abundant deionized water to take out all the soluble fibres released by this process. This same method has been carried out for the 70% ethanol solution. Then, we collected all the solution, and to estimate the solute, we freeze-dried the samples. After this processing, the amount of soluble fibres could be measured.

The results achieved on the fibre solubilization have not been as high as hoped, and for this reason, we decided to try a different approach using the ROTOCAV System (**Figure 17**), an industrial scale equipment capable of hydrocavitate thanks to a rotor-stator unit. The homogenizer has a cooling water seal to disperse the heat produced due to the mechanical rotation and has a working flow of 5.6 m^3/min.

Figure 17. ROTOCAV (e-PIc S.r.l.) cavitational system.

The homogenizer is connected to a 120 L tank with a circular setting. In this way, the processed matrix exiting from the homogeniser returns to the tank. The tank has a mechanical agitator that provides a high homogeneity of the matrix that enters the homogenizer.

7 kg of BSG flour has been placed in the tank with 70 L of water. During the processing, 50 mL samples have been taken at different times (0-0.5-1-2-5-10-20-30 min.) to evaluate how the soluble part increases during the processing. Since this process is based on the biomass disruption caused by the homogenization process, which creates hotspots, the temperature of the liquid has been monitored. At the start of the processing, the temperature was 22.4 °C, and in the end, after 30 min, it only reached 29.0 °C. The samples collected have been filtrated using a paper filter and divided into two aliquots. One sample was dried-freeze, and the other was lyophilised to evaluate the amount of soluble fibre released in the solvent. In **Figure 18,** a picture of the sample pre- and post-filtration is showcased, and a clear increase in turbidity and colour can be seen in the first samples.

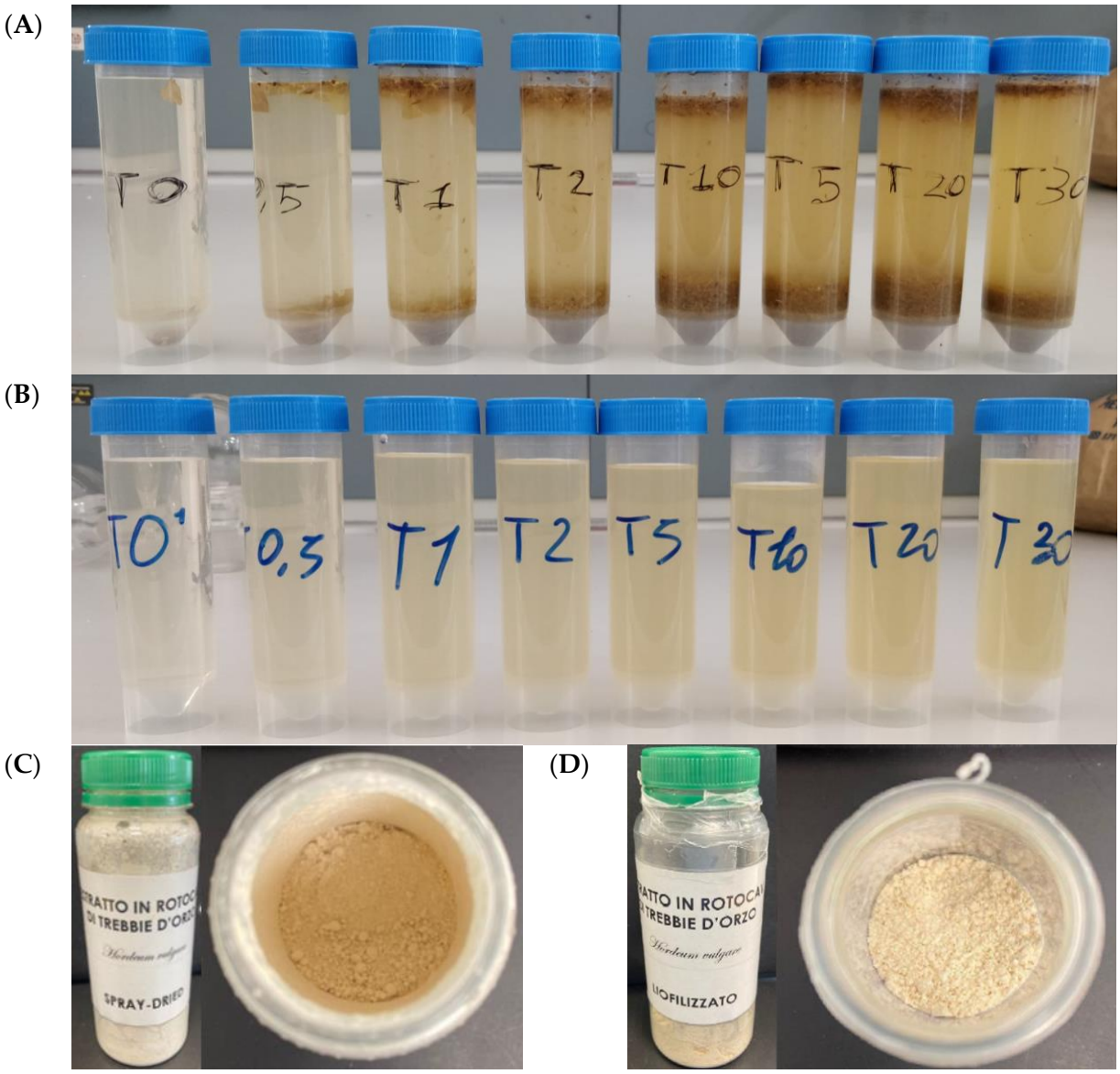


Figure 18. (**A**) samples after the homogenization processing; (**B**) samples after the paper filtration;(**C**) sample recovered and sprayed dried; (**D**) sample recovered and freeze-dried.

We used the Peleg model to evaluate the extraction kinetics [62] [63]. This hyperbolic model was used to evaluate the extraction kinetics of the phenols, the maximum achievable extraction yield, and the time to reach the plateau. The Peleg model is based on the following equation.

$$C(t) = C_0 + \frac{t}{k_1 + k_2 t}$$

$C(t)$ is the concentration of the studied compound at the t time, while C_0 is the starting concentration that has been calculated at t_0 before starting the homogenization. k_1 is known as the Peleg Initial Extraction Rate, and it is correlated to the initial extraction rate (B_0).

$$B_0 = \frac{1}{k_1}$$

This kinetic constant can be used to estimate the instant of the maximum extraction speed, which is an essential data for industrial transposition of process, and the extraction speed at any time of the extraction. Meanwhile, k2 is the Peleg Capacity Constant, which describes the highest extraction yields (Ys) that reached the plateau.

$$c_{eq} = Y_s = \frac{1}{k_2}$$

The Peleg equation can be linearized, as shown in the following equation. This arithmetic process provides a fast and easy way to extrapolate the k_1 and k_2 values, respectively the intercept and the slope of the straight line.

$$\frac{t}{C(t)} = k_1 + k_2 t$$

This method helps the operator find the concentration of the extract at each time and evaluate when it is appropriate to stop the extraction treatment to optimize the process.

The spray-dried sample and the freeze-dried one have been sent to Procemsa spa laboratories to evaluate the possible application as a source of fibre in energy bars or liquid integrators. Unfortunately, the extracts produced with the ROTOCAV had a persistent unpleasant flavour and smell that can be associated with old cereals and dust. All the prototypes of liquid integrators created had some significant problems in the flavour and colour department. Even in low concentrations of 1.5 gr/L, the extract added significantly shifted the beverage's colour, making it dark brown and with a high turbidity. Moreover, the beverage's flavour changed significantly, losing most of its freshness and aroma and shifting towards a sweet cereal cookie flavour that is undesirable in a refreshing beverage. The prototypes produced also showcased an undesired formation of a solid dark beige deposit. For all these reasons, we decided to implement the extracts produced in a solid product to reduce their impact on colour and flavour.

For the selective extraction of the arabinoxylan and β-glucan fraction after deep and exhausting research, we decided to use an enzymatic digestion approach. We decided to collaborate with "Heallo S.r.l.," a company specialized in the formulation of functional food starting from food industry wastes. They have already optimized and obtained a patent for the extraction process of the arabinoxylan fraction from BSGs [64]. Their extraction process can be divided into 2 phases: the enzymatic hydrolysis of the desired fibre and the separation of those fibres from the remaining components. In the first step, the BSGs are

charged in a tank with water in a weight ratio of 1:1 to 1:5 to moisturise the matrix. Then, a mix of 3 enzymes composed of endo-1, 4-beta-xylanase, alpha-amylase, and endo-1, 3 (4)-beta-glucanase are introduced. When the enzymes are introduced, the system gets heated at 45-65 °C for 1 to 6 h. in a slightly acidic environment with a pH between 4 and 6. After digestion, the enzymes are inactivated by thermal unfolding, and the system is heated up to 80 °C for at least 5 min.. When the digestion is completed, the compounds of interest are solubilized in the solvent, so at first, the suspension gets filtered to remove all the solid deposits remaining only with the solution of water and fibre. Since fibre content is not very high, it is necessary to concentrate the solution via evaporation. When the solute concentration reaches an optimal concentration, the solvent gets spay-dried and is collected. The powder recovered has a bright beige colour and a characteristic cereal flavour and odour. This extract is already on the market with the commercial name "JaxPlus®". This food integrator is already used in many products to reduce the post-meal glycaemicglycaemic peak and stabilize the cholesterol level thanks to the arabinoxylan β-glucan activity. The EFSA organization has acknowledged these compounds' positive activity when ingested in the correct doses (8g of fibre over 100g of available carbohydrates) [65]. The Heallo company has already introduced this innovative product in various alimentary goods, collaborating with many companies (**Figure 19**).

Figure 19. Showcase of the JaxPlus® powder and various food products, which have already been introduced with an industrial production scale.

We decided to implement this fibre in various goods, both beverages and solids. The JaxPlus® extract showed a much higher solubility than the previous extracts obtained via ultrasound and hydrocavitational treatments. Moreover, the organoleptic properties of this powder were less pervasive and intense, making it much easier to introduce without modifying the organoleptic properties of the good.

For the application of fibre to the production of functional juices, it was decided to select as the starting matrix (juices) those currently marketed by Baladin with the apricot, pear and peach flavours. The main reason to start the tests with the juices is that this product is already characterized by high turbidity and viscosity, reducing the resuspension problems of the powder. The experimentation was divided into two phases.

During the first phase, three different concentrations of fibre 1%, 1.5% and 3% were used to evaluate the variation in the colour of the product, the sedimentation of the fibres themselves and the pleasantness of the product by an expert panel of tasters present at the Food Technology sector of DISAFA while in the second phase, the products selected in the first phase were subjected to an approval test by a panel of consumers.

The colour analysis was carried out using the Konica-Minolta CM-5 spectrocolourimeter (CIE L*a*b* system) with a measurement area of 8 mm, an observation angle of 10°, a D65 illuminant and a wavelength spectrum between 360 and 740 nm. The colour measurement was carried out excluding the specular component, and three replicates were carried out for each sample.

Sedimentation was evaluated by leaving the product at a controlled temperature (+4 °C) for 24 h. and subsequently visual observation of any deposit.

For the sensorial evaluation, the panel of expert tasters was made up of 5 trained people; for the tasting by a potential final consumer, the panel was made up of 24 people. In both evaluations, 30 mL of juice was placed inside methacrylate glasses so the taster could evaluate the product using all the senses. As a taste test, in the case of the evaluation by the consumer panel, a satisfaction test with a 9-point hedonic scale was used where 1 indicates extremely unpleasant, 5 is neither pleasant nor unpleasant and 9 is extremely pleasant. The consumer was asked to evaluate the following parameters: appearance, smell, taste, aroma, structure and compressive satisfaction of the sample. The propensity to purchase was also assessed on a 7-point hedonic scale where 1 corresponds to definitely no, 4 I do not know and 7 definitely yes.

We also decided to test the JaxPlus® formulation in a sweet baked good like Panettone, a typical Italian dessert common during Christmas. Albertengo company was in charge of preparing two different batches of panettone. **Table 18** shows the recipe difference between the classic batch, called p-standard, and the enriched batch called p-rich.

The two batches produced have been used in a clinical trial to evaluate the change in the glycaemic curve and insulin concentration during the digestion of the panettone and the sense of satisfaction and fullness afterwards. The production process of p-rich and p-standard began with yeast production and ingredient dosing, followed by mixing and overnight leavening at 29-34 °C and 80% humidity. In the morning, the dough stayed for 6-8 h. at 36 °C, reducing its humidity to 75%. When the dough reached this moisture level, it

was baked for 58-63 min. at 165-175 °C. At the end of baking, the panettoni are packed in a closed box, sanitized with alcohol, and stored at room temperature in a dark environment.

Table 18. Composition of p-standard and p-rich per portion (per 100 g of product).

Ingredients	p-standard	p-rich
Dough		
Wheat Flour type 00 (g)	26.5	24.5
Sultanas (g)	18.4	18.4
Water (L)	9.8	9.8
Butter (g)	9.2	9.2
Egg yolk (g)	9.2	9.2
Fibre Jax Plus (g)	-	2.0
Raw cane sugar (g)	5.8	5.8
Natural yeast (g)	5.7	5.7
Raisin beer (L)	5.1	5.1
Fatty acid mono diglycerides (g)	2.8	2.8
Liqueur vine (marsala) (L)	2	2
Invert sugar syrup (g)	1.8	1.8
Glucose - Fructose syrup (g)	1	1
Fine sea salt (g)	0.3	0.3
Barley malt extract (g)	0.3	0.3
Glaze		
White sugar (g)	7.3	7.3
Fresh egg white (g)	2.7	2.7
Hazelnut flour (g)	1.4	1.4
Sweet almond flour (g)	0.9	0.9
Apricot kernel flour (g)	0.7	0.7
Sunflower oil (g)	0.1	0.1

For the clinical trial, ten Caucasian, non-smoking, healthy adult volunteers between 20 and 65 years were enrolled. The exclusion criteria were the presence of obesity, chronic diseases, pharmacological therapies or dietary restrictions potentially influencing glycaemic and/or insulinemic responses, and allergy or intolerance to the ingredients of panettone. Participants were instructed not to change their usual lifestyle the week before each test. The day before each test, every participant was provided with the same diet, explained in detail by the same dietitian (20% proteins, 30% lipids, 50% carbohydrates, fibre 20-26 g/day), and instructed to avoid alcohol, hard exercise, inactivity, and sleep deprivation. All tests were performed in the morning at 8:00, after 10 h. fasting. Participants were randomized to receive either the enriched panettone (p-rich) or the unenriched product (p-standard). The

order of administration of each product was randomized using a computer-generated sequence. On the day of each test, fasting individuals underwent a blood sample collection and weight and height measurements. Then, participants must consume 100 g of the product within 20 min. while sitting. Drinking 250 mL of tap water was allowed during the test. Blood samples were collected at 15, 30, 45, 60, 90, and 120 min. after the consumption of each food. Then, individuals could leave the room and move without carrying out exercise and eating for an additional 120 min., during which they continued to report their subjective feelings of appetite, subjective sensations of hunger, satiety, fullness, and propensity to eat were assessed at 60, 120, 180, and 240 min. after the consumption of the products by visual analogue scale (VAS).

Blood samples were immediately centrifuged, and aliquots of serum were stored at -20 °C. Serum glucose was measured in triplicate by the enzymatic hexokinase method commercialized by Beckman Coulter Ireland Inc. The kit has a sensitivity of 0.04 mmol/L and a range between 0.6-45.0 mmol/L (10-800 mg/dL). Insulin was measured by a chemiluminescent immunoassay assay kit produced by Beckman Coulter Eurocenter. This method has a sensitivity between 0.03 μIU/mL (0.21 pmol/L) and a range between 0,03-300 μIU/mL.

3.1.3 Results and Discussion

The oxidative process of the fatty acids of the BSGs has been analysed to evaluate the oxidative degradation speed of the matrix. As expected, the sample maintained at a higher temperature showed an increase in fatty acid degradation, leading to rancidity of the biomass. **Table 19** shows the results of the evaluation of the oxidative stability of the BSGs.

As can be seen in the table below, regardless of the storage condition, values significantly increase in the first three months of storage due to the hydrolytic oxidation of triglycerides with the consequent release of free fatty acids, which can, therefore, be subject to further oxidation. In fact, at the last sampling point, values decrease due to the oxidation of free fatty acids with the formation of volatile and non-volatile aromatic compounds. The decrease in acidity compared to the first three months of storage was equal to 6, 7, and 11 percentage points for storage at -18 °C, 20 °C, and 4 °C. At all sampling points, statistically significant differences between conservation methods were observed. In particular, after three months, the best conservation method (characterised by the lowest acidity content) was found to be that carried out at -18 °C while among the other methods, no statistical differences were highlighted while at the end of shelf-life, the best condition was found to be that at 4 °C (smaller quantity of FFA). However, it must be taken into consideration that the decrease in acidity is linked to the action of oxidative enzymes, which have not been blocked by the stabilization (drying) treatments of the threshers or by the storage temperatures.

Table 19. Values (mean ± standard deviation) of free acidity (FFA), peroxide number (PV), p-Anisidine value and Totox of grain samples stored under different conditions and related results of the Duncan test ($p< 0.05$).

		Months						
	Stock	**0**		**3**		**9**		*Signif.*
Free Acidity (FFA) (% oleica c.)	-18 °C	12.57 ± 0.96	a	20.44 ± 0.87	c,A	14.52 ± 0.19	b,B	***
	+ 4 °C	12.57 ± 0.96	a	24.26 ± 0.51	b,B	13.48 ± 0.24	a,A	***
	+20 °C	12.57 ± 0.96	a	23.26 ± 1.07	c,B	14.32 ± 0.10	b,B	***
Signif.				**		**		
Peroxide number (PV) (meq O_2/kg oil)	-18 °C	26.29 ± 0.18	b	28.42 ± 0.53	c,A	10.27 ± 0.23	a,B	***
	+ 4 °C	26.29 ± 0.18	b	39.98 ± 0.69	c,B	8.21 ± 0.06	a,A	***
	+20 °C	26.29 ± 0.18	b	113.7 ± 0.58	c,C	11.16 ± 0.04	a,C	***
Signif.				***		***		
***p*-Anisidine (AnV)**	-18 °C	6.12 ± 0.59	c	0.74 ± 0.42	a,A	2.93 ± 0.35	b,B	***
	+ 4 °C	6.12 ± 0.59	b	2.63 ± 0.41	a,B	1.90 ± 0.00	a,A	***
	+20 °C	6.12 ± 0.59	a	12.3 ± 0.12	b,C	6.73 ± 0.15	a,C	****
Signif.				***		***		
Totox	-18 °C	58.7 ± 0.83	b	57.59 ± 1.46	b,A	23.47 ± 0.13	a,B	***
	+ 4 °C	58.7 ± 0.83	b	82.59 ± 1.21	c,B	18.33 ± 0.12	a,A	***
	+20 °C	58.7 ± 0.83	b	239.6 ± 1.13	c,C	29.05 ± 0.23	a,C	***
Signif.				***		***		

The number of peroxides increased statistically significantly in the first three months of shelf-life regardless of the storage condition, demonstrating the activation of the oxidative enzymes which acted against the free fatty acids present from time 0. The storage condition storage that determines the most significant oxidation appears to be that carried out at 20 °C where there is a percentage increase equal to 335%, while the best values were found in the storage condition at -18 °C where the percentage increase was only 8%. Subsequently, as already indicated previously, the number of peroxides decreases due to the secondary oxidation reactions involving the hydroperoxides with the formation of aldehydes. As already observed for acidity, the lowest values were detected in samples stored at 4 °C followed by samples stored at -18 °C.

To evaluate the chemical stability of the matrix, we can analyse the results of the TOTOX parameter, which represents the history of the oxidation of the matrix. From the results, it is possible to underline how the raw material placed in conservation was already characterized by an ongoing oxidation process, perhaps due to the oxidation that occurred due to the drying temperatures and the non-total deactivation of the lipolytic enzymes. Keeping the dried raw material at a temperature of -18 °C prevents the propagation of these reactions. In contrast, storage at temperatures above 0 °C determines an increase in primary and secondary reactions, and this increase is positively correlated with the temperature of conservation (the higher it is, the greater the enzymatic activity). From the data obtained, it

seems that after nine months of storage, all the primary oxidation reactions are completed while the secondary reactions remain in a reduced mode. The fact that the reactions are slowed down should not, however, be seen as a positive aspect since the matrix appears to be completely damaged and difficult to use as a functional ingredient since it would bring unpleasant aromas and chemical products to the finished product that are dangerous for the final consumer.

Even if the BSGs have been processed in less than 24 h., they showed some oxidative degradation promoted by spoiling microorganisms at T0. So, It was unsurprising to find many different strains populating the BSGs. **Figure 20** shows the picture of the petri dish after the incubation time.

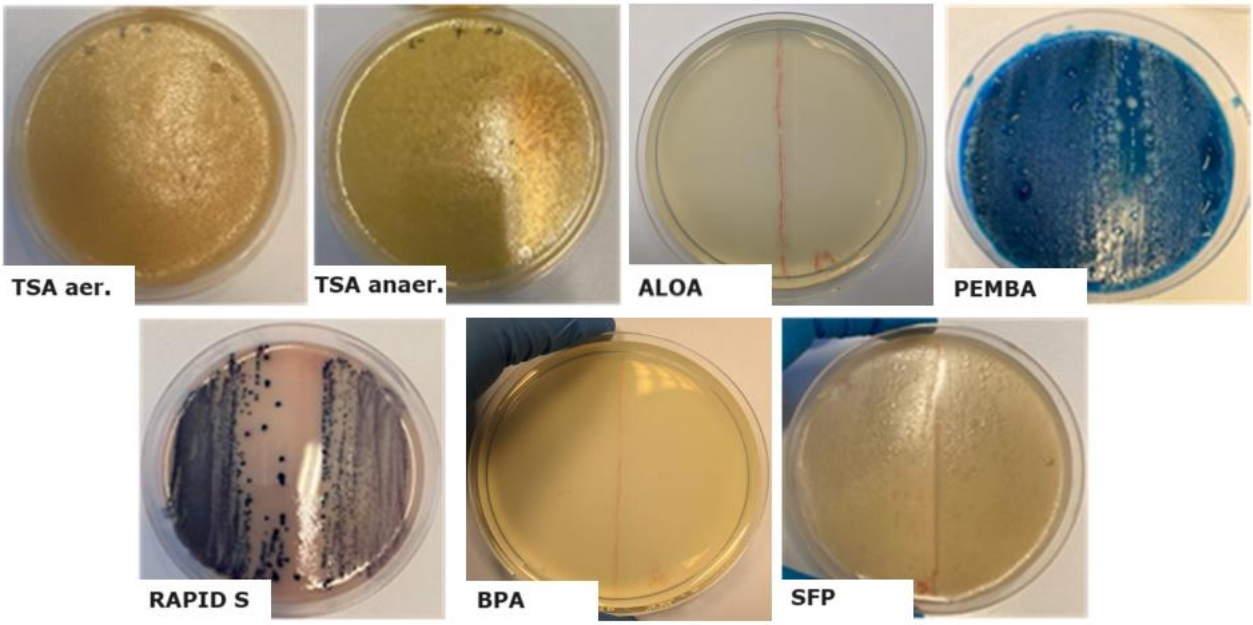


Figure 20. Petri dish with different selective terrain for the microbiological stability assessment of the BSGs.

Many colonies of different strains have grown on the selective terrain, and the qPCR analysis demonstrates the presence of the spoiling microorganisms. The results of the microbiological stability assessment are summarised in **Table 20**.

Table 20. Summary of the microbiological analysis made on BSG to evaluate the presence of spoiling microorganisms.

Spoiling microorganism	Selective terrain	Cell count on Petri	qPCR result
Total aerobic count	TSA (tryptic Soy Agar)	>3 $\log_{10}$ CFU/g	Not operated
Total anaerobic count	TSA (tryptic Soy Agar)	>3 $\log_{10}$ CFU/g	Not operated
Lysteria monocytogenes	ALOA	0 CFU/g	Negative
Bacillus cereus	PEMBA	>3 $\log_{10}$ CFU/g	Positive
Salmonella spp.	RAPID S	>3 $\log_{10}$ CFU/g	Positive
Staphylococcus aureus	BPA	0 CFU/g	Negative
Clostridium spp.	SFP	>3 $\log_{10}$ CFU/g	Positive

From the results obtained, it can be concluded that the treatments carried out do not have a bactericidal effect. Indeed, the total aerobic/anaerobic and the detection of specific microbial groups suggest a bacteriostatic effect. The detected microorganisms may have different origins, but the association of spore-forming microorganisms (*B. cereus, Clostridium spp*) with environments/habitats such as soil air. The hygienic-sanitary quality of stabilized BSGs could be improved. It would still need to be explored whether the contamination occurs during the stabilization process or whether this contamination occurs downstream of the process. Given stabilised grains' low water activity characteristics, the detected microorganisms cannot develop. This can happen in a subsequent integration phase into food products, if the water activity conditions vary and microbicidal treatments are not foreseen. For this reason, it is necessary to adopt an approach based on risk analysis and HACCP principles when using stabilized grains in the food production sector.

Regarding microbiological stability, the drying treatment only leads to a bacteriostatic and not bactericidal effect. Therefore, the possibility of using them in foods is feasible only in those food products characterized by low water activity unless a microbicidal treatment is provided in the production process.

The results from both the oxidative and microbiological stability assessment showed that the matrix is prone to spoiling. The moisture level of the BSGs when exiting the brewery makes them a perfect environment for contamination. It is possible to optimize the BSG processing even more, reducing the time between the biomass recovery and the flour production. To reduce the total aerobic and anaerobic count, the moisture level of the BSGs must be reduced when the matrix is still hot with a temperature around 80 °C and processed as soon as possible. Even when dried and stocked, some biomass can be degraded, so it is suggested to plan a fast use for the BSG flour when produced. Typical breweries produce BSGs daily during the year, so it is possible to schedule the processing very easily depending on the request of the partners involved in the processing of the by-product.

After these evaluations, we implement the flour as it is in a sweet biscuit recipe, replacing the increasing concentration of wholegrain flour (**Figure 21**). **Table 21** shows the data relating to the physical and humidity parameters of the wholemeal biscuit samples produced. As can be seen, applying the threshers determines statistically significant differences for all the parameters considered. In particular, it is possible to observe how the threshes can absorb the water present in the recipe, leading to wetter finished products, and at the same time, the fibre contained in it tends to hinder the formation of the protein network and the fat and sugar dissolution process. Therefore, biscuits tend to retain their original shape more.

Figure 21. Wholegrain biscuits obtained with increasing concentrations of BSGs.

As can be seen, the application of the BSGs determines statistically significant differences for all the parameters considered. In particular, it is possible to observe how the BSGs can absorb the water present in the recipe, leading to wetter finished products, and at the same time, the fibre contained in it tends to hinder the formation of the protein network and the dissolution process of the fat and sugar causing a net reduction of the spread value. Therefore, biscuits tend to retain their original shape more.

Table 21. Values (mean standard deviation) of the physical parameters of the biscuit samples and related Duncan results.

BSG%	Width (cm)	Thickness (cm)	Spread	Moisture (%)
0	35.69 ± 0.00 c	3.15 ± 0.07 a	113.5 ± 2.59 d	1.28 ± 0.09 a
25	34.96 ± 4.00 b,c	3.63 ± 0.15 b	95.72 ± 4.19 c	1.48 ± 0.18 b
50	34.58 ± 0.14 a,b	3.91 ± 0.04 b,c	89.72 ± 0.74 b,c	1.55 ± 0.08 b
75	34.34 ± 0.70 a,b	4.05 ± 0.18 c,d	84.12 ± 3.83 a,b	1.75 ± 0.06 c
100	33.89 ± 0.04 a	4.32 ± 0.18 d	79.89 ± 4.97 a	2.18 ± 0.08 d
Signif.	*	**	***	***

As shown in **Table 22**, replacing wholegrain flour with BSGs resulted in a statistically significant variation in all the colour coordinates examined. In particular, less bright products are obtained by increasing the percentage of grains used in the recipe (L*). In contrast, from a chromatic point of view, the replacement of wholemeal flour with grains determines a chromatic modification which remains statistically constant with the variation in the percentage of employment (a* and b*).

Table 22. L* indicates the brightness of the sample (0 – black; 100 - white); a* indicates the absorbance of the red spectrum red when positive and green when negative; b* indicates the absorbance of yellow when positive and blue when negative.

BSG %	L*	a*	b*
0	61.11 ± 2.29 c	10.43 ± 0.45 b	29.06 ± 0.96 b
25	53.96 ± 1.08 b	9.24 ± 0.54 a	24.18 ± 1.76 a
50	53.18 ± 2.47 b	9.48 ± 0.21 a	24.93 ± 1.50 a
75	49.11 ± 0.99 a	9.49 ± 0.62 a	24.97 ± 1.39 a
100	47.89 ± 1.47 a	9.57 ± 0.29 a	25.75 ± 0.79 a
Signif.	***	*	***

Table 23 shows the data relating to the analysis of the structure of the biscuits. As can be seen, the replacement of wholegrain flour with BSGs leads to harder biscuits in a statistically significant way only in the case of a total substitution and less crunchy biscuits, especially in the case of 100% substitution. A greater quantity of insoluble fibre causes this trend and determines a lower gluten network, which leads to a lower product expansion with consequent crunchiness loss. The latter also decreases as a result of the higher moisture content of the finished product.

Table 23. Evaluation of biscuits' hardness and crunchiness.

BSG %	Hardness (N)				Crunchiness (n. peaks)			
0	37.77	±	8.03	a	50.50	±	6.38	c
25	39.75	±	7.00	a	43.94	±	10.66	b,c
50	44.08	±	10.27	a	40.87	±	13.85	b
75	45.93	±	9.18	a	38.06	±	15.00	b
100	60.31	±	17.15	b	23.25	±	11.70	a
Signif.	***				***			

Table 24 shows the data relating to total polyphenols and their antioxidant capacity. As can be seen, the increased use of threshers in the finished product leads to obtaining biscuits characterized by a greater content of polyphenols with antioxidant action. Therefore, consuming such biscuits could lead to a functional benefit for the final consumer. The increase in concentration of polyphenols was proportional to the additions made, while the oxidizing capacity remained constant in the concentrations of 25 and 50%.

Table 24. Total phenolic count and DPPH measurement on the different biscuit batches.

BSG %	Total polyphenols (mg GAE/g)				Antioxidant capacirty (⚜mol TE/g)			
0	0.28	±	0.03	a	0.61	±	0.02	a
25	0.38	±	0.01	b	0.78	±	0.02	b
50	0.51	±	0.04	c	0.78	±	0.03	b
75	0.79	±	0.00	d	1.21	±	0.03	c
100	0.90	±	0.02	e	1.40	±	0.02	d
Signif.	***				***			

The panel test organized by DISAFA had some interesting, partially unexpected results. The panellists were enrolled, and before tacking the biscuit, they all had to answer specific questions, especially on their health and consumption habits. The different batches of biscuits have been evaluated on their aspects and the result is summarized in the histogram in **Figure 22**.

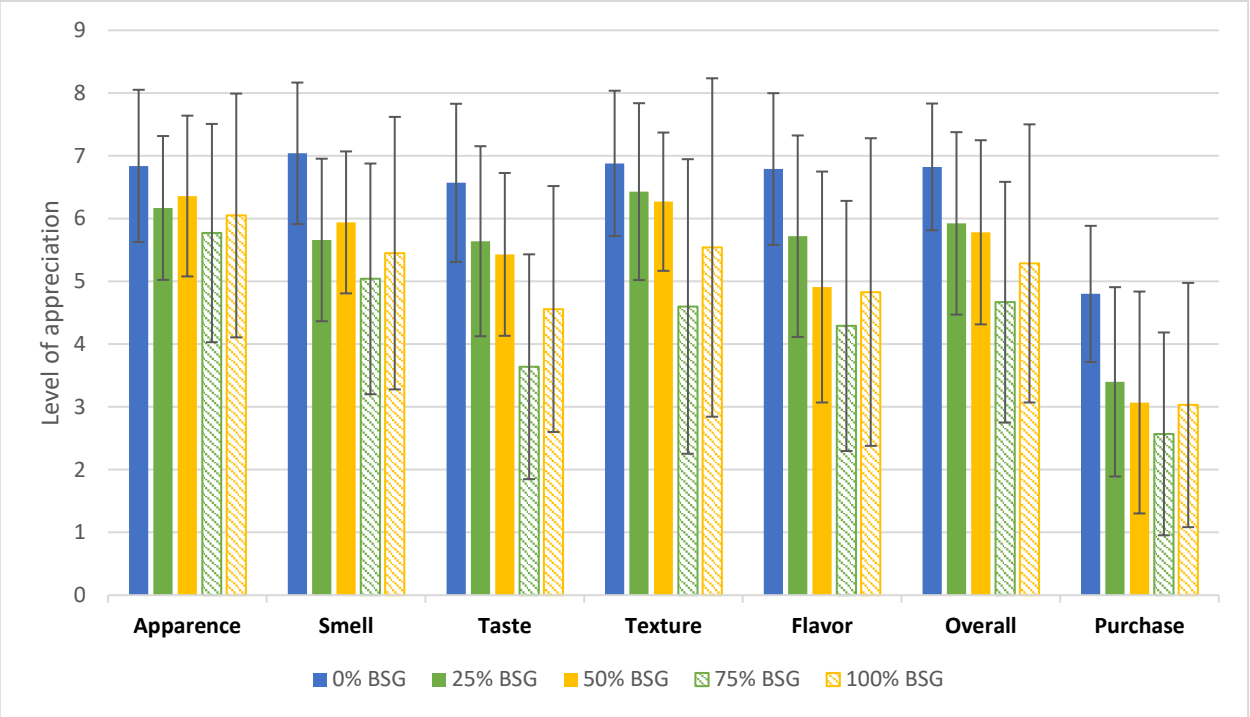


Figure 22. Panel test responses on the BSG sweet biscuits.

The panel test operated by the DISAFA department of the University of Turin aimed to foretell the market's response if the BSG biscuits were commercialized. At first glance, the enriched biscuits scored lower in nearly all categories compared to the 0% recipe already commercialized by Albertengo srl. The data collected from the panellists showed high heterogeneity in evaluating the different aspects of the product. The difference in people's tastes is also confirmed by the high standard deviation shown in the graph. If not evaluated deeply, the data collected would suggest that the consumers would not appreciate the biscuits made with BSGs. However, if we consider only the panellists that prefer and consume regularly wholegrain products, the results change significantly, as can be appreciated in **Figure 23**.

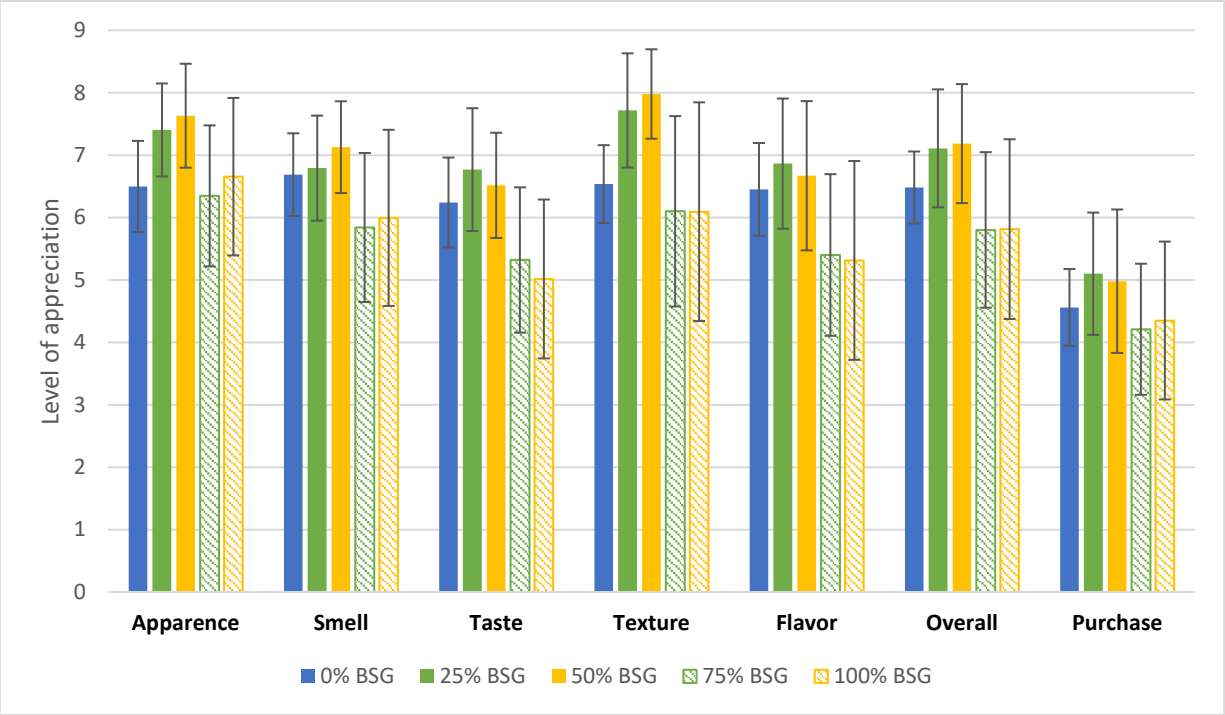


Figure 23. panel test response of the people that prefer and regularly consume wholegrain products.

When applying this filter in the product evaluation, we see a net increase in the appreciation of the BSG products, primarily in the 25 and 50% concentration. For the other two batches, the presence of BSG was too high and adverse for the whole tasting experience. Appreciating a net reduction of the standard deviation in all categories is also possible. This reduction is caused by the applied filter that selects only people with a common taste and commercial desire.

From the beginning of the formulation of this product, it was known that the biscuits would aim at a small niche market and not at broad consume. The market pocket of wholegrains and functional food has grown in the last decade but is still a small percentage compared to the whole market. However, consumers of this niche pocket are more inclined to buy goods that promote a healthier lifestyle and green and sustainable solutions. The biscuits produced are right on this trend. BSG flour reduces the amount of free starch, reducing the calories per serving while increasing the concentration of fibre and protein. A sustainable and circular approach also drives the project because a by-product of the food industry is reintroduced into the system and not wasted as compost or animal feed. The combination of those two aspects is enlighted by the fact that the population investigated was more prone to buy the BSG biscuits than the normal ones.

The BSG flour was also used in the production of some salty snacks. We produce three batches with increasing concentration of BSG flour and the other three batches with the flour produced by the RBSGs (roasted brewery spent grains). The salty snacks produced are shown in **Figure 24**.

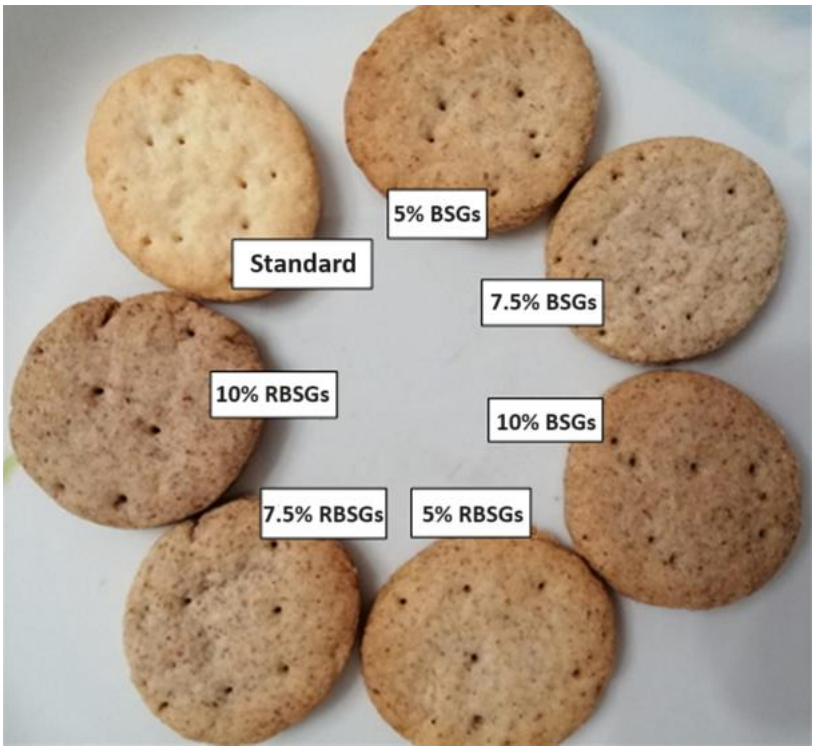


Figure 24. Salty snacks produced with increasing concentrations of BSG and RBSG flour compared to the standard product already on the market.

Table 25 shows the data obtained from the physical and structural analyses of the biscuits. As can be observed, the maximum replacement of flour with BSG (10%) regardless of the type of treatment carried out on the BSGs itself (drying or drying/roasting) is the condition that leads to statistically significant differences compared to the other functionalization conditions leading however, to obtain a biscuit that appears to be more similar to the standard from a statistical point of view. In general, as already observed in sweet products, the increase in the use of BSGs leads to obtaining wetter and, consequently, harder products. However, unlike what was observed in the sweet type, the crunchiness appears to be influenced less due to the use of the microbiological yeast capable of producing the gases necessary for the expansion of the product during cooking.

Table 25. Values (mean ± standard deviation) of the physical parameters of the biscuit samples and related Duncan results

	Hardness (N)	Crunchiness	Width (cm)	Thickness (cm)	Spread (cm)	Moisture (%)
Standard	26.34 ± 6.19 b	26.34 ± 6.19 b	3.58 ± 0.05	0.98 ± 0.03 d	3.62 ± 0.08 a	2.99 ± 0.17 c
5% BSG	28.88 ± 2.93 a	28.88 ± 2.93 a	3.65 ± 0.09	0.90 ± 0.04 c	4.08 ± 0.14 a	2.25 ± 0.12 b
7.5% BSG	24.92 ± 4.53 a,b	24.92 ± 3.36 a,b	3.68 ± 0.09	0.64 ± 0.07 a	5.72 ± 0.59 c	1.70 ± 0.10 a
10% BSG	26.62 ± 4.53 b	26.62 ± 4.53 b	3.62 ± 0.12	0.74 ± 0.06 b	4.93 ± 0.33 b	2.23 ± 0.03 b
Signif.	*	*	ns	***	***	***
Standard	26.34 ± 6.19 b	26.34 ± 6.19 b	3.58 ± 0.05	0.98 ± 0.03 a	3.62 ± 0.08 b	2.99 ± 0.17 b
5% RBSG	21.61 ± 4.14 a	21.61 ± 4.14 a	3.28 ± 0.67	2.30 ± 1.60 b	0.79 ± 0.03 a	2.93 ± 0.06 b
7.5%RBSG	21.23 ± 4.86 a	21.23 ± 4.86 a	3.6 ± 0.04	0.92 ± 0.09 a	0.78 ± 0.07 a	2.47 ± 0.20 a
10% RBSG	25.64 ± 4.30 b	25.64 ± 4.30 b	3.58 ± 0.04	0.83 ± 0.07 a	0.86 ± 0.08 a	3.05 ± 0.11 b
Signif.	**	**	ns	*	***	*

As shown in **Table 26**, using BSG leads to obtaining biscuits characterized by a colour shift. The biscuits in which the dried/toasted grains were used are characterized by a less intense brightness (L*) and colour tending more towards red than the biscuits in which only the dried grains were used. The variation in colour as a function of the increase in the use of BSG follows a more linear trend in the samples in which dried ones were used compared to toasted ones.

Table 26. Colour analysis of the different batches of salty snacks.

	L*	a*	b*
Standard	67.18 ± 0.45 c	6.25 ± 0.33 a	30.98 ± 0.29 b
5%BSG	59.06 ± 0.60 b	7.29 ± 0.49 b	27.55 ± 0.64 a
7.5% BSG	56.93 ± 1.76 a	7.82 ± 0.75 b	26.84 ± 1.29 a
10% BSG	57.46 ± 0.86 ab	7.86 ± 0.24 b	26.73 ± 0.70 a
Signif.	***	**	***
Standard	67.18 ± 0.45 c	6.25 ± 0.33 ab	30.98 ± 0.29 a
5% RBSG	61.79 ± 1.15 b	5.59 ± 0.07 a	25.23 ± 0.56 a
7.5% RBSG	58.70 ± 1.55 a	6.31 ± 0.58 ab	25.00 ± 0.67 a
10% RBSG	56.91 ± 1.22 a	6.99 ± 0.30 b	24.31 ± 1.23 b
Signif.	***	**	***

We create a panel test targeted to predict the possible response of the market, and the data collected is summarized in **Figure 25**. Only the 5% batches scored similar to the standard sample. The increase of BSG flour has a harmful effect on all the analysed compartments. As can be seen, the panellist did not receive the product well. The increase in fibre content only positively affected the product's texture. All the other aspects were penalized by its introduction. Even if considering only the panellist who regularly consumes wholegrain products, we do not see a clear improvement in the salty snacks scores. This

behaviour from the consumers indicates that it is risky to commercialise because not even the small market pocket we want to target is fully convinced by the product's organoleptic properties.

For this reason, we decided to stop the industrial production. The salty snacks produced have been offered as appetizers in various pubs owned by the Baladin Brewery, and the customer response was quite good. However, evaluation made in a characterized environment could cause an alteration in the purchase evaluation of a person, so further evaluation will be done.

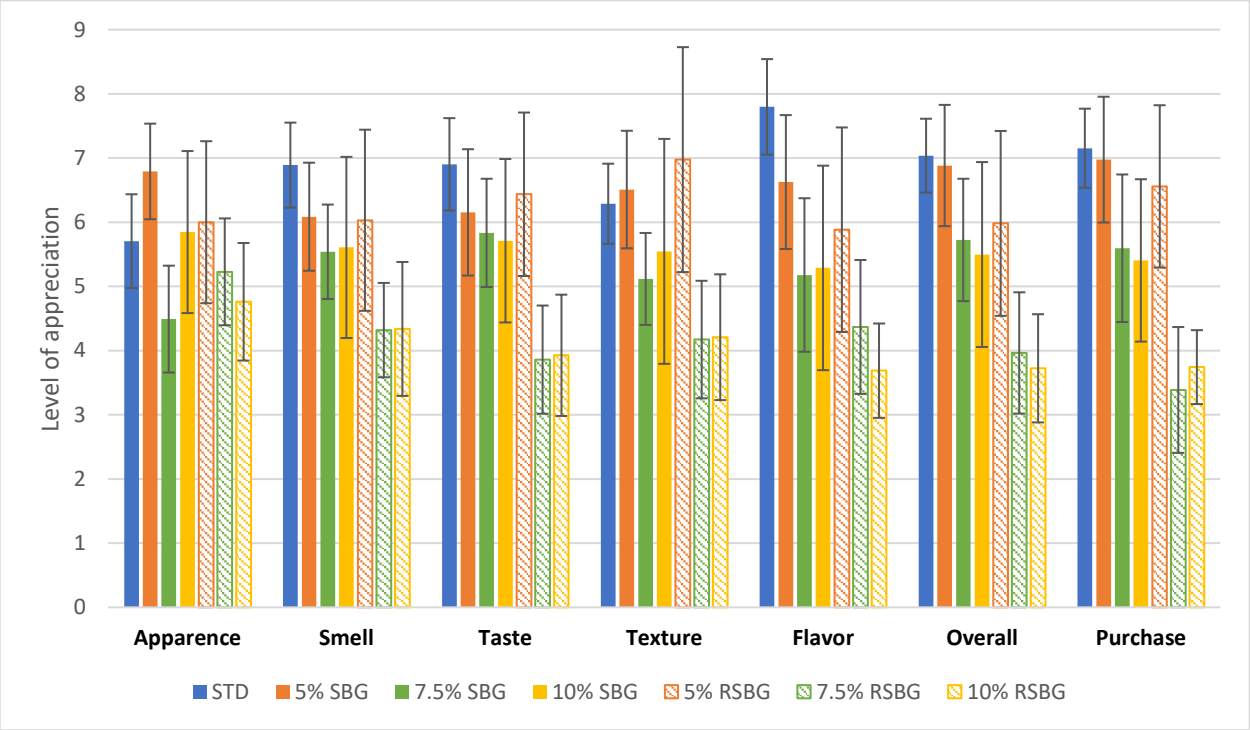
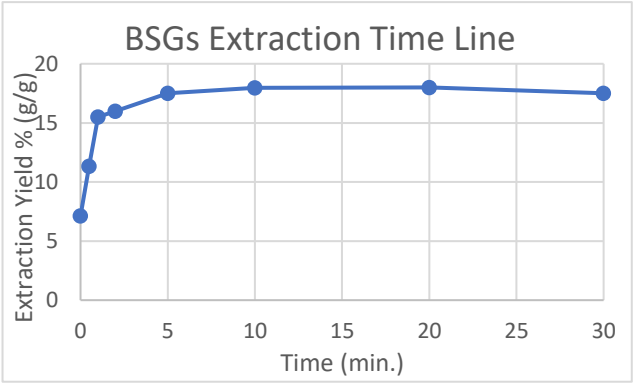


Figure 25. Panel test evaluation on the different batches of BSG-based salty snacks.

After this first attempt of using the BSG flour as is, we tried some extraction techniques to recover and concentrate valuable compounds. The extraction operated using the ultrasound system reached meagre yields. The use of different solvents did not significantly change the extraction yields; water and hydroalcoholic solution (70% v/v) reached 6.6% and 7%, respectively. Based on these results, we decided to change the extraction technology and treat the BSGs with the rotor-stator unit (Rotocavby EPIC srl). The samples taken during the extraction have been freeze-dried to estimate the solubilization of vegetal fibre. In **Table 28** are presented the data acquired.

Table 27. Extraction timeline of the BSGs homogenized with the corresponding graph.

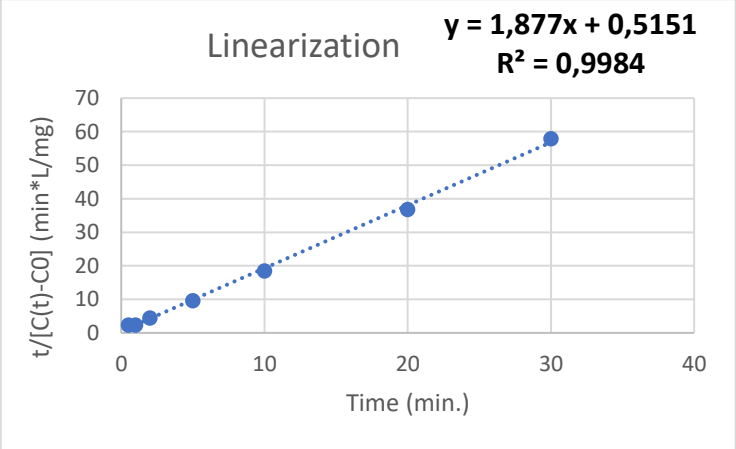
Sample time (min.)	Mass (mg)	Vol (mL)	Conc. (mg/mL)	Ext. Yield % (g/g)
0	35	98	0.357	7.143
0.5	30	53	0.566	11.321
1	38	49	0.776	15.510
2	40	50	0.800	16.000
5	42	48	0.875	17.500
10	44	49	0.898	17.959
20	45	50	0.900	18.000
30	42	48	0.875	17.500



For this extraction, we used the Peleg model to evaluate the extraction kinetics as described in the previous subchapter. In this case, to estimate the concentration of the extract, we used the mass recovered after the freeze-drying process. The linearization procedure had a high R^2 value, confirming that the model was a good choice, and the Ys was comparable to the final concentration reached after 30 min. (**Table 29**) (**Figure 26**).

Table 28. Linearization of the Table 29 BSGs data using the Peleg model.

Time (min)	*C(t) (mg/mL)*	t/[C(t)-C0] (min*mL/mg)
0,5	0.566	2.392
1	0.776	2.387
2	0.800	4.515
5	0.875	9.652
10	0.898	18.484
20	0.900	36.832
30	0.875	57.915
Constants		
k_1 = q = 0.5151	B_0 = 1.941	
k_2 = m = 1.877	Y_s = 0.890	



From the data elaboration, it is possible to see that after only 5 min. of treatment, the extraction yield reached is close to the Ys value. The maximum concentration reachable following the Peleg estimation was 0.890 g/L, and after 5 min., the experimental data acquired showed that the concentration of soluble fibres was 0.875, proving that we already reached the plateau. So from this analysis, extending the treatment time would only be a waste of time and energy. Unfortunately, all the tests made to implement the extracts collected from the ROTOCAV treatment have created many organoleptic and visual problems when introduced. We wanted to exploit the high solubility of the fibre produced, but the customers poorly received the beverage's change in colour and flavour. Even when

introduced in the protein bars, a significant recipe reformulation must be done to recreate a balance in texture and flavour. Unfortunately, all the tests made had a lower score than the products already on the market, so we decided to focus our work on a different approach.

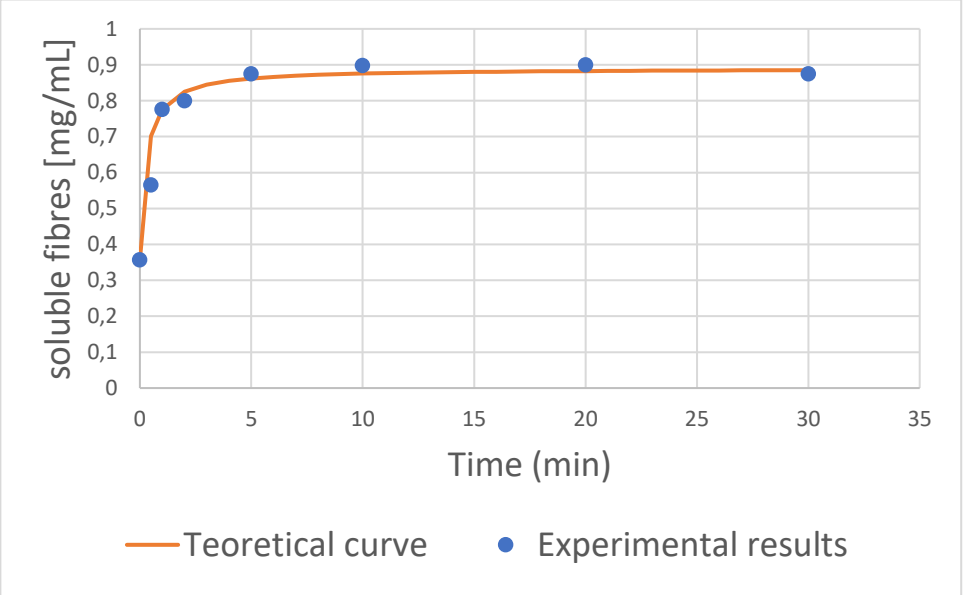


Figure 26. Superimposition of the theoretical curve created using the Peleg model (orange curve) and the experimental data acquired (blue dots).

The extraction technique operated by Heallo S.r.l produces a fine powder with a less prominent flavour, maintaining a high solubility even in a pure water solvent. The first application tried was on the fruit juices. The Baladin already produces a line of three juices, so we first tried implementing JaxPlus® in those recipes.

Figure 27 shows photos of freshly produced fruit juices. As can be seen, when the concentration of fibre used in the formulation increases, the colour darkens. The table shows the values obtained from the spectrocolourimetric analysis to understand these variations better. For all the juices studied, except for pear, statistically significant differences were observed for all the parameters analysed. Generally speaking, as the use of fibre increases, a decrease in brightness (L*) and an increase in the parameters a* and b* are observed with overall colours that tend more towards green and yellow respectively (**Table 29**).

From the results obtained, it was highlighted that pear fruit juice is not very suitable for its functionalization with fibre since the colour tends to change a lot even at the lowest concentration, but above all, unpleasant herbaceous notes are perceived in the finished product. Regarding apricot and peach, the highest concentration (3%) was discarded for both juices because the product's structure appears too gelled and bitter notes are perceived.

Figure 27. Fruit juices added with JaxPlus fibre.

Table 29. The colourimetric analysis results on the juices are summarized. L* indicates the brightness of the sample (0 – black; 100 - white); a* indicates the absorbance of the red spectrum red when positive and green when negative; b* indicates the absorbance of yellow when positive and blue when negative.

Pear												
	L*				a*				b*			
Standard	19.81	±	0.30		-1.25	±	0.15	a	4.50	±	0.38	a
1% Fibre	19.61	±	0.05		-0.69	±	0.15	b	7.57	±	0.15	b
1.5% Fibre	19.54	±	0.27		0.05	±	0.30	c	9.48	±	0.25	c
3% Fibre	19.51	±	0.54		2.45	±	0.03	d	14.04	±	0.14	d
Signif.	ns				***				***			
Aprocot												
	L*				a*				b*			
Standard	19.14	±	0.10	b	5.42	±	0.07	b	18.10	±	0.25	b
1% Fibre	17.94	±	0.21	a	4.79	±	0.13	a	17.32	±	0.24	a
1.5% Fibre	18.93	±	0.09	b	5.94	±	0.16	c	19.22	±	0.08	c
3% Fibre	17.97	±	0.28	a	6.37	±	0.08	d	17.60	±	0.26	a
Signif.	***				***				***			
Peach												
	L*				a*				b*			
Standard	17.62	±	0.23	a	0.78	±	0.02	a	11.94	±	0.36	a
1% Fibre	20.74	±	0.03	b	0.65	±	0.07	a	13.48	±	0.10	b
1.5% Fibre	20.41	±	0.52	b	1.15	±	0.10	b	13.41	±	0.56	b
3% Fibre	21.2	±	0.78	b	3.73	±	0.12	c	15.91	±	0.74	c
Signif.	***				***				***			

In addition to these chromatic variations, lower colour saturation is also observed, with shades that tend more towards grey rather than pure colour (also called dirtier colours - Chroma parameter of the colour). Regarding pear fruit juice, each addition of fibre leads to a statistically significant colour variation, while in the case of apricot and peach, similarities are observed between the sample at 1% and 3% fibre and between 1% and 1.5% fibre respectively.

Figure 28 shows the photos relating to the standard juice (not enriched with fibre) and the juice functionalized with the three different fibre concentrations after 24 h. of shelf-life. As can be seen, regardless of the juice used, as the fibre concentration increases, a darker finished product is obtained, but above all, a deposit is created due to the gelling of the fibres, which the final consumer does not appreciate.

Figure 28. Comparison of the different fruit juices functionalized with JaxPlus fibre after 24 h. (A) Pear; (B) Apricot; (C) Peach.

Therefore, in the second phase, where a group tasted the product of potential consumers, only apricot and peach juices were analysed in concentrations of 1% and 1.5% fibre. The panel test results are summarized in **Figure 29** for the apricot juice and **Figure 30** for the peach juice. Before tasting the juices, the panellist answered some questions about their shopping habits and interests. These questions showed us a high interest in sustainability and implementing circular practices. From the results collected, introducing the JaxPlus powder in the apricot juice clearly hurts the final product's organoleptic properties.

In contrast, the enriched peach juice had an overall score comparable to the standard juice. Peach juice better supports the fibre flavour, and together they create a new balance with some nut notes and the end. Unfortunately, even the best result scored lower regarding the desire to purchase. From these results, we understood that changing and creating a designed juice to introduce JaxPlus is necessary. It is essential to balance the flavour of the enriching powder. Otherwise, the beverage will not reach the organoleptic standard desired.

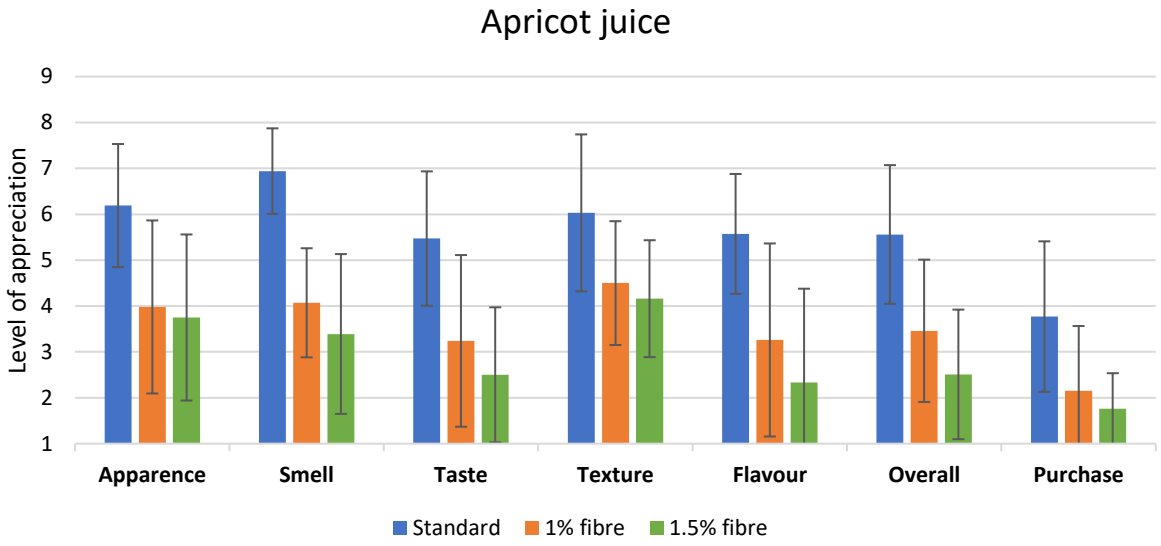


Figure 29. Result of the panel test on the apricot juices enriched with JaxPlus.

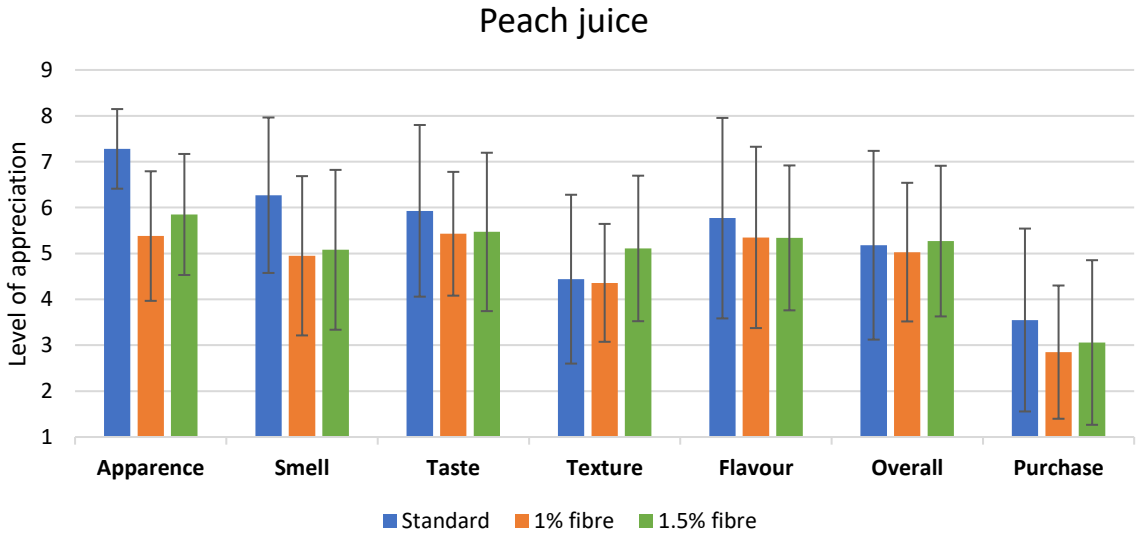


Figure 30. Result of the panel test on the peach juices enriched with JaxPlus.

The JaxPlus powder has also been introduced in some baked goods to evaluate its application on a solid good. From the starting test, it was clear that the JaxPlus powder, when introduced in high quantities, would change the texture of the panettone, reducing

the alveolus dimension of the dough causing a net reduction in fluffiness of the product. The recipe used (**Table 18**) reached the maximum quantity of JaxPlus without negatively affecting the product's texture. The p-rich product has been evaluated and did not show significant organoleptic differences compared to the p-standard version. When this result was achieved, we started the clinical trial. All the volunteers have followed a strict.

Consuming panettone enriched with soluble fibre obtained from a by-product of the brewing process determined lower glucose and insulin responses and higher satiety than a traditional panettone. The enriched product was well tolerated and did not cause any adverse effects.

Consuming foods enriched with arabinoxylans determined a lower increase in serum glucose and insulin values compared to the corresponding food not enriched with fibre. The data acquired from the blood samples are summarized in **Table 30**.

Table 30. Glucose and insulin average values after the panettone intake.

	P-standard	P-rich
Glucose (mg/dL)		
0	86.4±7.00	86.4±5.46
15	97.5±10.4	94.0±13.1
30	110.9±17.1	105.5±14.7
45	102.5±18.4	99.4±19.8
60	102.0±15.7	89.0±23.7
90	89.9±16.1	74.6±19.4
120	81.1±9.85	72.1±14.0
AUC (mg*min/L)	11520.0±1206.7	10453.5±1543.3
Insulin (μU/mL)		
0	4.85±2.37	4.36±3.31
15	9.44±3.63	8.78±3.07
30	17.8±9.86	16.3±3.67
45	21.2±8.33	17.0±6.20
60	21.2±10.2	14.1±4.62
90	18.7±11.0	15.0±7.52
120	12.4±6.01	10.1±5.19
AUC (μU*min/mL)	1985.8±724.5	1583.3±379.5

These results show that the p-rich version of the Panettone creates a lower and late glycaemic peak after the ingestion. From the Area Under the Curve (AUC) value, it is possible to evaluate the reduction of the described parameters during the whole digestion. The results are in line with many other studies investigating the interaction between the

presence of specific hemicellulose polymers, such as arabinoxylans, with the digestion activity. The consumption of foods enriched with arabinoxylans determined a lower increase in serum glucose and insulin values with respect to the corresponding food not enriched with fibre [66][67][68][69].

The consumption of p-rich compared to the standard product increased the satiety and reduced the desire to eat after 3-4 h. from the test. In **Table 31**, the data acquired from the volunteers is presented.

Table 31. Appetite scores following the tests.

	P-standard	**P-rich**
Hunger		
60	3.10±1.85	3.60±1.78
120	5.50±2.72	4.20±2.30
180	5.80±2.44	4.60±2.84
240	7.40±1.84	5.10±2.96
AUC (unit*min)	993.0±342.8	789.0±439.8
Fullness		
60	5.90±1.73	6.60±1.84
120	4.50±2.07	6.00±1.82
180	4.40±2.22	5.70±2.41
240	3.00±2.83	5.00±2.71
AUC (unit*min)	783.0±340.7	1050.0±367.7
Satiety		
60	6.70±1.16	7.30±1.49
120	5.80±1.99	6.80±1.48
180	5.40±1.58	6.90±1.60
240	3.40±2.07	6.70±1.49
AUC (unit*min)	975.0±245.9	1242.0±253.1
Desire to eat		
60	4.90±2.69	5.00±2.83
120	7.00±2.45	5.50±2.84
180	8.50±1.18	5.50±2.72
240	9.50±0.71	6.20±2.57
AUC (unit*min)	1362.0±279.4	996.0±484.1

Satiety is a complex and dynamic process influenced by multiple factors, including the physical and mental status of the patient, central and peripheral mechanisms, as well as the characteristics of food [70], such as macronutrient composition, dietary fibre content, energy density, portion size, and palatability [71][72]. Dietary fibre leads to satiety by adding bulk

and increasing the viscosity of the faecal mass along the gastrointestinal tract. Adding dietary fibre leads to decreased energy intake and enhanced expression and production of satiety hormones, such as PYY and GLP-1 [73]. However, few data are available about the effects of arabinoxylan-enriched products on satiety, with conflicting results [74][67][75][76].

The absence of short-term differences in satiety in our participants could be explained by the high-fat content of panettone, which delays the digestive process and makes it impossible to discern the effects of arabinoxylan per se from the effects of the food matrix. Additionally, the early stages of satiety are primarily influenced by cognitive factors, such as previous experiences with the same food, and by oro-sensory factors, i.e., taste, texture, flavour, aroma, and palatability [77]. However, the delayed effect on satiety and desire to eat is noteworthy as it may affect daily dietary energy and nutrient intake since satiety affects the period between meals and potentially the amount of food consumed at the subsequent meals of the day [78]. This suggests that including this small amount of soluble fibre in food products could potentially contribute to appetite control and better dietary management.

In conclusion, the integration of a fibre derived from BSG into panettone determined better metabolic control and satiety post-prandial. Further research with a larger sample size and extended intervention periods is warranted to confirm these preliminary findings and explore the long-term effects of arabinoxylans in different food products. Unfortunately, the post-meal glycaemic peak reduction is not significant enough to encourage the consumption of the p-rich product by a diabetic consumer. However, even at the formulation's start, we knew this was a hypercaloric meal. Thanks to the fat and simple sugar content, the panettone, both in the dough and the glaze, will inevitably create a big spike in blood glucose levels. The presence of high quantities of hemicellulose in a meal causes an alteration in the digestive system. The ptyalin enzyme of the saliva does not hydrolyse these fibres; they can even pass through the extreme conditions of the stomach without significant alterations. When the arabinoxylans reach the intestine, only a fraction of microorganisms have the right group of digestive enzymes, so the release of free sugars will only be partial. The fact that most of the fibre remains untouched increases water retention and reduces faecal mass, reducing the appetite.

3.1.4 Conclusions and Future Applications

After all the different approaches tried to exploit the BSGs, it is clear that the brewing industry has wasted this by-product for too long. The abundance of it and the fact that the by-product is produced daily make it easy to find and remove all the possible supply chain problems.

As explained in detail, the biomass's main problem is the low microbiological stability when not treated promptly. The optimal option would be to have an efficient drier

connected to the lauter tun of the brewery to ensure the fastest treatment possible. Of course, this solution would be recommended only for industrial realities with a production rate above 200 hL daily. For smaller realities, the only option would be to collect the BSGs of multiple micro-breweries and process them all together.

From the results achieved during this industrial test, it was clear that BSGs have far more applications in the food industry of solid goods than in beverages. The low solubility of the fibre makes it very difficult to implement them as an additive. Also, the increase of turbidity and the formation of a thick deposit on the bottle must be explained and accepted by the consumer; this is a difficult mission to achieve. Moreover, the taste of the BSGs is far more persistent and intense while driven in a liquid medium than solid goods. Usually, when drinking, a consumer wants something refreshing and the sweet cereal notes of the BSGs are in opposition. For this reason, we decided to invest most of our time and resources in developing and producing solid goods.

The clinical trial operated on the panettone enriched with JaxPlus fibre showed that the product cannot be commercialized as a safe meal for diabetics because the amount of fats and sugars is too high. Nevertheless, the industrial test was a massive success for both flavour and concept.

When given the possibility of choice, the customer would choose the panettone enriched with fibre. This decision is driven by the interest and desire to increase global awareness on the ecology problem and food waste. Of course, this result was achievable because the overall flavour evaluation of the p-standard and p-rich panettone was the same. After the commercial launch during the winter between 2023 and 2024, 2000 p-rich panettoni have been produced and sold in detail shops in Italy. Since the market's response has been so reassuring, the company plans to increase its production for next winter, reaching 8000 p-rich panettoni for the Italian and global markets [79].

The possible applications of the BSGs exceed the food industry and can be used in many other sectors thanks to their abundance and cost efficiency. The future studies the brewery will explore aim to use it as bio-feed for cellular cultivation of bacteria or fungi. This field is fascinating since those cultivations are exploited to produce valuable compounds such as drugs or bio-fertilizers. In the following years, we will further investigate the possibility of producing natural fertilizers from BSGs. The bio-fertilizers produced will be tested in the hops and barley fields used to produce the raw materials the brewery needs. The final desire is to create a complete circle for the raw materials used, reducing food production costs and using chemicals for the correct development of hops and barley.

The same approach will be tested for micro-algae propagation with fertilizing applications. Of course, in this case, since the microorganism operates the chlorophyll photosynthesis for its sustain, it will not be fed with the BSGs but use the CO_2 produced by the yeast during the fermentation process. Usually, the CO_2 gas is lost in the environment

but could be recovered. The gas developed the fermentor is a complex mixture composed primarily of carbon dioxide and ethanol followed by traces of other compounds like fermentation esters and hops EOs. It is important to note that while carbon dioxide is a desired byproduct for carbonation, some other gases, such as hydrogen sulfide, can be undesirable in excess amounts as they may impart off-flavours to the beer. Brewers take measures to control fermentation conditions, yeast health, and other factors to minimize the production of unwanted compounds and ensure a clean and flavourful final product.

3.2 Brewery spent yeast (BSY)

Brewery spent yeast refers to the residual yeast cells and other by-products generated during the fermentation process in beer production. This substance is a complex mixture of various organic and inorganic components, resulting from the metabolic activities of yeast cells as they convert fermentable sugars into alcohol and carbon dioxide. The composition of brewery spent yeast can vary depending on factors such as the type of beer being brewed, the fermentation conditions, and the specific yeast strain employed.

Towards the conclusion of fermentation, the yeast starts to segregate in the tank through flocculation. Thousands of yeast cells cause this event, aggregating into clusters known as 'flocs,' which then either ascend to the surface (in top-fermenting) or settle at the vessel's bottom (in bottom-fermenting) [80]. Effective flocculation is crucial in an industrial yeast strain as it allows for multiple reuses of the yeast during the brewing process [81]. A minor quantity of yeast from the preceding fermentation is employed to initiate the subsequent one, a practice referred to as re-pitching.

At its core, BSY primarily comprises spent yeast cells, which are the remnants of the *Saccharomyces cerevisiae* or other yeast strains used for fermentation. A high content of protein, nucleic acids, lipids, and cell wall components characterises these spent cells. In particular, the protein content is noteworthy, as yeast cells are rich in amino acids and peptides. The spent yeast may also contain traces of unfermented sugars, residual malt extract, hop compounds, and various fermentation by-products.

The cell wall of spent yeast is a complex structure composed of polysaccharides, glucans, and mannoproteins. These components contribute to the overall texture and viscosity of the spent yeast. The mannoproteins, in particular, are known for their ability to interact with other substances, impacting the yeast slurry's overall stability and rheological properties.

Furthermore, brewery spent yeast may contain non-cellular elements such as trub, which consists of coagulated proteins, polyphenols, and hop resins. Trub is typically separated from the spent yeast during brewing, but some residual material may remain.

Regarding inorganic components, brewery spent yeast can contain minerals and trace elements originally in the wort. These include potassium, magnesium, phosphorus, and

various metals. The concentration of these elements can vary based on the composition of the wort and the yeast's ability to assimilate and accumulate them during fermentation.

Managing BSY is a crucial aspect of the brewing process, as it represents a substantial by-product that requires proper handling to prevent environmental issues. Many breweries recycle spent yeast for various purposes, such as animal feed, nutritional supplements, or even as a source of bioactive compounds. Understanding the intricate composition of BSY is essential for optimising its utilisation and minimising its environmental impact in the context of sustainable brewing practices.

After the BSGs, the spent yeast is the second most abundant by-product exiting a brewery. It is estimated that for every 10'000 hectolitres of beer, 15 tons of BSY must be disposed of [82]. BSY is a valuable source of vitamin B complex, proteins and other compounds with nutraceutical applications. For this reason, it already found some applications in the food and non-food industry [83].

In essence, spent yeast, an often overlooked residue from the brewing sector, possesses numerous potential applications. As the focus on waste reduction and environmental impact grows among food and beverage producers, brewer's spent yeast emerges as a precious raw material, given its cost-effectiveness and rich nutrient content. This by-product holds significant potential for diverse industries, including creating nutritional supplements, functional food ingredients, and other value-added products.

3.2.1 BSY composition

The recovered yeasts are the remaining cells that have operated the fermentation process, transforming wort into beer. Fermentation is a stressful process for the microorganisms living in an anaerobic environment with an acidic pH around 4.5 and a high ethanol concentration. For this reason, the vitality of the cells recovered is relatively low; some have already started autolysis. In this final fermentation phase, the yeast starts to aggregate in a process called flocculation.

Flocculation is described as the tendency of yeast cells to bind together [84]. This bonding facilitates the convenient removal of yeast from the 'green' beer towards the conclusion of fermentation, either from the top or bottom of the vessel, depending on the yeast strain. Yeast flocculation must occur at the appropriate time, typically near the end of the bulk fermentation in the brewing process. Untimely or incomplete flocculation can adversely affect the fermentation process, resulting in a slower and 'sluggish' fermentation[85].

Yeast flocculation is a complex phenomenon influenced by the expression of specific genes, namely FLO1, FLO5, FLO8, and FLO11 [80]. However, these genes exhibit high variability and differ among yeast strains. Additionally, flocculation is affected by factors influencing the composition and morphology of the cell wall, such as nutrient availability, dissolved oxygen levels, fermentation temperature, and yeast handling and storage

conditions. Conditions that increase cell collisions in the medium, such as stirring, can also impact flocculation [80].

In the food industry, brewer's spent yeast is predominantly employed as a flavour enhancer or a nutritional supplement. Its considerable nutritional value makes it versatile for various applications within the functional food industry. BSY is a by-product with a substantial protein content, ranging from 45% to 60%, and is generally recognised as safe (GRAS). According to a study conducted by Vieira et al. in 2016, the protein content in BSY reached up to 64.1% (dry weight), with a notable proportion of essential amino acids, as indicated in **Tables 32** and **33**. The study found that 40% of the total amino acid count consisted of essential ones, aligning with the requirements established by FAO/WHO (1990) for adult humans [86]. Typically, sulfur-containing amino acids (methionine and cysteine) serve as limiting factors; however, this investigation revealed that S-amino acids surpassed the target set by FAO/WHO [87].

Additionally, the study identified a high concentration (34%) of flavour-enhancing amino acids, including glutamic acid, aspartic acid, glycine, and alanine. The elevated levels of these specific compounds suggest the potential to utilise BSY or BSY extracts to enhance flavour.

The carbohydrates, after the proteins, are the second most abundant component in the BSY. This family is composed of some traces of unfermented sugars that remained in the wort at the end of the fermentation process, but the majority are membrane polysaccharides. A significant portion of BSY polysaccharides, totalling around 83% of the overall polysaccharide content, is insoluble. Even after a rigorous alkaline treatment, these polysaccharides can only be partially dissolved, resulting in an insoluble fraction that accounts for 24% of the total polysaccharides [88]. The primary components of BSY polysaccharides include β-glucans, mannoproteins, and glycogen. Although chitin is present in the cell walls in a small concentration, it significantly contributes to the insolubility of the polysaccharides. In the context of Saccharomyces cerevisiae, the cell wall constitutes approximately 25–50% of the volume and 15–30% of the cell's dry weight. According to Pinto et al. [88], the cell wall is composed of 50–60% β-glucan, 35–40% mannoproteins, 1–3% chitin, and 1–23% glycogen [89].

The cell wall's β-glucan component primarily consists of a β-(1,3) backbone, constituting approximately 85% of the yeast glucan component, along with a minor β-(1,6) fraction (up to approx. 3%) [90]. β-glucans have been associated with various health benefits, including prebiotic, anti-inflammatory, anti-diabetic, anti-cancer, and immune-modulating effects [91]. In terms of functionality, β-glucans alter the texture, mouthfeel, and rheological characteristics of food products. They are also effective stabilisers, emulsifiers, and potential fat replacers [92]. Notably, β-glucans recovered from BSY, when compared with commercially available β-glucans from baker's yeast, demonstrated better physical

properties such as higher water holding capacity, increasing the apparent viscosity and emulsion capacity [93].

Table 32. Chemical composition of BSY from different sources

Chemical Composition	Vieira et al. [87]	Cabellero-Cordoba and Sgarbieri [94]	Marson et al. [95]	Mathias et al. [96]	Jacob et al. [97]	Average
Protein	64.1±0.2	47.19	40.8	45.6	74.3±0.5	54.4
α-amino	12.9±0.1	n.d.	n.d	4.09±0.04	5.81±0.10	7.9
Ash	14.0±0.2	8.55	7.0±0.1	5.9±0.05	13.5±2.3	9.8
Fat	1.32±0.04	3.53	0.21	n.d.	0.67±0.01	1.4
Moisture	7.70±0.12	n.d.	n.d	n.d.	6.8	3.6
Carbohydrate	12.9±0.1	21.52	n.d	n.d.	14.7	16.4
RNA	4.00±0.16	7.04	1.9±0.1	n.d.	5.518±0.10	4.6
Soluble Fibre	n.d.	9.65	6.6±0.1	n.d.	n.d.	8.1
Insoluble Fibre	n.d.	2.57	n.d	n.d.	n.d.	2.6

Table 33. BSY amino acids composition [87].

Non-Essential Amino acids	% (w/w) ± St. dev.	Essential amino acids	% (w/w) ± St. dev.
Alanine	9.29 ± 0.31	Lysine	3.16 ± 0.23
Arginine	6.00 ± 0.79	Leucine	3.51 ± 0.20
Aspartic acid	5.98 ± 0.22	Phenylalanine	3.01 ± 0.01
Cysteine	2.19 ± 0.01	Isoleucine	3.23 ± 0.03
Glutamic acid	15.0 ± 0.39	Threonine	2.60 ± 0.00
Asparagine	2.00 ± 0.01	Tryptophan	Nd
Glutamine	3.13 ± 0.21	Methionine	2.28 ± 0.15
Glycine	3.69 ± 0.60		
Histidine	11.9 ± 0.80		
Proline	2.65 ± 0.03		
Serine	4.60 ± 0.21		
Tyrosine	2.15 ± 0.00		
Valine	4.49 ± 0.08		

The BSY are also a valuable source of minerals and salts. These micronutrients are essential for maintaining life; the minerals are often essential cofactors of enzymes, and without their presence, the enzymatic activity would cease. These minerals are crucial in synthesising ribosomal proteins and are vital for maintaining acid-base balance and facilitating water retention in the cell. The quantification of these compounds is presented in **Table 34** with the Recommended Dietary Allowance (RDA).

These micronutrients are essential for correct biological activity. As shown in the table above, the integration of BSY into a standard diet would be almost enough for the daily

intake of minerals. Moreover, the BSY also have quite a high concentration of vitamin B complexes. To become bioavailable, these valuable compounds must be extracted from the yeast cells' cytosol. Vitamin B complex refers to a group of eight water-soluble vitamins that play essential roles in numerous biological processes in the body. These vitamins include thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7), folic acid (B9), and cyanocobalamin (B12).

Table 34. Mineral Composition of BSY, n.q. not quantified.

Macrominerals	**Vieira et al.** [87] **mg/100 g dw**	**Jacob et al.** [97] **mg/100 g dw**	**RDA** [98]
Sodium (Na)	1228±22	88.1±0.001	1600 mg
Potassium (K)	9148±69	6248.7±0.001	2000 mg
Calcium (Ca)	27.1±0.40	16.4±0.001	800 mg
Magnesium (Mg)	273±2.31	210.2±0.001	375 mg
Trace Elements			
Chromium (Cr)	0.019±0.00	n.q.	40 μg
Iron (Fe)	1.76±0.03	3.672±0.01	14 mg
Manganese (Mn)	0.564±0.013	0.15±0.01	2 mg
Cobalt (Co)	0.030±0.001	0.252±0.01	n.a.
Molybdenum (Mo)	0.003±0.00	n.q.	50 μg
Zinc (Zn)	11.9±0.29	9.963±0.01	10 mg
Copper (Cu)	0.364±0.001	0.221±0.011	1 mg
Selenium (Se)	0.030±0.00	n.q.	55 μg

Thiamine (B1) is vital for turning carbohydrates into energy and supporting neurological functions. Riboflavin (B2) plays a crucial role in energy production, cellular function, and antioxidant defence. Niacin (B3) is involved in cellular metabolism, DNA repair, and maintenance of skin health.

Pantothenic acid (B5) is necessary for synthesising coenzyme A, which is involved in energy production, fatty acid metabolism, and the synthesis of various compounds. Pyridoxine (B6) supports brain development and function, neurotransmitter synthesis, and the metabolism of amino acids and lipids.

Biotin (B7) plays a critical role in various metabolic processes, including the breakdown of carbohydrates, proteins, and fats. Folic acid (B9) is essential for DNA synthesis, cell division, and red and white blood cell formation. Cyanocobalamin (B12) is involved in DNA synthesis, nerve function, and the production of red blood cells.

Together, the B complex vitamins act as coenzymes in numerous enzymatic reactions throughout the body, enhancing energy production, metabolism, and the synthesis of essential molecules. They are crucial for the proper functioning of the nervous system, brain

health, the formation of red blood cells, and the maintenance of overall health and well-being. The quantification of these compounds is presented in **Table 35** with the RDA.

Table 35. Vitamin B composition of BSY compared to the RDA. n.q. not quantified.

Vitamins	**Vieira et al.** [87] **(mg/100 g dw)**	**Jacob et al.** [97] **(mg/100 g dw)**	**RDA** [98]
Thiamine (B1)	n.q.	7.46±0.5	1.1 mg
Riboflavin (B2)	n.q.	10.6±0.5	1.4 mg
Nicotinic acid (B3)	77.2±1.1	78.6±2.0	16 mg
Pyridoxine (B6)	55.1±2.5	5.90±0.5	1.4 mg
Folic Acid (B9)	3.01±0.02	5.29±0.1	200 μg
Cyanocobalamin (B12)	n.q.	0.16±0.1	2.5 μg

3.2.2 Material and Methods

The Baladin brewery supplied the company Agroindustria srl with approximately 1,000 litres of spent yeasts collected at the end of fermentation contained in an IBC. The biomass appeared as a relatively dense liquid with a quantity of water around 85-90%. When the biomass arrived at the Agroindustria facility, it was spray-dried in less than 24 h. to prevent any degradation.

The spray-dryer used can remove 150L/h, consuming 2.6kW per litre removed. The high viscosity of the biomass made this process more consuming energetically and time-wise compared to other biomasses with the same water concentration. The powder produced by the spray-dryer has a beige-brown colour with a small dimension of particles and a characteristic yeast flavour. From this state, it was clear that the major problem to face for using BSY was the organoleptic balance of the final product.

We conducted some microbiological tests to evaluate the presence of spoiling microorganisms. The evaluation uses traditional microbiology techniques such as selective cultivar terrain and selective molecular biology tests like qPCR to verify and double-check the result. We evaluated the presence of *Lysteria monocytogenes*, *Bacillus cereus*, *Salmonella spp.*, *Staphylococcus aureus*, and *Clostridium spp*. Also, a total aerobic and anaerobic count has been operated using Tryptic soy agar as terrain. For each analysis, 10g of sample were weighed and introduced in a solution of Buffered Peptone Water and Listeria Enrichment Broth for 24 h. at 37 °C. After this time, 1 mL of the broth was used to plate the petri dish, and then the Petri was stocked for 24 h. at 37 °C. After one day from plating, the cell growth was evaluated, and the results were checked using the TaqMan® methodology for the selective species we wanted to screen. **Table 36** summarises the microbiological analysis made on the BSGs.

After these tests, the BSY powder samples were sent to the Procemsa R&D group, where some formulations of BSY inside protein bars were tested. Proprietary recipes have been adopted as a screening starting point.

Table 36. Tests operated on the BSG flour to evaluate the microbiological stability.

Microorganism species	Selective terrain	qPCR double check
Total aerobic count	TSA (tryptic Soy Agar)	No
Total anaerobic count	TSA (tryptic Soy Agar)	No
Lysteria monocytogenes	ALOA	Yes
Bacillus cereus	PEMBA	Yes
Salmonella spp.	RAPID S	Yes
Staphylococcus aureus	BPA	Yes
Clostridium spp.	SFP	Yes

Using the same approach applied to the BSGs, we tried to use the BSY without any treatment and as a starting material to selectively extract valuable compounds. In particular, the most abundant and valuable molecules in this biomass are the vitamin B complexes. The Procemsa R&D team tested different approaches concerning both the lisys of the cellular membrane and the purification of the proteins. A cellular lysis must be operated to free the cytosol to release the desired component. The spray-dried extract (1g) was mixed thoroughly with 10 mL of mobile phase A (consisting of 50 mM ammonium acetate/methanol in a ratio of 99:1). The resulting mixture was subjected to 30 min. of sonication at 27±3 °C using an US water bath After sonication, To evaluate the extension of the lysis we spread 10 μL of lysate on a microscopy slite to see the morphology of the cells and we plate 1 mL on an agar petri dish. After the lysis, the sample was centrifuged for 15 min. at 6000 RPM and passed through a 0.22 μm membrane filter before being injected into the HPLC system following a consolidated method for quantifying vitamin B complex [87].

Unfortunately, the purified vitamin B complexes resulted too expensive compared to the synthetic version already on the market. After these tests were conducted in collaboration with Procemsa Laboratories, we understood that the flavouring features of our by-product was too high for direct application. The BSY collected by the brewery showed a high bittering power when introduced into the goods. It can be explained by the presence of hops particles in the slurry. As explained in chapter 2.3.2, some beers are dry-hopped after fermentation, and the hops introduced tend to precipitate in the conical part of the fermentor with the yeast themselves. This co-precipitation makes the BSY collected highly homogeneous and so challenging to separate. Since most Baladin beers use this process to enhance the product's flavour, it is not the best type of BSY for reintroduction in the food industry.

3.2.3 Result and Discussion

The result of the microbiology test conducted on the spray-dried BSY showed that the final product still had some vitality. All the selective terrain used to evaluate the presence of risky pathogens resulted negative. However, the PCA petri dish showed contamination in both the aerobic and anaerobic growth with a high colony count. The results are summarised in **Table 37**.

Table 37. Summary of the microbiological analysis made on BSy to evaluate the presence of spoiling microorganisms.

Spoiling microorganism	**Selective terrain**	**Cell count on Petri**	**qPCR result**
Total aerobic count	TSA (tryptic Soy Agar)	>3 $\log_{10}$ CFU/g	Not operated
Total anaerobic count	TSA (tryptic Soy Agar)	>3 $\log_{10}$ CFU/g	Not operated
Lysteria monocytogenes	ALOA	0 CFU/g	Negative
Bacillus cereus	PEMBA	0 CFU/g	Negative
Salmonella spp.	RAPID S	0 CFU/g	Negative
Staphylococcus aureus	BPA	0 CFU/g	Negative
Clostridium spp.	SFP	0 CFU/g	Negative

The treatments carried out do not have a bactericidal effect. In fact, the total charge determined in the sample suggests a bacteriostatic effect. It should be underlined that the charge is considered relatively high, but yeasts can develop on the soil used for the total charge, therefore, this high charge is not surprising. It must still be considered that, in some cases, the morphology of the colonies can be traced back to microorganisms belonging to the *Bacillus* group (**Figure 31**).

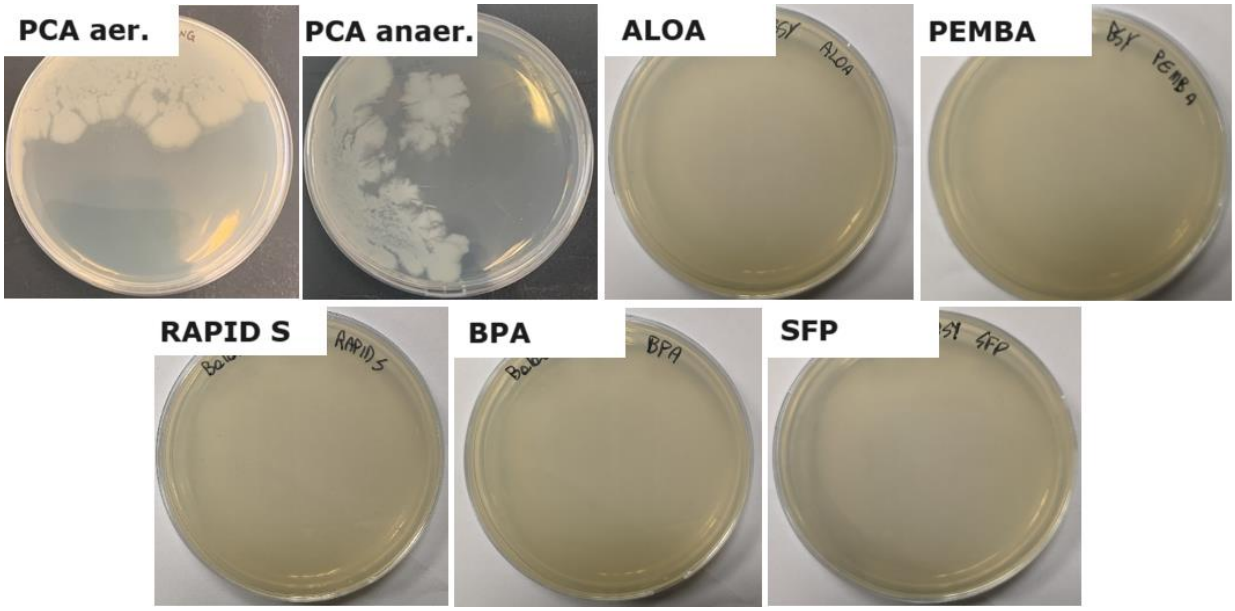


Figure 31. Petri dish with different selective terrain for the microbiological stability assessment of the BSY.

The *Bacillus* microbial group members are ubiquitous microorganisms considered environmental contaminants, often associated with air and soil. Furthermore, they are spore-forming, and their endospores resist adverse conditions. They contaminate matrices with relatively low charges. It could be interesting to explore the stability over storage time and the variability in load between different batches of different strains of *Saccharomyces cerevisiae* used in brewing.

Unfortunately, the production tests operated by Procemsa were not successful. The amount of BSY introduced in the protein bars to have a relevant supplement of proteins was detrimental to the final flavour of the product. All the internal testers preferred the classic recipe of the protein bars, and for this reason, the research was stopped.

Unfortunately, the research line on the purification of the vitamin B complex showed the impossibility of using this matrix as a source of those valuable compounds. The time and the cost needed for this process were too high compared to the already standardised methods for vitamin synthesis.

3.2.4 Conclusion and Future Applications

As presented in this chapter, the possible innovative applications of the BSY have found a dead end. The only potential application in the food industry is the production of a unique condiment used primarily in the United Kingdom and Australia (known as Marmite and Vegemite, respectively), characterized by a strong flavor and saltiness, usually eaten with bread. These products are primarily composed of BSY from local breweries and additioned with salt and some flavouring.

The strong features of Marmite/Vegemite is usually loved by people who have consumed it since the childhood, but is not appreciated by other cultures. Since the brewing industry is well spread across all the continents, all the Vegemite and Marmite industries prefer to collect the BSY from local producers, and for us, it is impossible to introduce a product like it in the Italian market. Thus, we decided to use the BSY for biogas production since it has a good carbon-nitrogen ratio for the anaerobic digestion of methane. This process starts with the anaerobic digestion operated by selective strains of bacteria and archaea. They break down complex organic compounds in the feedstock into simpler molecules in the absence of oxygen.

During anaerobic digestion, microorganisms metabolise organic matter through a series of biochemical reactions that can be summarised in the following steps:

- *Hydrolysis*: Complex organic compounds are broken down into simpler compounds such as sugars, amino acids, and fatty acids.
- *Acidogenesis*: Further breakdown of these compounds into volatile fatty acids.
- *Acetogenesis*: Conversion of volatile fatty acids into acetic acid.

- *Methanogenesis*: Methane-forming microorganisms convert acetic acid and hydrogen into methane and carbon dioxide.

All these metabolic activities transform the feedstock into biogas. The primary components of the biogas produced are methane (typically 50-70%) and carbon dioxide (typically 30-50%). Small amounts of other gases, such as hydrogen sulphide and traces of nitrogen and oxygen, may also be present. The biogas generated during anaerobic digestion is collected and stored for later use. Storage can be in gas holders, tanks, or other suitable containment systems.

Biogas is a versatile and renewable energy source that can be used for various applications since it has high energetic power and can be used easily as a fuel.

The remaining material, known as digestate, is rich in nutrients and can be used as a fertiliser or soil conditioner after anaerobic digestion.

Biogas production is considered a sustainable and environmentally friendly way to convert organic waste into valuable energy, reducing greenhouse gas emissions and providing a renewable energy source for various applications.

This process is not very remunerative compared to the reintroduction of the BSY in the food industry, but it is still a suitable way to reduce food waste and enhance the circular economy.

4 EXTRACTION OF FLAVOURING COMPOUNDS

The story of extractions is a rich and diverse narrative that spans centuries, cultures, and industries. The extraction of flavouring compounds from natural sources has been a practice deeply rooted in human history, with applications ranging from perfumery and medicine to food and industrial processes.

These procedures have ancient origins, with ancient civilisations such as the Egyptians, Greeks, and Romans using botanicals and resins for perfumes, incense, and medicinal purposes. Techniques like distillation were employed to extract EOs from aromatic plants. The knowledge of flavours recovery continued to evolve during the Middle Ages and the Renaissance. Alchemists and herbalists explored various methods of distillation and maceration to extract EOs and tinctures for medicinal and alchemical purposes. The refinement of distillation techniques in the 9th century by Islamic alchemists, such as the invention of the alembic still, played a crucial role in the efficient extraction of EOs. This knowledge was later transmitted to Europe, influencing the Renaissance alchemical practices.

The Industrial Revolution in the 18th and 19th centuries marked a significant turning point in the extraction of aromatic compounds. Steam distillation and other advanced techniques were developed, allowing for mass production of EOs and other volatile extracts, opening new possibilities for entrepreneurs. The 19th and 20th centuries witnessed the growth of the perfume and cosmetics industries thanks to scientific progress. Aromatic extractions became central to perfumery, with perfumers using a wide array of natural and synthetic compounds to create unique fragrances. The recovery of those compounds from biomasses became integral to drug development in the pharmaceutical industry. Many traditional remedies were studied, leading to the isolation of active compounds. The extraction and purification of active molecules have been essential for developing modern drugs.

The evolution of this topic continues with ongoing scientific research and exploration of new sources of interesting components. This includes discovering unique plants and microorganisms that produce novel aromas, expanding the possibilities for various industries. In essence, the story of flavours extractions is a tale of human ingenuity, exploration, and a deep appreciation for the sensory qualities of nature. It has shaped industries, cultures, and how we experience the world. From ancient practices to modern innovations, the extraction of aromatic compounds remains an art and science that connects us to plants' essence and diverse fragrances.

Today, the variety of extraction techniques employed is vast, including steam distillation, solvent extraction, cold pressing, and supercritical fluid extraction. These methods are used in food, cosmetics, pharmaceuticals, and aromatherapy industries. With a growing

emphasis on sustainability and natural products, there is a renewed interest in traditional extraction methods and sustainable sourcing of aromatic compounds. The awareness of the environmental impact of certain extraction practices has led to the investigation of eco-friendly methods. It remains vital that the new green extraction reaches comparable extraction yields with the traditional methods. Otherwise, they will not be economically significant and interesting for the industries.

One of the primary drivers behind the increasing popularity of green extraction is the global emphasis on sustainability. Traditional extraction methods often involve the use of large amounts of solvents, which can have detrimental effects on the environment. Green extraction methods aim to minimise these impacts, making them more environmentally friendly. As consumer preferences shift towards natural and eco-friendly products, industries such as cosmetics, pharmaceuticals, and food are adopting green extraction to meet this demand. Green extracts align with the growing consumer awareness of the impact of industrial processes on the environment and human health. Stringent environmental regulations have driven this new trend, and increasing scrutiny of industrial practices has pushed companies to adopt greener alternatives. Green extraction methods often align with regulatory requirements, providing an ethical and compliant approach to obtaining valuable compounds. Continuous research and technological advancements have led to the development of novel green extraction techniques. These methods utilise alternative solvents, such as supercritical fluids or water, and energy-efficient processes, showcasing the potential for innovation in sustainable extraction practices.

One of the primary challenges green extraction methods face is the economic feasibility of adopting these techniques on a large scale. Initial setup costs for equipment and technology may be higher, and industries need to assess the long-term economic viability of transitioning to green extraction. Green extraction methods often involve complex processes that require optimisation for specific applications. Achieving the desired yield and purity of extracted compounds while maintaining environmentally friendly practices poses a significant scientific and engineering challenge. Scaling up green extraction processes from laboratory to industrial levels can be challenging. Ensuring consistent and efficient extraction on a larger scale without compromising the green principles requires innovative engineering solutions and process optimisation. There is a need for increased education and awareness within industries about the benefits and techniques of green extraction. Many companies may resist change due to established practices, and overcoming this inertia requires concerted efforts in education and industry collaboration. Green extraction methods have gained substantial popularity as industries strive to align their practices with environmental sustainability. However, overcoming economic, technical, and scalability challenges remains crucial for the widespread adoption of green extraction. The ongoing collaboration between scientists, engineers, and industries is essential to drive

innovation, optimise processes, and address the significant challenges associated with implementing sustainable extraction practices.

This trend has also been verified in the panel tests made for both the panettone and the biscuits using BSGs. The customer prefers to buy and consume products that have been produced with a green and sustainable approach.

In this research, the focus has been driven towards the extraction of the EOs derived from hops because they are the most valuable biomass for a brewery. As explained in the subsection 2.3.2, the traditional method for EO extraction is called dry hopping. This methodology can be summarised as an extended maceration of the biomass in the beer at the end of the fermentation, so it has a low ethanol concentration. Of course, this technique became popular during the Middle Ages when the technical advances and equipment were limited. Nevertheless, now it is possible to use innovative technologies to extract and purify the desired compounds, augmenting the possible industrial application.

This study focuses on extracting lipophilic aromatic compounds from the matrix commonly used in breweries: hops, citrus peels and coriander seeds. For economic reasons of possible implementation on an industrial scale, the technologies tested were optimised for hops extraction.

Every year, more than 130,000 tons of hop cones are processed into hop pellets to supply the growing brewing industry [99]. Hop cones are the inflorescences of the female plant *Humulus lupulus* L., which belongs to the *Cannabaceae* family. This plant is a perennial climbing vine, native to Europe, Southwest Asia and North America, and requires a temperate climate [100]. There is only one harvest per year, and this occurs between the end of August and the first weeks of September. Almost 97% of hops produced are destined for brewing purposes [101]. The USA leads world production with 44.3 tons, followed by Germany and the Czech Republic with 39.0 tons and 6.1 tons, respectively [9]. The glands of the female plant produce and secrete lupulin, a small yellowish resinous powder found at the base of the inflorescence brackets. These glands have a high concentration of EOs and resins, which are responsible for the organoleptic properties of beer [5]. They serve as storage for the resins and EO synthesised by the plant [6]. The production of these compounds depends on the hop variety and plays a crucial role in the final fragrance [104]. At the end of the harvest, the hop cones have a water content of over 75%. In this state, decomposition and mould growth can occur very quickly, so it is essential to dry the material as soon as possible. Keeping the temperature as low as possible throughout the process is essential to preserve the EO and avoid losses. When moisture content reaches 10%, the matrix can be stored for one year in a controlled atmosphere (ca. 6 °C) to prevent oxidation. Typically, however, the cones are converted into pellets, as they have a much higher density and are more practical to use. Pelletization is carried out in two successive

steps: first, the cones are crushed and then conveyed to an extruder, which produces the typical cylindrical shape of 2-3 cm long and 5 mm in diameter [8].

Hop cones are of complex chemical composition, as listed in **Table 38** [2,9,10]. Since the cones are mainly composed of bracts, the amounts of proteins, cellulose and polyphenols are very high compared to those of resins and EO, which are the active aroma molecules. This fact shows how potent these molecules are in terms of flavour, especially in beer, where the hops represent less than 5% of the ingredients used but can still characterise the final product to such an extent.

Table 38. Average chemical composition of dried hop cones.

Constituent	Content (%)
Cellulose	45
Total resins	15-30
Proteins	15
Moisture	10
Ash	8
Polyphenols (tannins)	4
Essential oil (EO)	0.5-3
Monosaccharides	2
Pectins	2
Amino acids	0.1

The most critical constituents for brewers are resins and EO. The first class of compounds can be divided into hard and soft resins, depending on their solubility in hexane. The latter are divided into α- and β-acids. The α-acids are primarily responsible for the bitter taste in beer [11].

The EO are, by definition, the lipophilic volatile fraction of the hops and are responsible for the aroma imparted to the beverage. The aroma can change significantly depending on plant variety and growing environment [12]. Many studies have reported that this volatile fraction is a complex mixture of more than 200 components [13], and even more recently, the use of comprehensive multidimensional gas chromatography and a flame ionisation detector (FID) has led to the detection of about 1000 different compounds [14].

In addition to their flavouring features, the components of hop EO can be exploited for a wide range of purposes, including therapeutic, cosmetic, and nutritional applications [108][109]. Furthermore, particular interest has been shown in their several biological properties; they demonstrate antioxidant, antimicrobial and antiviral attributes [110][109][111], as well as antitumoral effects [112] and uses in pest control [113].

The profile of the volatile fraction of hops mainly depends on genetic and environmental factors and the extraction protocol applied. The traditional method suggested by ASBC

(American Society of Brewing Chemists) consisted of steam distillation lasting up to 7 hours. This method is highly time and energy-consuming and is only applied to analyse hop quality. The conventional methods generally applied to recover the EO are steam distillation, hydro distillation, maceration and absorption [114][115].

The conventional extractions used to recover natural products generally include several unitary operations, including plant pretreatment (drying, comminution, etc.), material extraction, and downstream processes (separation, evaporation, etc.). These steps are generally time and energy-consuming when not optimised, but the extraction stage, in particular, can involve considerable water and/or solvent consumption, generating a large amount of waste materials [116]. The conventional extraction of EO from the hops consists of steam distillation that lasts up to 5 h. [14]. This process is very time and energy-consuming and uses a traditional heating method. In the last decade, companies have invested considerable resources into optimising their effective practices thanks to an increasing concern about the environmental situation [117]. These new strategies, which follow the "green extraction" approach, must enhance process intensification and extraction efficiency while using safe and sustainable solvents.

With the increasing concern for environmental issues, companies have adapted their ethical values and productive practices in recent years. The pursuit of global sustainability and "green industry" requires real solutions that can minimise environmental impact while maintaining product quality [118][117]. In this context, process intensification is imperative, enhancing extraction efficiency, producing safer extracts of higher quality and reducing unitary operations. These great expectations rely on enabling technologies, such as microwave (MW), ultrasound, and super- and sub-critical fluid extractions [119][63][120][121], together with the application of new green solvents [122][123][124]. This type of approach, which has emerged over the last decade, has now suitably evolved towards up-scaling [125][61][126][127][128]. Protocols that have been optimised on the lab scale require pilots and pre-industrial prototypes, which are crucial steps to bridge the gap between academia and industry and can consistently pave the way towards process sustainability.

Several works have reported the application of supercritical CO_2 (sc-CO_2) as part of transposing the awareness of ecological and environmental issues to the recovery of the hop volatile fraction. This technique exploits the peculiar polarity and viscosity that CO_2 achieves in the supercritical state. In this form, it behaves similarly to ethanol and methanol, extracting both α-acids and the lipophilic fraction (also volatiles) [129][130][131]. The extract obtained using this technique is stable for long periods and can be introduced directly into the brewing process as CO_2 is desired in the final product. Nevertheless, the recovered product represents a mix of several classes of compounds and is not purely composed of volatiles.

The main objective of this research line is to provide a new way to aromatise beverages using natural ingredients. We had to aim for good extraction yields both for yields and the quality of the extracts produced. From the start, we knew that this extract would be used for the aromatisation of both alcoholic and non-alcoholic beverages. Applying an aromatising agent to an alcohol-free product creates more problems for the risk of contamination. Ethanol, even in low concentrations above 3% vol/vol, makes the proliferation of the majority of risky pathogens impossible. Every time a new ingredient is introduced during the production of a beverage batch, it is a possible source of contamination. Not only because to introduce the ingredient, you have to expose the batch to the environment but also because the ingredient itself can be a carrier of spoiling microorganisms, which can also be applied to extracts.

We decided to investigate the possible applications of technologies that would not use organic solvent during the extraction but only water. This decision makes optimising the processes much harder because the extraction yields have to be comparable to other less eco-friendly technologies. However, the extract produced has a broader range of applications since it can be introduced more easily in solid foods and beverages, even alcohol-free ones, which is one of the few markets with net growth in the last decade.

4.1 Multiple Effect Fractional Condensation Under Vacuum (MEFCUV)

The extraction was carried out using Multiple-Effect Fractional Condensation Under Vacuum (MEFCUV) (**Figure 32**), produced by the company Tropical Food Machinery (Busseto, Italy). The extractor comprises two major components: the charging tank and the condensation apparatus. The charging tank has an internal volume of approximately 150 L and a mechanical paddle mixer with scrapers. The main tank has an outside jacket connected to a vapour generator to heat the vessel. The vessel has the purpose of creating a vapour that is rich in flavour compounds and is able to work under a vacuum, thus reducing the boiling temperature. The charging tank is connected to the condensation apparatus, which is composed of four condensation columns refrigerated at the top and heated at the bottom to enhance fractional distillation. The whole system is connected to a vacuum pump that can reach −900 mbar, which is connected to the charging tank and the first three columns. A simplified scheme of MEFCUV is reported in **Figure 33**.

Figure 32. Multiple-Effect Fractional Condensation Under Vacuum (MEFCUV) (Tropical Food Machinery srl, Busseto IT).

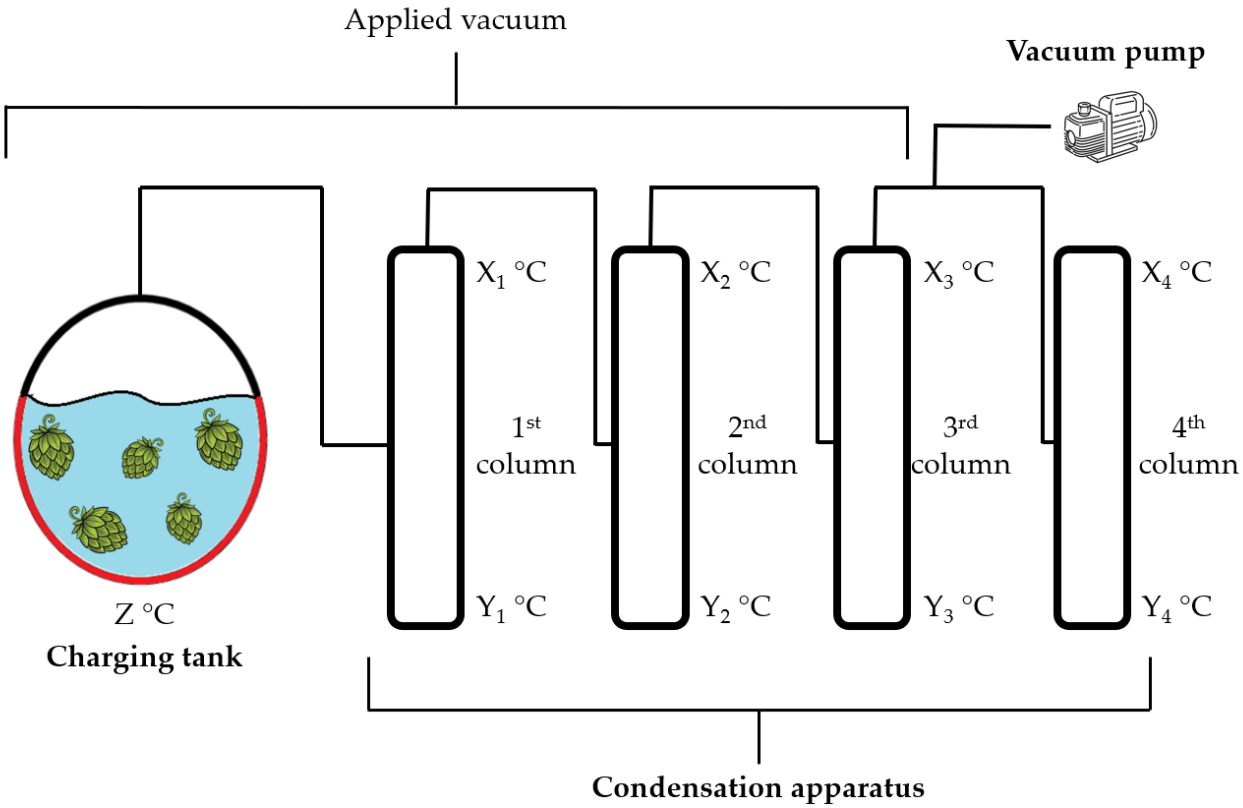


Figure 33. Scheme of the MEFCUV system. Starting from the left: charging tank; condensation apparatus; vacuum pump. Temperature in each part of the system can be regulated independently.

The system described works as a refinery tower that separates the aromatic compounds depending on their boiling point. When the aroma recovery unit is activated, the volatiles extracted by the matrix loaded in will start to separate themselves in the different condensation columns depending on their boiling point. If the system is appropriately set for the biomass, it is possible to concentrate the majority of water in the first condenser and all the aromatic compounds in the last three columns. This technique can selectively extract the terpenes, and it can also separate the volatiles into two fractions, one enriched in sesquiterpenes, such as caryophyllene, farnesene, and humulene, and the other enriched in monoterpenes. This separation is caused by the difference boiling points of these two families of compounds. The production of separated extracts increases potential applications in brewing as well as in other beverages and perfumes. Myrcene, for example, is often used in the perfume industry, especially in men's fragrances, because of its pungent aroma. In this case, when the floral and grassy scents of sesquiterpenes may be undesirable, a heavily enriched fragrance with myrcene would be preferable.

4.1.1 Materials and Metods

The hops used belong to the Citra and Chinook varieties. The Chinook hops were harvested at the end of August 2020 in the Piedmont region (Italy). After harvesting, the cones were dried for 12 h at 42 °C, which decreased the average humidity to 8%. The material was left at room temperature for 24 h. before pelletization. Dried cones were left to rest for 24 h., and their humidity rose to 10/11%. The hops then underwent pelletization. This process started with a fine mincing of the cones, and the collected powder was then pressed through an extruder to create the pellets. The temperature increased substantially during pelletization and reached 50 °C. At the end of processing, the hops were stored under a nitrogen atmosphere at 6 °C for their preservation. Citra hops were bought from the Yakima Chief Hops company and kept at 6 °C during storage. The hops present themselves in pellet form, a small cylinder of 1/2 cm in length and 0.5 cm in diameter. The water content was evaluated via thermogravimetric analysis by leaving an average weight of 1.5 g of biomass at 100 °C overnight and measuring the relative weight loss. The tests were performed in triplicate, and the results are shown in **Table 39**. The humidity level of the Citra variety is in line with the technical sheet of Yakima Chief Hops for this product.

Table 39. Results of the humidity level analysis performed on the pellet samples in triplicate.

Hops variety	Humidity (w/w%)	S.D. (w/w%)
Citra	9.12	± 0.13
Chinook	11.21	± 0.32

For the first batch, 12 kg of Chinook pellet hops were loaded into the charging tank, and 12 L of water was added to rehydrate the biomass (1:1 S/L ratio). Soaking was accelerated by homogenisation for 20 min. When all the hops were suspended, the chamber was heated to 50 °C. The condensation columns were set at different temperatures, as shown in **Table 40**. After 50 min of extraction, the temperature inside the columns started to increase due to chiller-dimensioning issues. The extraction was stopped to contain the leakage of volatiles from the last column. The water from the condensation apparatus was recovered and stored at 6 °C. The extracts collected from the second column were labelled as HCH (Heavy volatiles Chinook), whilst LCH (Light volatiles Chinook) represent the merged product of the third and fourth columns. The sample recovered from the first condensation step was discarded due to excessive dilution.

Table 40. Temperature settings of each column for the Chinook extraction.

	1st Column	2nd Column	3rd Column	4th column
Top	25 °C	5 °C	-8 °C	-8 °C
Bottom	50 °C	25 °C	-8 °C	-8 °C
Pressure	-500 mbar	-500 mbar	-500 mbar	0 mbar

The second extraction was performed at a higher average temperature in each condensation column, as shown in **Table 41,** to reduce the demand on the chiller. A total of 10 kg of Citra hops was loaded with 10 L of water for rehydration and were left to soak for 20 min under agitation. The extraction was carried out for 90 min. The water from the condensation apparatus was recovered and stored at 6 °C. The extracts collected from the second column were labelled as HCT (Heavy volatiles Citra), whilst LCT (Light volatiles Citra) represent the merged product from the third and fourth columns. The sample recovered from the first condensation step was discarded due to excessive dilution.

Table 41. Temperature settings of each column for the Citra extraction.

	1st Column	2nd Column	3rd Column	4th column
Top	30 °C	10 °C	-5 °C	-5 °C
Bottom	50 °C	30 °C	-5 °C	-5 °C
Pressure	-400 mbar	-400 mbar	-400 mbar	0 mbar

We decided to use the HS-SPME technique to analyse the recovered extract since the terpenes were already suspended in a watery solvent, so a direct injection in the GC-MS column was impossible. This technique is based on the principle of adsorption/absorption and desorption, headspace solid-phase microextraction (HS-SPME) uses a coated fibre to trap and concentrate volatile and semi-volatile analytes from the vapour phase above a sample in a static or dynamic headspace process [132]. HS-SPME is a simple aroma

extraction technique that extracts a wide boiling-point range of volatile compounds without artefact formation and integrates sample preparation, extraction, concentration, and the introduction of the sample into gas-chromatography (GC). This technique, generally applied to samples with concentrations in the ppb–low ppm range, has been validated for numerous applications and, in particular, for reliable quantitative analysis since a direct proportion between the amount of analyte extracted by the fibre and its initial concentration in the sample matrix has been demonstrated [133]. In order to overcome problems with extraction reproducibility and therefore optimise analyte recovery, the polymeric fibre coating and coating volume, sample preparation, extraction time (sample/HS equilibrium), equilibration time (HS/fibrefibre equilibrium), and desorption time must be optimised. Several studies have investigated the aroma profile of hops using SPME extraction, in which the semi-quantitative analysis of compounds is performed using the total peak area [134], relative peak area (%)[135][136][137], and peak-area ratios of analyte internal standards (IS) [138][139]. In 2021, Su and Yin quantitated hop aroma compounds using a combination of stable isotope dilution analysis (SIDA) and standard addition method (SAM) in order to eliminate the matrix effect [140].

The analysis has been operated on the condensed water collected from each sample following the described method. The extracts have been placed inside a separation funnel for two hours at room temperature. This procedure was performed to ensure that the suspension volatiles inside the water were stable and did not create a second lipophilic phase at the top. The SPME holder for manual sampling and the Divinylbenzene/Carboxen/Polydimethylsiloxane SPME fibre (1-cm 50/30, DVB/CAR/PDMS) used were purchased from Supelco (Bellefonte, PA, USA). Clear crimp top glass vials (20.0 mL) and silver aluminium caps with PTFE/silicone septa were obtained from Agilent Technologies (Palo Alto, CA, USA). The fibre was first conditioned in the GC injection port at 250 °C to remove fibre contaminants and cleaned prior to each extraction in the hot injection port for 15 min. Analyses were performed in triplicate and expressed as means ± standard deviation (S.D.).

An aqueous toluene solution was used as an internal standard (IS). It was prepared at 86.7 mg/L for the HS-SPME analysis. Distilled fractions (500 μL) (HCH, LCH, HCT, LCT) were placed into a headspace 20-mL vial followed by the addition of 3.00 mL of toluene IS solution at 86.7 mg/L in water, while 3 mL of a 1:1 diluted toluene IS solution (43.4 mg/L) was added to the ground pellets (10 mg). The vials were clamped and equilibrated for 10 min in a hot water bath at 50 °C. After equilibration, a preconditioned 1-cm 50/30 DVB/CAR/PDMS fibre was exposed to the headspace of the clamped vial for 30 min at 50 °C [140]. The accumulated analytes were recovered via thermal desorption directly into the GC injector port for 10 min at 250 °C.

The GC-MS analyses were performed in an Agilent Technologies 6850 Network GC System, using a 5973 Network Mass Selective Detector, a 7683B Automatic Sampler (Santa Clara, CA, USA), and a Mega 5-MS capillary column (5% Phenyl, 95% Methyl Polysiloxane, 30 m × 0.25 mm i.d., 0.25 X m film thickness) (Mega S.r.l., Legnano, Italy). The injector was kept at 250 °C in split mode with a 10:1 split ratio. The oven temperature was programmed to start from 40 °C, was held for 2 min, then moved to 200 °C at 4 °C/min, and then from 200 °C to 260 °C at 20 °C/min and held for 5 min. Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min (average velocity 40 cm/s).

Conditions for the MSD were as follows: detector temperature at 280 °C, MS source at 230 °C, MS quadrupole at 150 °C, mass range, 15–400 amu.

Compounds were considered to be positively identified when electron-impact (EI) mass spectra matched with Wiley7n and NIST11 libraries with a minimum quality of 90%. The compounds that failed to meet the above criteria were considered to be tentatively identified. Myrcene, caryophyllene, farnesene, and humulene were identified based on standard retention times. The semi-quantitative analysis of flavour compounds (FC) in hop samples was performed based on toluene amount (mg), used as an IS [138], using the following formula:

$$\mu g_{IS} \times Area_{FC}/Area_{IS}$$

4.1.2 Results and Discussion

The volume of the condensed water for each column at the end of the extraction is reported in **Table 42**.

Table 42. Recovered condensed extract volume for each column at the end of the extraction process.

Hops	Fraction volume (L)			
	C1	C2	C3	C4
Chinook	3.3	4.5	1.8	0.5
Citra	4.5	4.8	1.2	0.4

As can be seen, there is no proportion between the volume collected in each column between the Chinook and Citra extraction. During the first process, the temperature inside the columns increased significantly, causing a net loss of the condensed water. From **Table 42**, it is clear that the loss of temperature control caused a higher recovery on the last columns due to lower condensation in the first and second columns, which were unable to condense the vapour stream efficiently.

The first organoleptic impressions of the condensed extracts showed that the first column had no flavour in the Citra and Chinook samples. On the other hand, the condensed extract from the second, third and fourth columns had a pleasant aroma, much more potent for

Citra. This difference can be explained by the issue encountered by the recovery unit during the first extraction, leading to an overall aroma loss. During the extraction, the hops lost almost all the water added at the beginning of the process. Vacuum evaporation at low temperatures resulted in hops deposition on the internal surfaces, enhancing the extraction rate by increasing the exchange area. The final moisture content of the hops was 16.9 ± 0.2 (w/w %), and no burnt areas were observed. The aroma of the residual hops was very faint and grassy, without the peculiar characteristics. The water collected during the Chinook extraction was utterly transparent in the first two columns, while light yellow turbidity was found on the samples collected from columns 3 and 4. The samples collected from the Citra extraction showed a much higher turbidity and yellow colour with some opalescent reflex on the surface. The flavours were much more potent, and the extract collected from the third and fourth columns were indistinguishable, while they were more piney and pungent compared to the second column.

As abovementioned, during Chinook extraction, the temperature increased in the last column, with a significant loss of volatile compounds. This phenomenon was visible since a thick hoppy-smell vapour cloud started to exit from the head of the last section of the condensation apparatus. During the second extraction, the protocol was modified, setting columns temperatures sustainable for the chiller. Hence, the energy demands for the chiller was reduced and the desired results were achieved. In fact, during all the process only a very light scent of hop was perceivable. At the end of the extraction process, the hops maintained their vivid green colour and had a low concentration of humidity, although no burnt biomass was found. The spent hops lost their characteristic flavour in change of a slight scent of grass. This empiric evaluation suggested that the aroma extraction was successful, and few volatiles remained in the biomass. Further tests can be done to optimise the extraction, reducing the overall treatment time.

According to several articles, the DVB/CAR/PDMS fibre was chosen for the analyses of hop samples[134][139][140]. Toluene was used as an IS for the semi-quantitative analyses because of its good solubility in water (526 mg/L at 25 °C, Pub Chem) and based on several experiments performed by the authors in the semi-quantitation of the profile of fruits aromatic fractions extracted with the same equipment. Procedure parameters, such as the sample amount (mg or mL), IS concentration (mg/L) and amount (mL) added to the samples, were optimized to get the best reproducibility results.

The data recovered from the triplicate analysis is available in the supporting materials (Tables S4, S5 and S6). **Table 43** reports the quantitative results for the HCH and LCH samples with S.D. of each compound. For the sake of comparability, the study highlighted the key molecules responsible for the peculiar flavour found in both extracts. **Figure 34** shows a graphical representation of the abundance of each compound in the HCH and LCH

samples and helps to underline the significant disproportion between the abundance of recovered compounds.

Table 43. Chinook compounds distribution between HCH and LCH samples showing the triplicates' average (AVG.) and standard deviation (S.D.).

Chinook Compounds	HCH AVG. ± S.D. (ppm)	LCH AVG. ± S.D. (ppm)
β-Myrcene	14.1 ± 2.3	286.3 ± 44.9
Linalool-L	16.1 ±1.2	90.6 ± 4.8
Geraniol	2.9 ± 1.6	28.7 ± 2.1
α-Copaene	3.3 ±0.3	119.4 ± 23
trans-Caryophyllene	41.6 ± 1.5	992.4 ± 165.9
α-Humulene	87.4 ± 2.5	1955.4 ± 346.9
α-Amorphene	5.9 ± 0.3	350.5 ± 71
β-Selinene	6.6 ± 1.7	313.2 ± 64.8
α-Selinene	9.8 ± 1.5	388.5 ± 81.3
δ-Cadinene	7 ± 0.2	390.8 ± 81.7
Isoledene	2.1 ± 0.1	189.7 ± 41.5

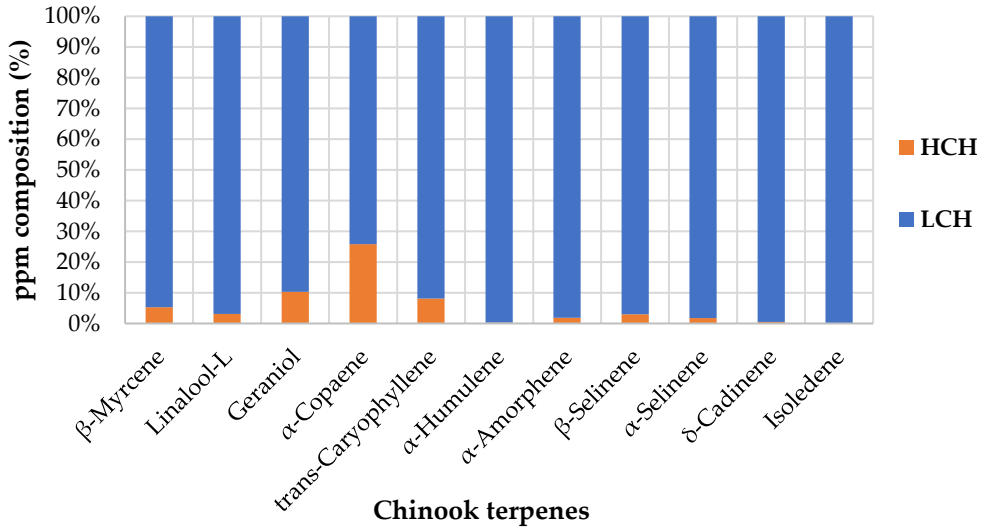


Figure 34. 100% stacked column representation of the Table 43 average ppm data.

The quantity of volatiles condensed in the HCH fraction is poor, due to the reduced extraction time and the chiller issues faced during the procedure. The heavy volatiles that condensed in column 2 shifted again to the vapour state when the temperature increased thus moving to the following columns. The lower concentration of compounds in the HCH fraction caused a decrease in the matching quality of the less abundant peaks by MS libraries (lower than 90%), compared to LCH and both HCT and LCT. In addition, triplicates did not show a complete overlapping of peaks present only in traces, although, as can be appreciated in **Figure 35**, there is a clear overlap of the major peaks in all three samples. This

detail, alongside the comparison with the compounds recovered from the Chinook pellet (PCH), proves that the extracts have maintained the volatile compounds present in the starting biomass.

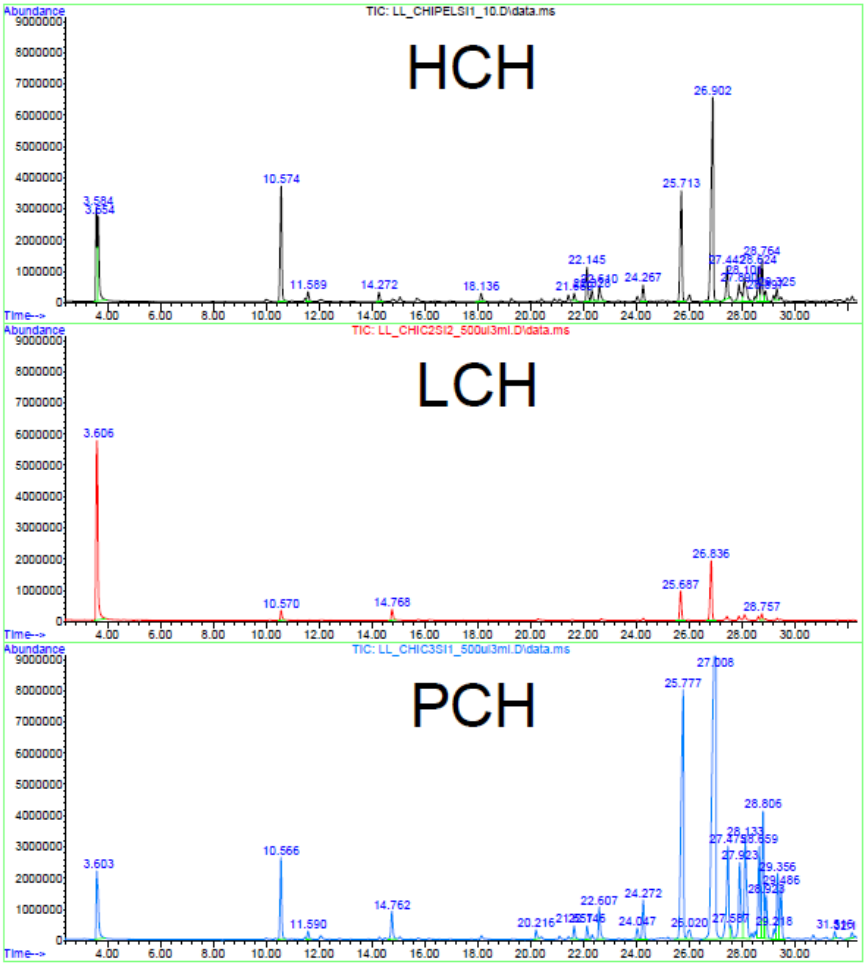


Figure 35. Superimposition of HS-SPME/GC-MS chromatograms of the HCH, LCH and PCH samples with the respective retention time for each compound.

Table 44 reports the semi-quantitative results for the HCT and LCT samples with the standard deviation of each compound. As for Chinook samples, the key molecules responsible for the peculiar flavour, found in both extracts, have been highlighted for sake of comparison. The whole data collected can be visioned in the supporting material (Tables S1 and S2).

Figure 36 reports the average percentage abundance of each compound found in the HCT and LCT samples. The lighter monoterpenes compounds placed on the left side of the graph have a higher concentration on LCT samples. Meanwhile, the sesquiterpene fraction that starts from the α-Copaene has a higher concentration in the HCT samples. The recovered extracts have a high correspondence with the peaks of the pellet biomass.

Table 44. The distribution of Citra compounds between HCH and LCH samples shows the triplicates' average (AVG.) and the standard deviation (S.D.).

Citra Compounds	HCT AVG. ± S.D. (ppm)	LCT AVG. ± S.D. (ppm)
β-Myrcene	54.6 ± 1.2	117.5 ± 11
Isobutyl isopentanoic acid ester	4.8 ± 0.7	12.5 ± 2
DL-Limonene	1.4 ± 0.1	8 ± 0.3
2-Nonanone	2.6 ± 0.3	6.2 ± 0.6
Linalool L	91.8 ± 12.5	267.5 ± 8
2-Decanone	2 ± 1	3 ± 0.4
Geraniol	6.4 ± 1.3	43.1 ± 3
2-Undecanone	25.1 ± 12	33.3 ± 1.6
2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester	97.6 ± 17	100.5 ± 9.2
α-Copaene	9.5 ± 2.6	5.7 ± 1.1
trans-Caryophyllene	213.8 ± 36.9	155.2 ± 27.7
α-Humulene	392.6 ± 60.8	291.8 ± 40.4
α-Amorphene	25.5 ± 6	11.4 ± 2.4
β-Selinene	46.5 ± 4.5	32.9 ± 5.3
α-Selinene	51.7 ± 10.3	30.8 ± 5.5
α-Farnesene	12.1 ± 4.5	4.9 ± 0.7
Geranyl propionate	4.8 ± 1.2	0.7 ± 1.1
δ-Cadinene	26.4 ± 4.9	10.9 ± 2.2

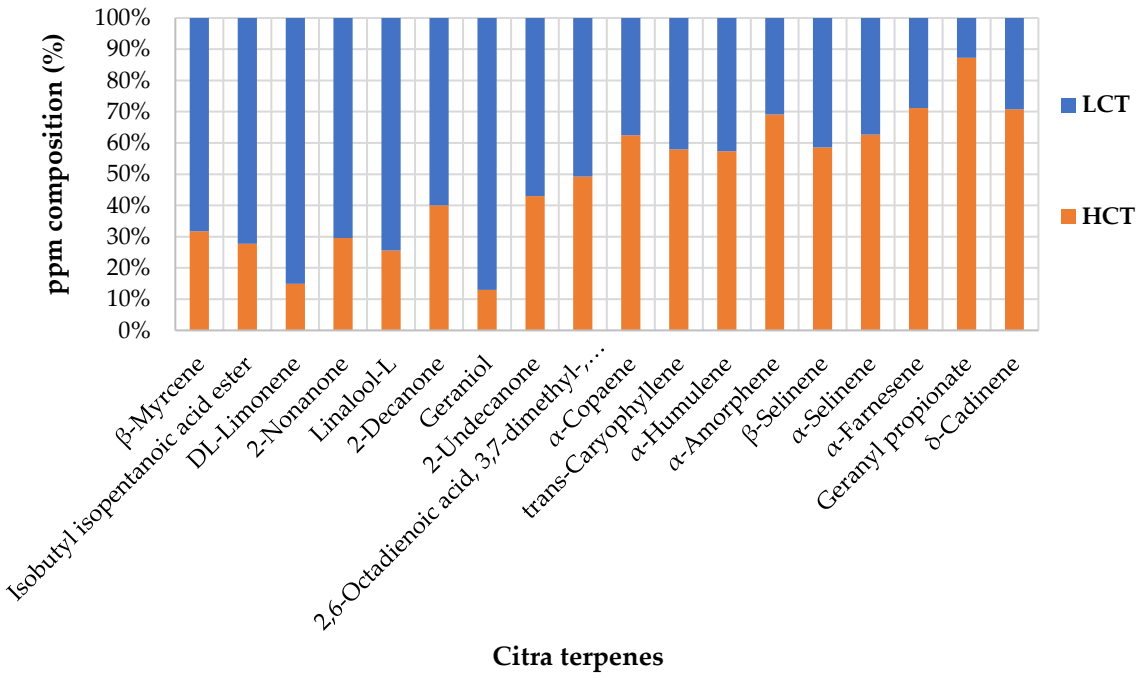


Figure 36. 100% stacked column representation of the Table 44 average ppm data.

From this histogram, it is clear that there is a high disproportion between the compared data. As presumed, there is a higher concentration of monoterpenes in the pellet. This result is unsurprising since the monoterpenes exhibit a lower boiling point and thus are the hardest to condense and the easiest to lose. During the extraction, the slightly pungent flavour that came out of the head of the fourth column can be associated with the lighter compounds (β-myrcene, isobutyl isopentanoic acid ester and DL-limonene). The higher abundance of the sesquiterpenes on the HCT-LCT samples is caused by the loss of monoterpenes, due to the stacked normalization of the graph. Given that the volatiles loss mainly concerns lighter compounds, the disproportion of sesquiterpenes fraction is less visible. In conclusion, it is possible to state that the whole extract produced generally maintained the original hops composition and the peculiar fragrance, without losing the major components responsible of hop's volatiles fingerprint.

This study tested a MEFCUV system to recover the volatile compounds from two commercial hop varieties. The results herein reported highlight the number of potential applications in flavouring areas. The strength of this technology lies in the possibility of extracting volatiles from biomass under mild conditions and without organic solvents. This environmentally friendly approach is beneficial for applications in the food industry, where organic solvents are strictly regulated. For what concerns the brewing application, the classic dry-hopping involves the soaking of the hop pellets at low temperatures (generally below 15 °C) for two to three weeks at the end of fermentation. This time-consuming process is inefficient and not cost-effective. Standard steam distillation for EO recovery is also time- and energy-consuming; the obtained volatiles entail several drawbacks for the dissolution. The lipophilic nature of EO poses two major problems for its application in beer products: low solubility and reduced foam stability. These two problems are closely related because, since the EO are not soluble, their lower density causes them to migrate upward. When they reach the surface, the formation of a second lipophilic phase prevents the foam stabilisation. These problems would not occur when LCT and HCT are used. This behaviour thanks to the stable water suspension achieved during the evaporation and condensation cycles in the distillation columns. Thus, no lipophilic layers would be generated after the addition into beer.

4.1.3 Conclusions

The MEFCUV is highly versatile thanks to its industrial scalability and its ability to produce extract fractions with different organoleptic properties. In a typical working day, a single operator can easily process 50-100 kg of hop pellets, and the extract collected can be easily stored and transported. This production yield is sufficient to supply all types of craft breweries. For implementation in an industrial brewery, a more powerful chiller is needed and the volume of the feed tank is increased or two extractors could be used in parallel. It is

also important to emphasise the economic relevance of producing different extracts fractions for original blends. The dispersion of terpenes in water also allows the extracts to be used in non-alcoholic beverages such as soft drinks, juices, integrators or non-alcoholic beers. These aromatic terpene fractions can also be used in the cosmetic and perfume industries.

The technique HS-SPME /GC-MS was used to identify and semi-quantify volatiles in collected hop samples. This analytical approach proved to be very efficient, with grey fibre application meeting the expectations [141].

To properly evaluate the microbiological and chemical stability of the extracts collected we stored 3 samples at three different temperatures: room temperature, 4 °C, and 6 °C for 12 months. Unfortunately just after 2 months the extracts stored at room temperature and 4 °C started to display mold inside the bottles that started colonising the whole sample. The concentration of the EO in the water was too low to block the proliferation.

The microorganism growth also significantly changed the flavour of the extract, which became more pungent and unpleasant with some distinct notes that were not perceivable when collected. This stability problem is quite challenging to overcome because the main pro of the MEFCUV technology was to have already the lipophilic volatiles resuspended in water to simply the industrial application. However, for a specific use in a brewery where the hops are harvested once per year, it is important to guarantee at least 12 months of storage. Of course, it would be possible to freeze all the extract, but this operation would significantly increase the energy and equipment costs. Moreover, the fact that the contaminants can not proliferate and colonize the extract when frozen does not mean that they do not survive in that specific environment. This means that every time you introduce some freeze extract in a product, you bring a high risk of future contamination of the product itself.

Of course, this risk can not be taken by any industrial producer; the benefits are not sufficient to take such a high risk, especially if the extract wants to be introduced in an alcoholic drink.

4.2 Microwave-Assisted Hydrodistillation (MAHD)

After the first approach was tried, we decided to investigate a new technology that would give us pure EO. In this way, the problems of extract contamination and stocking space would be reduced to their minimum. As explained previously, the traditional methods of extracting EO are energy and time-consuming, and the quality of the extract could be sub-optimal. To avoid these problems, we decided to collaborate with Milestone S.r.l. company to optimize a hydrodistillation assisted by microwaves.

Applying a MW field to heat the system is far more efficient than standard methods. First, the irradiation can be easily homogenized and interact with the water molecules inside the

reactor, reducing the possibility of hot spot formation. Moreover, it has been proven that the activity of MW on cell membranes and cell walls causes a reduction of stability and resistance, making all the valuable cytosolic compounds more accessible.

At the start of this project, the company Milestone srl already had a lab-scale hydrodistillator (ETHOS X) on the market and was completing the development of the industrial-scale version (ETHOS XL). Some tests conducted during this PhD Thesis was made on the prototype of this latter, being one of the first production tests performed for the Milestone company.

4.2.2 Material and methods

Hops, used for the hydrodistillation protocols, belong to the cascade variety. After harvesting, the cones were dried for 12 h. at 42 °C, decreasing the average humidity to 8%. The material was left at room temperature for 24 h. before pelletization. Dried cones were left to rest for 24 h., and their humidity rose to 10/11%. The hops then underwent pelletization. This process started with a fine mincing of the cones, and the collected powder was then pressed through an extruder to create the pellets. The temperature increased substantially during pelletization and reached 50 °C. Afterwards, the pellets produced were small cylinders of 1/2 cm in length and 0.5 cm in diameter. The final humidity reached by hop pellets at the end of the process was 12%. The materials were identified as fresh hops (FH), dried hops (DH) and pellet hops (PH) in accordance with the different post-harvesting processes. All the biomasses were stored under vacuum at -18 °C for their preservation. The water content was evaluated via thermogravimetric analysis by leaving an average weight of 1.5 g of biomass at 100 °C overnight and measuring the relative weight loss. The test was performed in triplicate and the results are reported in **Table 45**.

Table 45. Hops water content.

Hops	Water content (w/w, %)	S.D. (w/w, %)
Fresh	70	2.32
Dried	12	0.41
Pellet	12	0.41

MAHD was carried out using ETHOS X and ETHOS XL extractors (Milestone srl, Bergamo, Italy, see **Figure 37**).

Figure 37. ETHOS X (right) and ETHOS XL (left) reactors (Milestone srl, Bergamo, Italy).

The first tests were performed using ETHOS X, with a 5 L vessel, with a Standard Operating Procedure (SOP). The SOP is made up of a moistening phase and a standardized MAHD protocol, with fixed MW-irradiation power and time, as reported in the supporting material (Table S8). In the SOP, the water/cone ratio of the moistening pretreatment depends on the matrix type: 0.5 L/kg for fresh biomass and 1 L/kg for dried biomass (DH and PH). In addition, this study evaluated different moistening ratios for fresh hops: 0.25, 1 and 2 L/kg. The vegetal material (up to 1200 g) was soaked directly in the 5 L ETHOS X vessel for 15 min. with mild manual agitation. The extraction procedure required at least ¼ empty space in the vessel.

The effect of milder MW-irradiation on the hop volatile-fraction yields and quality was investigated employing a dedicated MAHD protocol, which we had previously optimized on sensitive biomass (*Cannabis sativa* L.) [142]. The hydrodistillation conditions are summarized in **Table 46**.

Table 46. Mild irradiation MAHD protocol

Step	Time (min)	Power (W)
1	3	500
2	3	1100
3	14	1600
4	90	1500

As MAHD had the best performance on fresh hops, the extraction scale-up of this matrix was investigated. In detail, fresh-cone cascades were processed in a 12 L vessel, which allowed approximately 3 kg of biomass to be treated. The MAHD protocol reported in **Table 46** was applied, with the last step being extended up to 6 h. to increase sample collection.

In order to accelerate the distillation process, a different approach that used a customized ETHOS X setup was investigated. Suitable glassware facilitated the incorporation of a vacuum pump into the system, while the vapour-collecting region was also equipped with a thermocouple. Similarly to ETHOS XL, this setup enabled direct temperature control to be performed with automatic adjustments to the power output being made by the system. Lastly, an additional liquid-nitrogen trap was added to avoid losses of the volatile fraction (see **Figure 38**). In detail, 700 g of fresh cascade hops were loaded with 700 mL of water into a 2 L vessel. Two different extraction protocols were applied: a mild one that reached 95 °C with a maximum 1200 W power delivered (protocol A); and a harsher one that reached 99 °C with a maximum 1600 W power delivered (protocol B). Both protocols are reported in the supporting material (Table S10). Due to the high evaporation rates during processing, 400 mL of water was introduced into the vessel in 4 steps. The extraction was stopped every time 100 mL was sampled, and an equal volume of fresh distilled water was introduced slowly into the vessel.

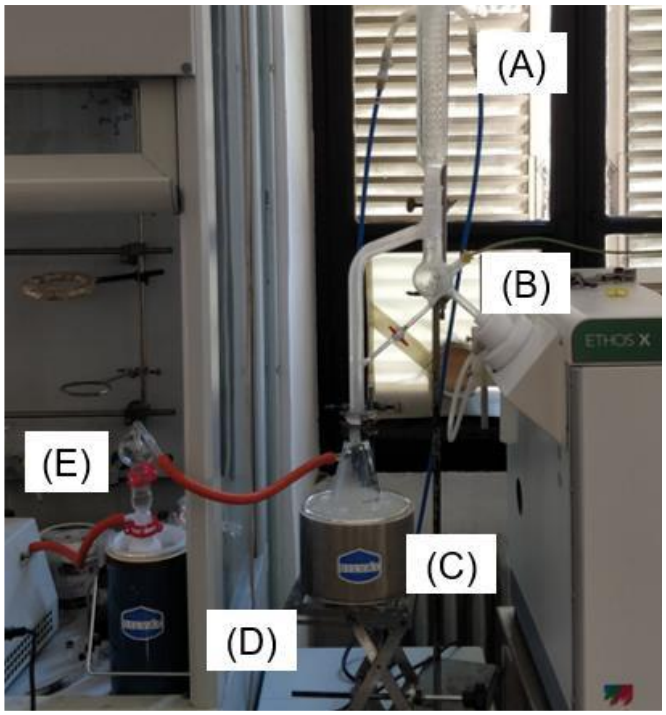


Figure 38. ETHOS X vacuum setup; (A) condenser, (B) thermocouple, (C) collecting flask, (D) liq-uid-nitrogen trap (E) pump.

For the first time, a new prototype reactor (ETHOS XL, Milestone srl, Bergamo, Italy, **Figure 36**) was tested to investigate the feasibility of pilot MAHD further. The ETHOS XL presents itself as a cube with 0.5 m sides. The bottom of the reactor has a depression that is filled with water during processing. A 45 L rotating drum, which is capable of maintaining a homogenized moistening level and the homogenized diffusion of microwaves over the treated biomass, is located in the centre of the reactor. The reactor has three magnetrons of 1.5kW of power each, although this is not the only heating source the ETHOS XL provides. Each internal panel has a resistance of 1.8 kW, and these maintain a constant temperature inside and enhance microwave activity in the biomass. The system has a recycling loop that prevents the reduction of the overall moistening level. The vapours released during the extraction are collected from the top of the reactor and sent to three parallel condensers to condense the vapours released. The extracted EO is stored in a chilled tube where the aqueous solvent is separated. A fourth condenser is placed on top of the separating tube to ensure no volatile compounds are lost. The chiller paired with the ETHOS XL requires at least 5 kW of power to ensure good refrigeration across all condensers. The reactor is equipped with a thermocouple in the vapour-collecting region, enabling direct temperature control via variable power output.

Furthermore, a 9 L water pool is inside the cavity to avoid combustion phenomena and strongly decrease energy consumption. This type of setup allowed a reduced water/cone ratio (0.5 L/kg, as SOP reports) to be used. The reactor was run at both half (low loading, LL) and full (full loading, FL) capacity to test pilot performance. In the first case, the water

pool required only a 5 L charge. Due to the cavity pre-heating system and magnetron-power variation, a dedicated irradiation protocol was tested, while step duration was modified according to reactor loading. Conditions are summarized in Table S9. The protocols were used on fresh, dried and pelletized hops.

The GC-MS qualitative analyses of the volatile fractions obtained using MAHD were performed in an Agilent Technologies 6850 Network GC System fitted with a 5973 Network Mass Selective Detector, a 7683B Automatic Sampler and a capillary column Mega 5MS (length 30 m; i.d. 0.25 mm; film thickness 0.25 _m, Mega s.r.l., Legnano, Italy). Each sample was prepared by mixing 5 _L of the volatile fraction with 1 mL of chloroform, with this being repeated in triplicate. The adopted chromatographic protocol is reported in the supporting material (Table S11). The identification of the individual compounds was performed using two approaches: (1) by comparing the retention time and mass spectrum with standard compounds, and (2) using the GC-MS Wiley275 and NIST05 GC libraries from the acquired chromatograms and only considering matching levels of over 95%. The summed areas of the relevant peaks were normalized to 100%. Calculated as percentages, relative peak areas were used to evaluate extract composition.

The MAHD extraction tests and their respective extraction yields are summarized in the Supplementary Material (Table S12 and Figure S1).

4.2.3 Results and discussion

The SOP was applied to evaluate the suitability of different types of biomasses for the recovery of the volatile fraction. As reported in **Table 47**, dry hops appear to be the best-performing matrix (9.25 mLVE/kg dry matrix of DH vs. 5.00 and 3.30 mLVE/kgdry matrix of FH and PH, respectively). Nevertheless, normalization that was performed on biomass water content shows that working with fresh material led to better volatile-fraction recovery (16.67 mLVE/kg dry matrix of FH vs. 10.51 and 3.75 mLVE/kgdry matrix of DH and PH, respectively).

Table 47. MAHD, SOP protocol screening.

Entry	Extraction Material	Hops (g)	L/S (L/kg)	Volatiles (mL)	Yield (mL_{VF}/kg)	Dry Yield ($mL_{VF}/kg_{dry\ matrix}$)
1	FH	1200	0.5	6.0	5.00	16.67
2	DH	400	1	3.7	9.25	10.51
3	PH	1000	3	3.3	3.30	3.75

Accordingly, the influence of the fresh-hop moistening process was evaluated. Thus, it was possible to evaluate even limited recovery variations. In detail, this study evaluated soaking ratios of 0.25, 1 and 2 L/kg. As reported in **Table 48**, using a ratio of at least 0.5 L/kg

for fresh biomass is necessary. Lower amounts of water (0.25 L/kg) triggered combustion phenomena, leading to the unsuccessful recovery of the volatile fraction. Conversely, higher additions of water (1 L/kg) did not appear to modify the extraction yield, with there being a little production decline for 2 L/kg (16.67 mLVE/kg dry matrix vs. 15.28 mLVE/kg dry matrix, entries 5 and 6, respectively).

Table 48. MAHD, moistening screening. Extraction material: Fresh hops (FH).

Entry	Hops (g)	L/S (L/kg)	Volatiles (mL)	Yield (mL_{VF}/kg)	Dry Yield ($mL_{VF}/kg_{dry\ matrix}$)
4	1200	0.25	Burnt biomass, no recovery		
5	1100	1	5.5	5.00	16.67
6	1200	2	5.5	4.58	15.28

According to our previous experience on volatile-fraction recovery utilizing MW irradiation, the three different hop typologies were tested by applying mild MAHD (see **Table 49**).

Table 49. MAHD, mild irradiation protocol and 12 L vessel scale-up. L/S ratio: 1 L/kg.

Entry	Extraction Material	Hops (g)	Volatiles (mL)	Yield (mL_{VF}/kg)	Dry Yield ($mL_{VF}/kg_{dry\ matrix}$)
7	FH	1300	8.0	6.15	20.51
8	DH	1000	7.5	7.50	8.52
9	PH	1300	3.2	2.46	2.80
10	FH – Scale-up [a]	3000	15.9	5.30	17.67

This protocol can save the lighter volatile fraction by avoiding condensation problems and leakages due to overheating. At the same time, this approach reduces the formation of the hot-spots that lead to biomass degradation and interfere with the final flavour of the recovered product due to degradation and compound alteration. As reported in **Figure 39**, mild MAHD provided a better performance on the fresh hops, reaching an approximate 23% rise compared to SOP, with the fresh hops again being

confirmed as the most promising matrix. Conversely, dried hops and pellets suffered average yield decreases of 18% and 25%, respectively. This trend supports the abundance of highly volatile compounds present in recently harvested hops, and these molecules are partially lost with excessively harsh extraction protocols. On the other hand, dry biomass, such as dried cones and pellets, have already been depleted of this light fraction during post-harvesting treatment. The L/S was maintained at 1 L/kg as in the reference protocol [142], as it has already been demonstrated that moistening does not significantly affect the

yield. In summary, mild MAHD appears suitable for FH extraction, while slight yield contractions are observed for DH and PH.

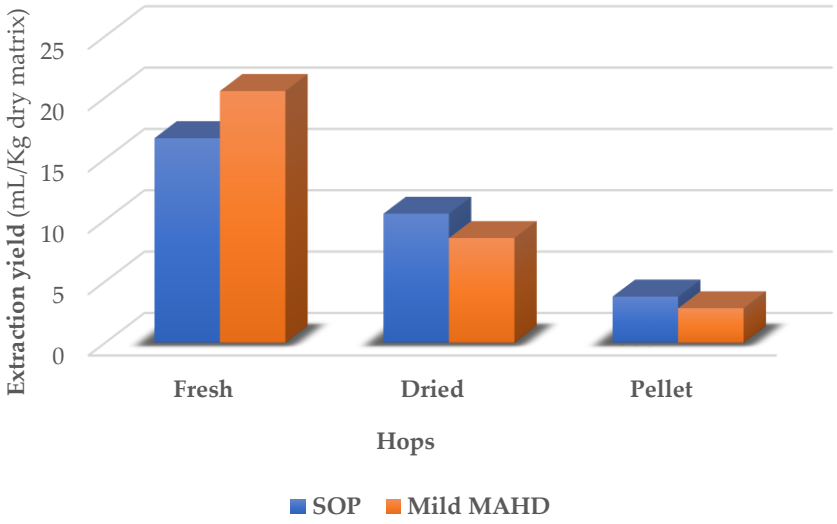


Figure 39. Volatile-fraction yield according to MAHD protocols.

Interest in volatile-fraction production and exploitation has driven the development of systems suitable for industrial-scale production. In our work, the very first step was taken by investigating the effects of scale-up on MAHD yield and by simply employing a larger extraction vessel. For this purpose, the starting load was tripled and tested in the same MW device.

To better understand process evolution, the volatile-extraction rate was monitored on FH applying Mild MAHD protocol (**Figure 40**). At the end of the mild MAHD protocol, only 9.4 mL of product was extracted (18.5 mL expected), although linear output was maintained. Thus, the process continued until a plateau was reached, with the hydrodistillation period extended to 360 min.. In the end, 15.9 mL of volatiles were collected (**Table 49**, entry 10), with a moderate loss of 16% compared to the small-scale expected yield, and an overall yield of 17.67 mLVE/kg dry matrix was achieved. From a physical point of view, the biomass was mainly similar to previous samples at the end of the six hours of hydrodistillation, except for a small combusted area near the vessel's surface. From this detail, it is possible to assume that higher biomass loadings require more homogeneous irradiation inside the reactor chamber.

Nevertheless, it is still remarkable that only a negligible fraction suffered from this effect. This test proved that treating higher biomass quantities using a larger vessel is possible, although the time required to reach comparable extraction yields increases proportionally. Our investigation highlighted the possibility of processing more biomass per extraction batch, though some adjustments to the extractor set up are required.

According to the results and observations gathered during the experimental setup and extraction screenings, the main issue for pilot-scale hydrodistillation is poor mass transfer. Large quantities of material interfere with vapour-flow dynamics because of the strong packing, which is then worsened by biomass texture. Due to their coarse size and heterogeneous composition, fresh hops show better behaviour, whereas dried cones and pellets suffer considerably from this effect. For pellets, in particular, this phenomenon is visually embodied by a swelling of approximately 15–20% (with respect to the loading volume) during the process, due to trapped vapours. Thus, the higher yield for fresh hops is not explained solely by the higher concentration of volatiles, which are lost in the drying process for dried and pelletized cones. In standard production practices, breweries usually exploit hops in the pellet form, making this format the most available and attractive for pilot-scale applications, despite being the most troublesome. A pilot reactor has been developed specifically to solve the scalability issues by focusing on enhancing mass transfer, which is a bottleneck in the scale-up of the hydrodistillation process. Moreover, the MW-distillation apparatus was merged with a hybrid technology to reduce energy consumption. The screening approach used to investigate pilot performance involved an initial set of extractions for all three typologies of biomass, with adjustments being made to the lab-scale reactor loadings (2–3 kg, LL, **Table 50**, entries 11–13).

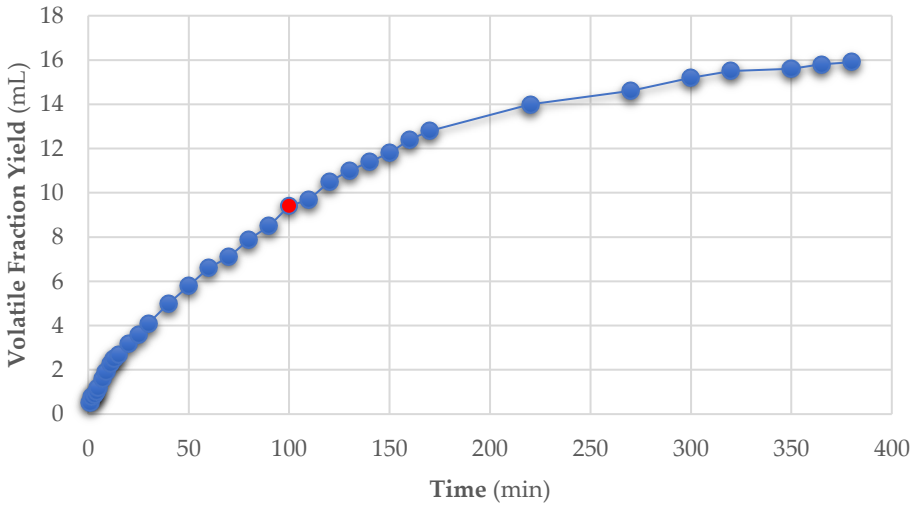


Figure 40. Extraction timeline, the red dot shows the end of the small-scale protocol.

The introduction of a mobile body provided a mass-transfer enhancement, homogeneous wetting, and exposure to MW. A direct effect can be observed on the dry cones and pellets, with the average mLVE/kg dry matrix yield increasing (8.52 vs. 11.76 and 2.80 vs. 6.25, respectively, entry 8 vs. entry 12 and entry 9 vs. entry 13). Fresh hops maintained comparable productivity.

Table 50. MAHD pilot scale. [a] The extraction has been carried out for 70 min. after reaching 100 °C inside the reactor chamber; [b] The extraction has been carried out for 120 min. after reaching 100 °C inside the reactor chamber.

Entry	Extraction Material	Hops (g)	Volatiles (mL)	Yield (mL_{VF}/kg)	Dry Yield ($mL_{VF}/kg_{dry\ matrix}$)	Label
11	FH [a]	2500	13.0	5.20	17.33	
12	DH [a]	2000	20.7	10.35	11.76	*Low Load*
13	PH [a]	2000	11.0	5.50	6.25	
14	FH [b]	8200	36.0	4.39	14.63	
15	DH [b]	3820	50.0	13.09	14.87	*Full Load*
16	PH [b]	4000	38.0	9.50	10.80	

Finally, the irradiation chamber was tested for a FL (**Table 50**, entries 14–16), whose weight was strictly related to the type of biomass. In detail, volatiles yield for FH are roughly unchanged, while a slight increase can be observed for DH, reaching a dramatic enhancement for pH, almost doubling the process outcome. In **Figure 41**, we can see how the pilot prototype was able to enhance the hydrodistillation yields for dried and pelletized hops, compared to the small-scale projections

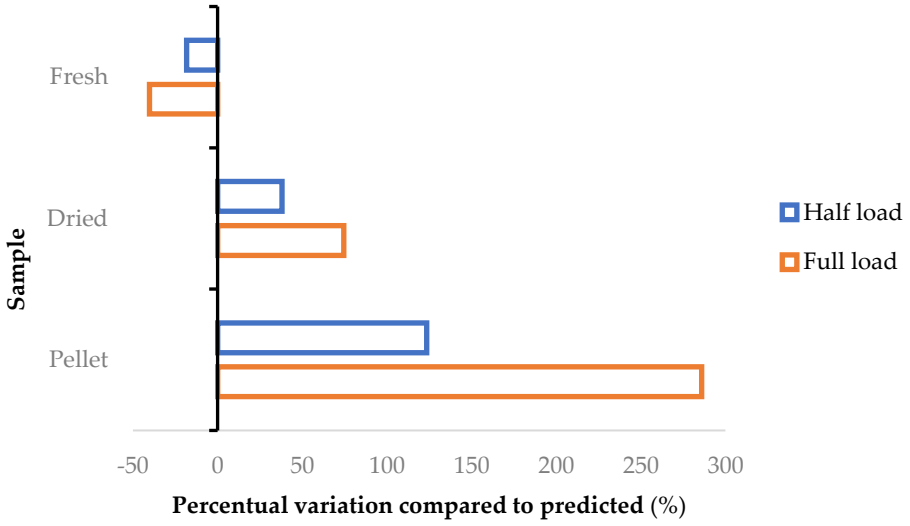


Figure 41. Percentage variation in mL of volatile fraction, according to the small-scale projections.

In particular, pellet productivity was approximately quadrupled, reaching 10.80 mLVE/kg dry matrix (entry 16), from the 2.80 mLVE/kg dry matrix (entry 9) obtained from the small-scale process. Similarly, dry cones nearly doubled the overall outcome, increasing from 8.52 (entry 8) to 14.87 mLVE/kg dry matrix (entry 15). On the other hand, fresh biomass suffered slight decreases with process scale. This effect can be explained by the soaking/charge step, whose relevance rises proportionally with matrix load. Fresh hops are very attractive thanks to the high amount of light volatiles lost during the desiccation and pelletization protocols. Extending early-stage protocols (such as soaking, mixing and

loading) contributes to wasting this fraction. Nevertheless, it is unreasonable to process huge volumes of newly harvested plants without expensive measures (cold chains, either inert atmospheres, vacuum systems or in combination) precisely because of their instability (in addition to fermentation and degradation). A practical example is the strategic choice of breweries to work with pelletized hops, which can provide a constant and stable supply. Thus, it is possible to consider that large-scale extraction for industrial purposes will focus on pellets and dried cones, as they are simply available for storage. Smaller applications (still approximately 3 kg) can be envisaged for high-quality processes using fresh biomass to recover the most volatile compounds.

While the vacuum setup was customized in order to find a reliable approach to switching from the typical power-control to a temperature-control system. These systems are able to detect the temperature of the extraction vessel using either IR probes or optical fibres. Nevertheless, these two approaches suffer from a certain degree of uncertainty; IR can only properly detect superficial temperatures, meaning that increasing vessel dimensions inevitably leads to large deviations, whereas optical fibres are difficult to insert inside the system due to sealing issues. Moreover, speaking of a static device, the detected T may be not representative of the whole vessel. For these reasons, the most common MAHD devices are set to power-control, without a PID associated with temperature evolution. The proposed vacuum setup was tested in order to verify the possibility of adjusting power irradiation according to vapour temperature. The details of the dedicated glass apparatus are shown in **Figure 42**.

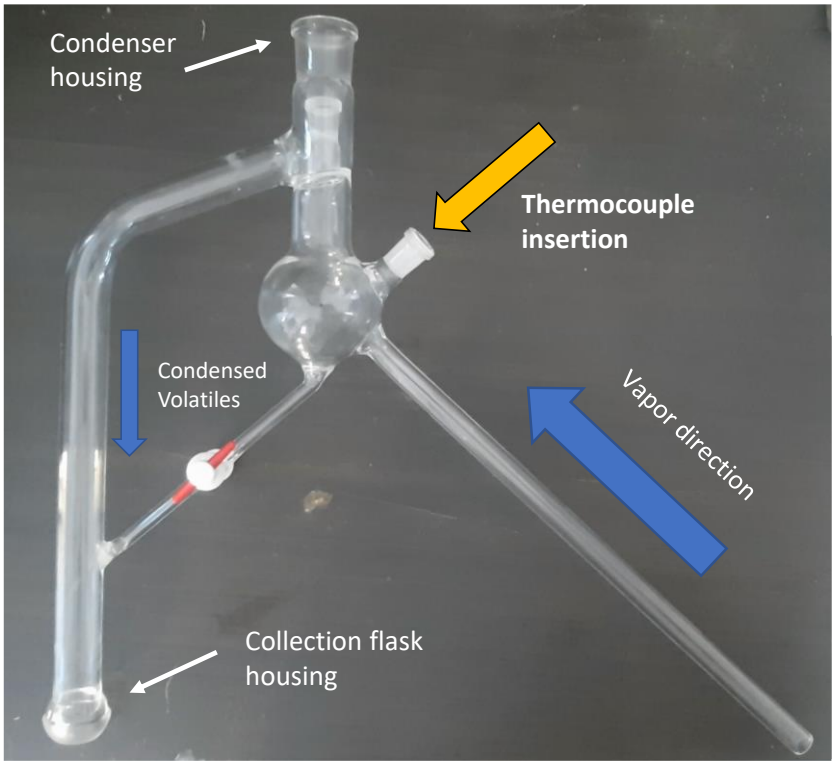


Figure 42. Customized glass apparatus implemented during MAHD under vacuum.

The optical fibre is placed right at the vapor output, whilst a slight vacuum (approximately 0.05 MPa) is applied to enhance the distillation, allowing higher extraction yields to be reached with lower energy consumption. The MAHD were tested at two different temperatures, namely 95 and 99 °C (**Table 51**), to screen the system's flexibility. From an engineering point of view, the heating profile and irradiation trend accurately followed the set parameters. The wattage emissions were strongly reduced, which decreased the energy consumption accordingly. For example, in the ramp stage, mild MAHD absorbed 1245W. Hence, vacuum protocols saved approximately 40% and 20.6%, at 95 and 99 °C, respectively (entries 17 and 18).

Unfortunately, the volatile fraction recovered by means of the vacuum setup was found to be smaller than that of SOP MAHD (16.67 mLVF/kg dry matrix). An average reduction of approximately 45% was observed, with a minimum gap of 4% when comparing the 95–99 _C protocols. These results can be explained if we assume that the performance of the trap section, which serves to avoid losses due to pump suction (**Figure 38**, elements C,E), was inefficient. Furthermore, the tests were performed on the most characterized matrix, namely, the fresh hops, which is, on the other hand, the richest in extremely volatile compounds, and presumably the most difficult to trap. On the basis of these considerations, future evaluations would involve a more sophisticated cooling system before the pump, such as a vertical condenser with a collecting flask that is not directly connected to the pump. Since additional customization is required, further investigations with this setup are suspended for the moment. Nevertheless, a promising approach to introduce temperature control as an instrumental parameter during MAHD is reported herein.

Table 51. Vacuum MAHD. Biomass: Fresh hops (700 g); L/S: 1 L/kg.

Entry	Temperature (°C)	Ramp irradiation[a] (W)	Volatiles (mL)	Yield (mL_{VF}/kg)	Dry Yield ($mL_{VF}/kg_{dry\ matrix}$)
17	95	747	1.7	2.43	8.10
18	99	989	1.9	2.71	9.05

Process energy consumption is a critical factor that must be evaluated for suitable scale-up design. We therefore monitored the average Watts absorbed by the ETHOS X and ETHOS XL. Furthermore, chillers were taken into account due to their significant impact on total energy costs. This data made it possible to calculate the overall Joules used over the entire process. It is important to remember that the applied protocols treated different quantities of matrix and produced different amounts of EO. For this reason, extraction energy consumption was normalized to the volumes of volatiles collected. As shown in **Figure 43** (Table S13), FH submitted to the mild irradiation protocol is characterized by the lowest energy consumption when using the ETHOS X. All the other extractions showed

higher consumption when carried out on the same system, especially when using pellets, due to the lower extraction rates. Conversely, the energy demands of the pilot reactor appear to be far better than those achieved using the lab-scale device, in particular when the instrument was used at full capacity (pilot-FL). This trend proves the effectiveness of the MW hybrid approach, together with the design of appropriate mixing/soaking apparatus.

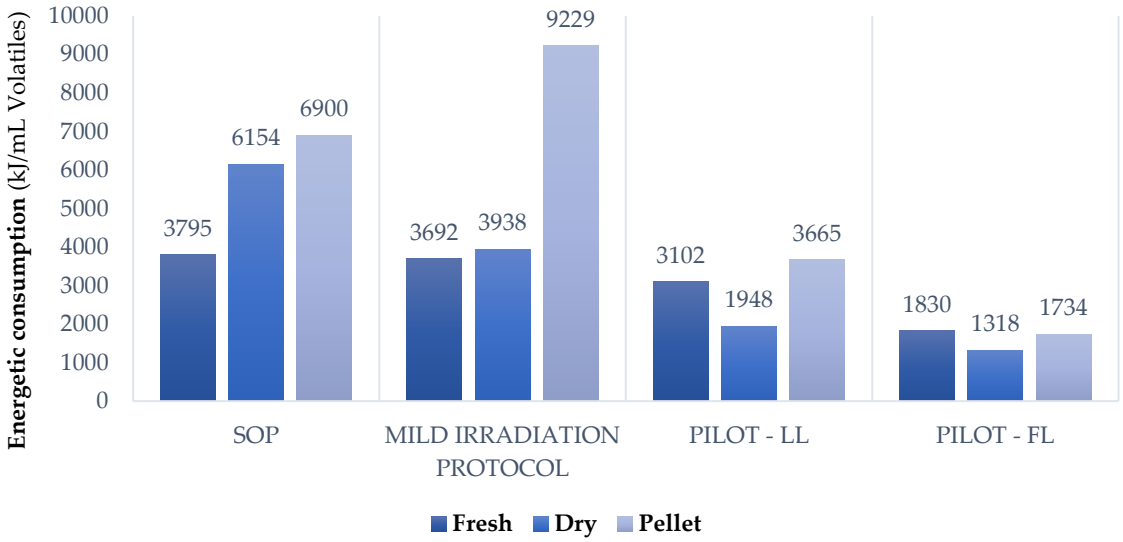


Figure 43. Energy consumption comparison. Chiller systems are computed.

During the GC-MS analysis, we focused our attention on the four characteristic hop compounds: myrcene, caryophyllene, farnesene and humulene. These terpenoids can be categorized by their chemical structure; monoterpenes (myrcene) and sesquiterpenes (caryophyllene, farnesene, humulene). The components of this last group have higher molecular weight and boiling points. As expected, myrcene is the most abundant terpenoid recovered from hops, followed by humulene, caryophyllene and farnesene. This trend was observed independently of matrix nature (FH, DH, PH). This is in line with the literature data on the cascade variety of hops [143]. An overview of this component distribution across the screened samples is reported in the Supplementary Material (Figure S2).

In the extraction carried out using SOP, it is possible to notice a high similarity between fresh- and dried-hop volatile fractions. (**Figure 44**). On the other hand, PH are characterized by a higher sesquiterpene-fraction concentration than the other matrices. The pelletization process probably causes this difference, as the mincing and extrusion steps occur at high temperature. Although it is considered to be a fast procedure, it can affect the terpenoid compounds. Since myrcene is the most volatile component, it is particularly prone to heat-related loss. It is possible to reduce pelletization temperature using a refrigerated system in order to lessen this phenomenon. For example, several industries exploit a liquid nitrogen system during mincing and extrusion. As these two steps are the crucial points, using a coolant afterwards would not be effective.

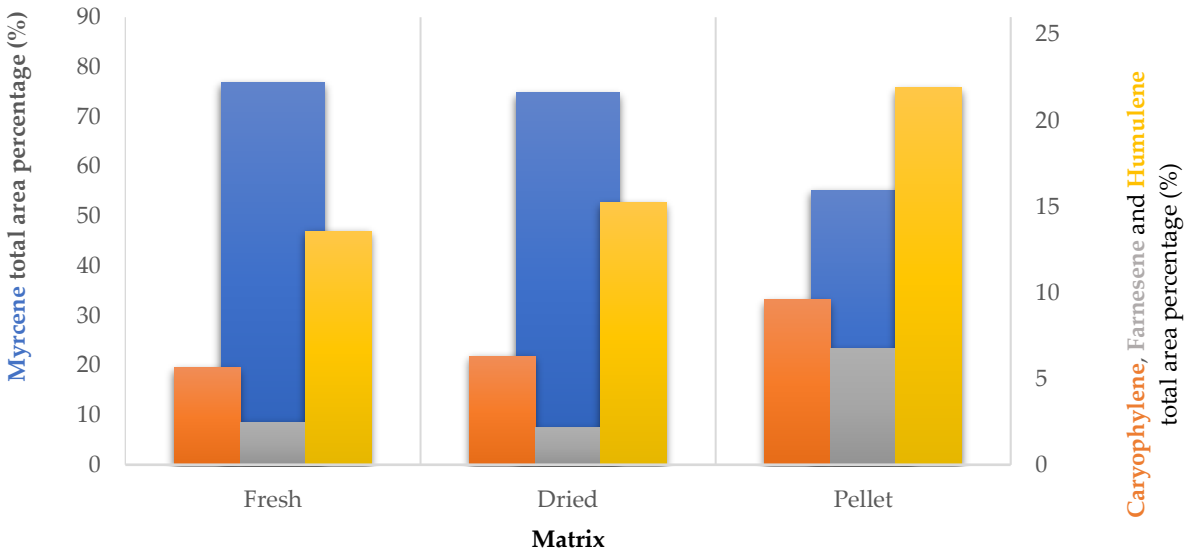


Figure 44. Main terpenoid distribution across SOP MAHD screenings, GC-MS analysis.

Much higher resemblance between the different biomasses was observed in the analysis of the volatile fractions recovered using the mild irradiation protocol, as can be appreciated in **Figure 45.** However, DH and PH still have a higher concentration of sesquiterpenes and a lower one of myrcene. The scale-up test that was carried out using the 12 L vessel (**Table 49**, entry 10) showed no considerable differences in sesquiterpene ratios, compared to the small-scale experiment, and a contraction in myrcene yield (approximately 8%). This reduction reflects the mild decrease in overall dry yield (17.67 mLVE/kgdry matrix vs. 20.51 mLVE/kgdry matrix) and can be attributed to FH being very sensitive to the soaking/charge steps that are necessarily extended due to the higher amount of starting material. Nevertheless, this loss can be considered negligible when we consider the three-fold increase in productivity (up to 3 kg). Thus, it is reasonable to employ the larger vessel without appreciably affecting the composition of the volatile fraction collected.

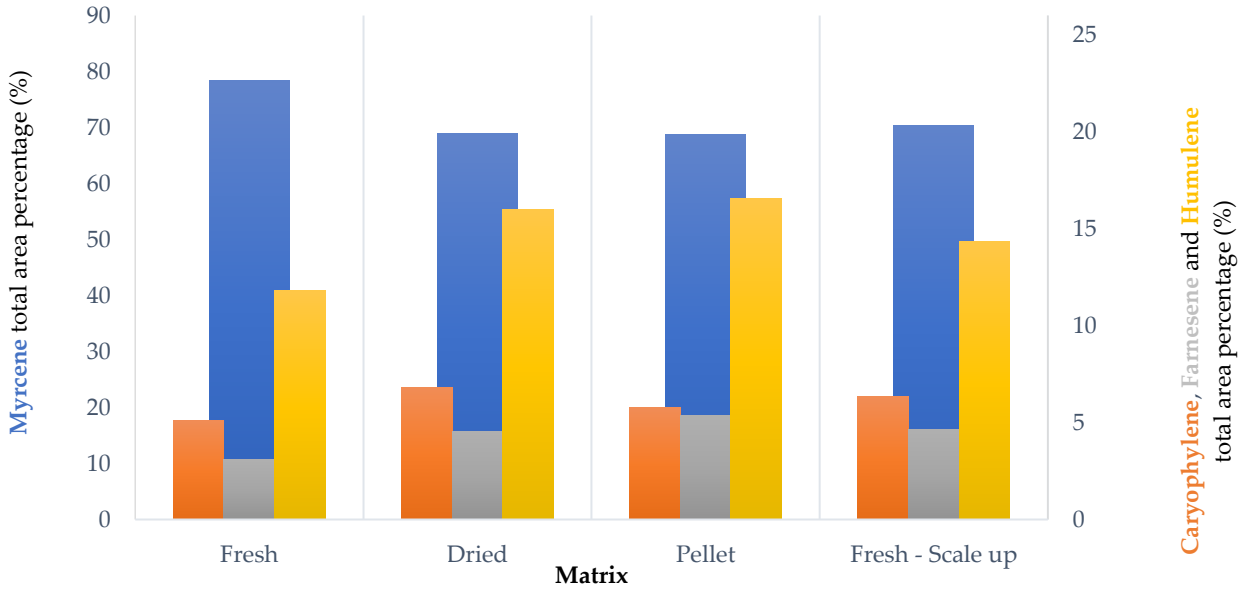


Figure 45. Main terpenoid distribution across mild irradiation MAHD and scale-up screenings, GC-MS analysis.

The analysis of the volatiles recovered from the ETHOS XL confirmed negligible fluctuation between LL (entries 11–13) and FL (entries 14–16) (see Figure S2), which confirms the interesting flexibility of the process. In addition, the relative composition of the main terpenes seems not to be influenced by the transposition to a larger scale (see **Figure 46**), meaning that the pilot scale-up not only reached comparable or enhanced extraction yields (**Table 49**) but also guarantees the collection of a high-quality product.

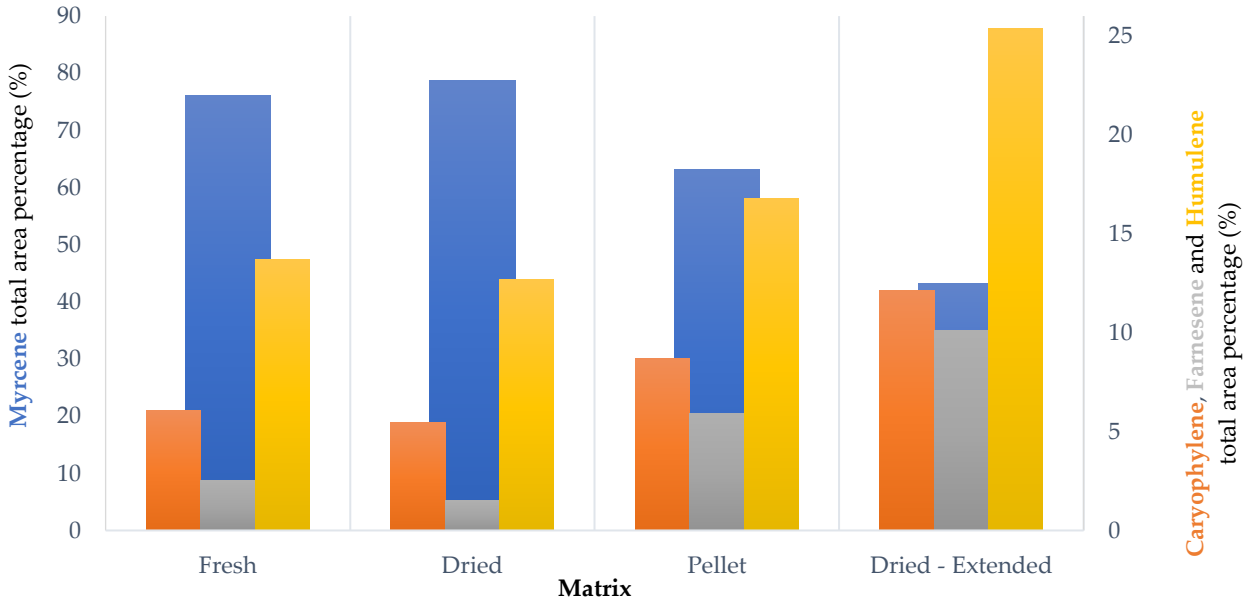


Figure 46: Main terpenoid distribution across ETHOS XL - MAHD (Full Load, FL), GC-MS analysis.

In order to study the evolution of volatile composition across the pilot protocol, the MAHD carried out on DH was extended by 20 min., and GC-MS also characterized the recovered product. As shown in **Figure 46**, it is interesting to note that the relative terpene

ratios appear to be unbalanced towards sesquiterpenes in the "extended" process. This is an important point indicating that the extraction is not linear during the entire MAHD process. It is plausible that the biomass is almost depleted of myrcene, due to its higher volatility, in the final phase of extraction. Thus, the collected product is progressively enriched with the sesquiterpene fraction by continuing the distillation. A simple evaluation of the percentage variation of terpene yield for the normal and extended protocols can support this hypothesis. To graphically depict the above reported discussion, it is possible to refer to the waterfall chart in **Figure 47** depicting how the accumulation of sesquiterpenes perfectly balances the myrcene contraction, and highlights the progressive enhancement of the first elements. It is reasonable to think that this type of behaviour may be exploited to select different product fractions, according to the most desirable flavour.

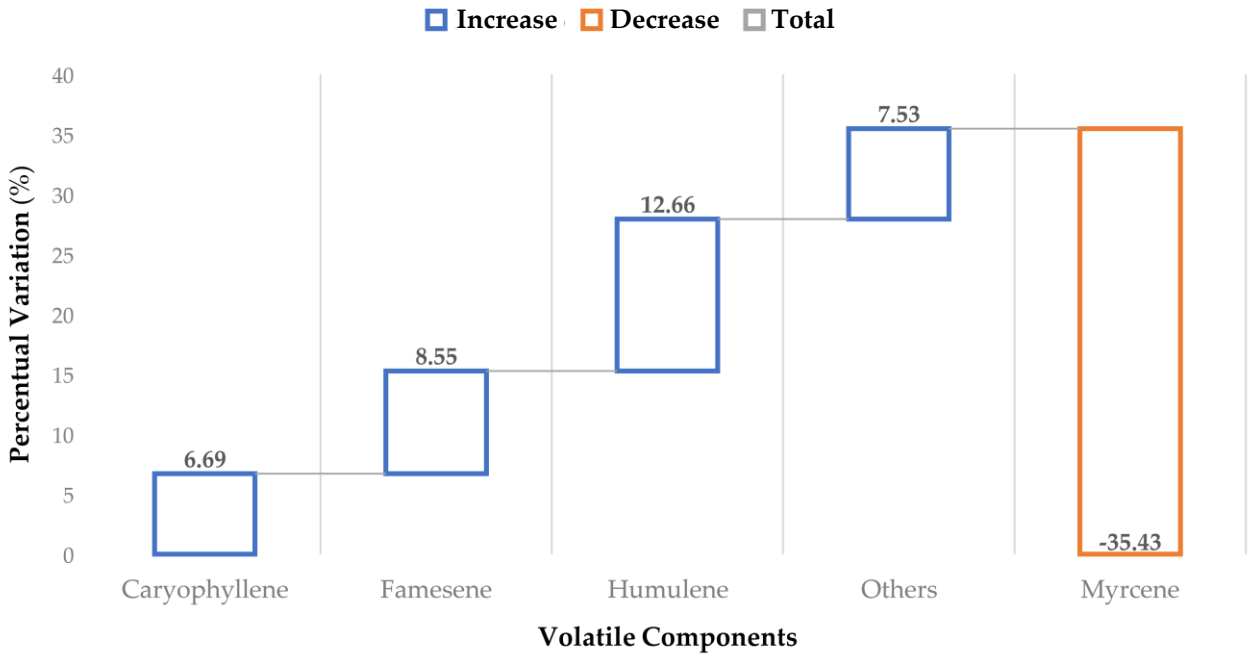


Figure 47. Waterfall chart of main-terpene percentage variation, according to normal and extended MAHD, performed with ETHOS XL.

In this study, we have applied MAHD to different types of hops (namely, FH, DH and PH). We first evaluated the variation in total volatile yields provided by several extraction protocols. Considerations as to overall MW irradiation and the soaking approaches were made, and we demonstrated that it is the possible to optimize the extraction procedure according to matrix nature. After this early-stage screening, the study continued by focusing attention on the scale-up transposition. Lab-scale tests with a 12 L reactor vessel and a new temperature-detection method paved the way for a pilot reactor (ETHOS XL, Milestone srl, Bergamo, Italy), which is able to process up to 30 kg on each run. The ETHOS XL reached extraction yields that are comparable with those of the laboratory-scale extractor. This instrument can treat more than 6 times the amount of biomass in a similar period, making

this process far more efficient. A single ETHOS X production run for a whole working day is 40 mL of volatile fraction and a consumption of 6.5 kg of fresh hops. An ETHOS XL in the same working time can produce 144 mL of volatiles and process 32.8 kg of fresh hops. Furthermore, we have verified how the pilot scale can significantly enhance the hydrodistillation yields for DH and PH, compared to small-scale projections; pellet productivity was approximately quadrupled, whilst that of dry cones nearly doubled. This feature is considerably valuable as the industry prefers to use dried and pelletized hops as this facilitates storage and preservation. Finally, the GC-MS analysis evaluated the variation of the four main volatile compounds. No significant variations were observed, while MAHD protocols were altered. On the other hand, hop preservation considerably influenced myrcene concentration, with the highest levels found in the fresh matrix. Finally, it was observed that the average quantity of sesquiterpenes (caryophyllene, humulene and farnesene) increased, with respect to monoterpenes, upon increasing the extraction time. It is reasonable to assert that this trend may be exploited to select different volatile fractions, according to demand.

4.2.3 Conclusions and Industrial Applications

The results collected using the ETHOS XL prototype were very promising. We decided to test this interesting equipment for the purification of many lipophilic volatile compounds from different biomasses such as citrus peels, coriander seeds and various inflorescence. The results collected showed very high extraction yields, especially for the softer biomasses like peels and inflorescences. Sensory experts tasted the terpene fraction collected from all the biomasses to verify the quality of the extract compared to other options on the market, and the results have been very positive. Moreover, the fact that the extract can be stored at -18 °C under a nitrogen atmosphere for long periods of time, maintaining its chemical and microbiological stability, makes it perfect for its implementation in the food industry. The low contamination rate of EOs makes them perfect even for applications on more susceptible goods like unbaked products or alcohol-free beverages.

The Baladin Brewery was developing the first 100% Italian craft alcohol-free beer and decided to utilize this approach to boost the aromas of the new product. The main problems faced during the development of an alcohol-free beer are the yeast/bacteria strain selection to operate the fermentation while remaining under 0.5% v/v of ethanol and the low aroma intensity of the product. The last problem occurs because is necessary to brew a wort with a very low concentration of fermentable sugars; thus, the aroma is quite lower compared to the standard product. However, the direct introduction of herbs or fruit peels to aromatise is not recommended since the absence of ethanol during maceration greatly increases the risk of contamination. For this reason, the Baladin Brewery decided to purify the desired

aromatic compounds from the inflorescences of *Cannabis sativa* L. using the ETHOS XL equipment and to introduce only the EO recovered for aromatization.

The NDA signed on this project prevents me from disclosing many details on the actual practices operated for the industrial production of Baladin Botanic (commercial name of the alcohol-free beer). The fermentation has been operated by a selected strain of yeast of the *Saccharomyces* family selected by the brewmaster from a wide range of commercial options. The extraction procedure followed the same steps of the fully loaded procedure described in section 4.2.2. The terpenes collected were introduced in the beer at the end of its maturation phase, right after the centrifugation, creating a staple emulsion between the EO and the alcohol-free beer. This step had many problems to overcome; for their chemical nature, the terpenes have a very low solubility in water, making the homogenization of the extract in the beverage very difficult.

To do it, we developed specific equipment implemented in the Baladin brewery for the stabilization of pure EO in an alcohol-free beverage. The machinery and the process implemented are currently under patent evaluation, so it is not possible to disclose many details on the procedure. Hopefully, in the next year, the three patents submitted will be accepted, and all the specifications will be published. Until then, I can just say that this procedure does not use any chemical solvent for the resuspension and has an insignificant energetic cost compared to the beer production.

Using this method, 16.000 cans of beer have already been produced for the Italian market and 32.000 bottles for the global market for a total of roughly 150,000 €. The use of this procedure has shown that is possible to implement new technologies to reduce risks and costs in old practices (**Figure 48**).

Figure 48. Baladin Botanic beer in can and bottle.

The press and the specialists of the sector have appreciated the beer well. It is not always easy to overcome the stubbornness of entrepreneurs attached to consolidated and trusted practices because there is often an investment to be made for new technologies. The implementation of unconventional technologies can significantly increase the extraction yields compared to standard methods. Reducing the total amount of raw materials used has a positive effect on the production and environmental costs of the process.

The strength of this extraction process relies on the simplicity of operation and wide range of applications. The lipophilic nature of the terpenes extracted makes them easy to purify, stock, and chemically stable in a controlled environment. The fact that the extract recovered is pure makes it a perfect candidate as an aromatizer in alcohol-free drinks.

The world's trend shows a sensible increase in healthier behaviours and habits such as the consumption of alcohol-free options. The extract produced using the MAHD is not only a valid option in this growing market but also promotes a green and sustainable approach which are recognized as important values for the customers.

5 FINAL SUMMARY AND PERSPECTIVES

This Thesis exemplifies the potential for optimisation in industrial practices. As leaders of industrial ventures, entrepreneurs often face challenges when considering changes to established operating procedures within their organisations. A multitude of technical, psychological, and practical factors influence this reluctance. One significant factor is the inherent risk of altering operational protocols. Industrial procedures are typically refined over time, incorporating lessons learned and optimised for efficiency and reliability through years of trial and error. Any deviation from these established practices introduces uncertainty, potentially disrupting production schedules, compromising quality standards, or increasing operational costs. Entrepreneurs, mindful of the delicate balance between risk and reward, may be hesitant to jeopardise the stability and success of their ventures.

Resource allocation presents another significant obstacle to procedural change. Implementing new operating protocols demands time, capital, and human resources investments. Entrepreneurs must weigh the potential benefits of such changes against the costs involved. In many cases, the perceived return on investment may not justify the expenditure, particularly if the existing procedures are deemed satisfactory or resources are limited. However, it's important to remember that the potential rewards of these changes can be significant, leading to increased efficiency, improved quality, and reduced costs. Psychological biases also play a crucial role in entrepreneurs' resistance to change. Humans naturally gravitate towards the *status quo*, favouring familiarity and predictability. Like everyone else, entrepreneurs may find comfort in the routines and structures guiding their operations thus far. Deviating from these established norms can evoke feelings of uncertainty and discomfort.

Moreover, entrepreneurs may not understand the necessity or potential benefits of altering operational procedures. Without clear insight into industry best practices, emerging technologies, or evolving market dynamics, they may struggle to justify the need for change. Limited awareness of alternative approaches or a failure to recognise inefficiencies within existing procedures can further impede efforts to instigate meaningful change.

Organisational culture also plays a pivotal role in shaping entrepreneurs' attitudes towards procedural modification. Established protocols often become deeply ingrained within an organisation's fabric, reflecting its values, traditions, and collective identity. Employees accustomed to familiar routines may resist changes perceived as disruptive or threatening. Entrepreneurs, mindful of maintaining workforce cohesion and morale, may be hesitant to challenge entrenched norms or confront resistance to change.

Finally, short-term considerations frequently overshadow long-term strategic objectives in entrepreneurial decision-making. While altering operational procedures may yield potential benefits in the future, the immediate costs and uncertainties associated with such

changes can deter entrepreneurs from focusing on short-term profitability. Maintaining stability and consistency in the face of uncertainty may thus take precedence over embracing innovation and adaptation.

In conclusion, entrepreneurs' reluctance to change industrial operating procedures stems from a complex interplay of risk aversion, resource constraints, psychological biases, limited awareness, organisational culture, and short-term focus. Overcoming these obstacles requires a concerted effort to foster a culture of innovation, promote open-mindedness, provide education and training on new technologies and practices, and incentivise experimentation and adaptation within the organisation.

However, this behaviour cannot be followed forever. When technology advances far enough, if only one entrepreneur decides to take the risk and implement a new production procedure, it will create a new golden standard. This change will make all his competitors obsolete, giving him a significant advantage in taking on the market. Unfortunately, this type of approach is prevalent in industries strictly connected to cultural aspects like food and craft productions.

The same can be said for the brewing industry, especially in the craft world, which is deeply connected to old procedures. The challenge we face is transferring the knowledge of chemical and physical principles to daytime procedures to simplify or shorten the required time without changing the final result. New technologies must be modelled and declined in different ways depending on the new field of applications.

For centuries, the by-products produced during brewing have been underrated and wasted. The increasing global awareness of food waste and the need for sustainable approaches is very inspirational. The lifestyle promoted by Western culture has induced the population to underestimate by-products or the environmental impact of your procedure. Only in the last decades, we can see a shift in this general behaviour. Our society needed to face this problem and its consequences before responding correctly. The importance of a sustainable approach has also been demonstrated by our panel test made on the different products enriched with BSGs. The customers were driven to buy the baked goods with BSGs because they value the effort to reduce food waste. This trend is more evident when you see panellists who gave a lower overall evaluation of the organoleptic properties but a higher desire to purchase the specific product.

The innovation effort made by the entrepreneur has shown many valuable returns; during the three years of research and development, many connections have been created between workers, industries and university departments. These new connections have already created collaboration projects between the realities, even on subjects not directly connected to the originating field. Moreover, the company's image is much stronger because it showcases its desire for innovation and the courage to try new and unexplored alternatives to traditional brewing. This is especially true for a craft brewery, which bases

much of its storytelling on the importance and respect given to the raw materials produced and harvested. This is the main difference understood and valued by customers between traditional industrial breweries and craft ones.

Moreover, Baladin Brewery can use its 20 pubs and restaurants to promote these new products, give them an excellent display, and explain efforts and philosophy. The salty snacks can be added to the aperitif in combination with the classical chips and peanuts so that the client will drink the beer produced from the cereals and pair it with the BSG crackers made with the whole grain remaining after the mashing phase so that in the exact moment the entire grain will feed you.

However, the most innovative and technologically demanding application of the BSGs relies on the selective extraction of the arabinose-xylans and β-glucans fibres. The purification of these components can modify the destiny of the BSGs from waste or animal-fed to integrators for nutraceutical products. The BSGs are an expense for traditional breweries because they must be disposed of; at maximum, they can be sold for less than 20 euros per ton (if any farmer is close by the brewery) exploiting BSGs as animal feed. The dedicated chapter explains that this is a very profitable biomass with many possible applications. The only problem it faces is the microbiological stability; to properly exploit all the BSGs produced by a brewery, a dedicated plant to stabilise the by-product is required. In this case study, we demonstrate that it is possible to transform the BSGs in flour and add it to a small percentage of baked goods. When used in small percentages (under 10% of total flour), there are no significant shifts in taste or texture, just a slight browning shift in the colour. In this case, the BSGs introduced in substitution of wheat flour can be valued as the flour itself, which is more than 40 times higher than BSGs. The return on investment should not be evaluated only on the increase of money gained using the by-product but also by the brand image upgrade.

The same thoughts can be made on the selective extraction and purification of the arabinoxylans and β-glucans. Those compounds gain even more value when introduced in nutraceutical and pharmaceutical products than simple flour. However, the logistic problems faced for its production were far higher. The main issue was the displacement from the drying facility to the Heallo compound, where those fibres were extracted and purified. The cost of the whole procedure is influenced primarily by the distance between the two facilities rather than other factors, such as the cost of raw materials, energy, and specific enzymes used to release the fibres.

For this reason, it seems essential to have an extraction facility near the brewery as for the drying one. Since a brewery's BSG production is massive, even smaller plants could benefit from using one of the described processes to transform a cost into a new range of possibilities. Of course, the investment needed to purchase a suitable drying machine and the extraction apparatus can be too high for small companies. In this case, the more

straightforward solution is to create a hub capable of processing the BSGs from different local breweries. In this way, the starting investment would be divided by the breweries, and the facility can be built to treat higher volumes, increasing the overall optimisation of each phase.

The BSG production of the Baladin brewery is estimated to be around 3 tonnes of wet biomass per day. It is impossible to convert all the BSG produced into flour for baked goods because the production rate exceeds the bakery's request. So even if this by-product processing proved itself economically profitable and sustainable, not all the BSGs produced can be exploited in that way. For this reason, we continue to investigate new possible applications that have not yet been tested.

Luckily, the possible applications of this exciting biomass are even more extensive than those investigated in this study. For example, two new research projects emerged from the collaboration that started during this project. The University of Turin, especially the Department of Food Science, is investigating the possibility of using the BSGs as a terrain for cultivating different fungi or bacteria. The study is just in the earlier stages, but the BSGs have been used to create a solid terrain and a liquid broth. The solid terrain shows promising results for cultivating many varieties of fungi, while the liquid broth has a lower replication cell rate than commercial competitors.

Fungi, often overshadowed by plants and animals, represent a vast reservoir of untapped potential. Their ability to thrive in diverse environments and metabolise various substrates makes them invaluable in multiple applications. Through controlled cultivation techniques, fungi can be tailored to fulfil specific needs, offering sustainable and efficient solutions to pressing global issues. Fungi can find applications in many fields; in agriculture, they can establish symbiotic relationships with plant roots, enhancing nutrient uptake and resilience to environmental stresses. Additionally, fungi-based biopesticides offer eco-friendly alternatives for pest control, reducing reliance on chemical agents and minimising ecological impact. Fungi possess remarkable capabilities for biodegradation and detoxification, making them a new horizon in the bioremediation field. Certain species can degrade complex pollutants, including hydrocarbons, pesticides, and heavy metals, thereby mitigating environmental contamination and restoring ecosystems. Fungi cultivar offers cost-effective and environmentally friendly solutions for wastewater treatment, soil remediation, and pollution control. However, fungi cultivations can also be exploited to produce specific compounds. Filamentous fungi such as *Aspergillus* and *Penicillium* species are employed in producing enzymes, organic acids, and flavour compounds used in food processing and biotechnology. Additionally, fungal fermentation processes yield valuable metabolites such as antibiotics, enzymes, and biofuels, contributing to sustainable bioproduction practices. They have long been esteemed in traditional medicine and are a rich source of bioactive compounds with therapeutic properties. Cultivation of medicinal

mushrooms such as *Ganoderma lucidum* and *Cordyceps sinensis* has garnered considerable attention for their immunomodulatory, antioxidant, and anticancer effects. Furthermore, producing fungal enzymes and metabolites holds promise for drug discovery and pharmaceutical applications.

Despite the myriad applications of fungi cultivars, several challenges persist, including optimising cultivation techniques, enhancing productivity, and addressing regulatory constraints. Advances in genetic engineering, synthetic biology, and bioprocess optimisation offer exciting avenues for overcoming these hurdles and unlocking the full potential of fungi in various industries. Collaborative interdisciplinary research efforts are essential to harnessing fungi's capabilities and translating scientific discoveries into practical solutions. Fungi cultivation represents a cornerstone of biotechnological innovation, offering diverse applications in agriculture, medicine, environmental remediation, and bioproduction. Researchers and practitioners can develop sustainable solutions to address global challenges and promote a more resilient and prosperous future by harnessing fungi's biochemical diversity and metabolic prowess.

The head of this new research will be again the Baladin Brewery, an agricultural brewery that farms most of its raw material used for brewing. For this reason, we decided to focus our attention on researching fungi capable of growing on BSGs and can be used as fertilisers or biopesticides. In this way, the brewery can use innovative products to increase field yields and reduce the use of chemical pesticides. Moreover, with these applications, the brewery already directly uses the products made without the need to find clients or enter a market where the brand is less known and weak. If this new project is successful, it will also positively affect the brand image. The brewery could become the first example of a total conversion of by-products produced since the BSY has already been converted completely into biogas. Marketing-wise, it is crucial to communicate to consumers that the food waste produced during beer production is wholly transformed into fertiliser for the fields where those raw materials were made. Implementing all these processes to treat the by-products of the brewery would reduce by nearly 90-95% the food waste produced during brewing. As shown in **Figure 49**, the BSGs are converted into baked goods to boost the revenue and into fertiliser for the barley fields, increasing the production rate for the next season; meanwhile, the BSY are digested into biogas that can be used to power the brewery itself.

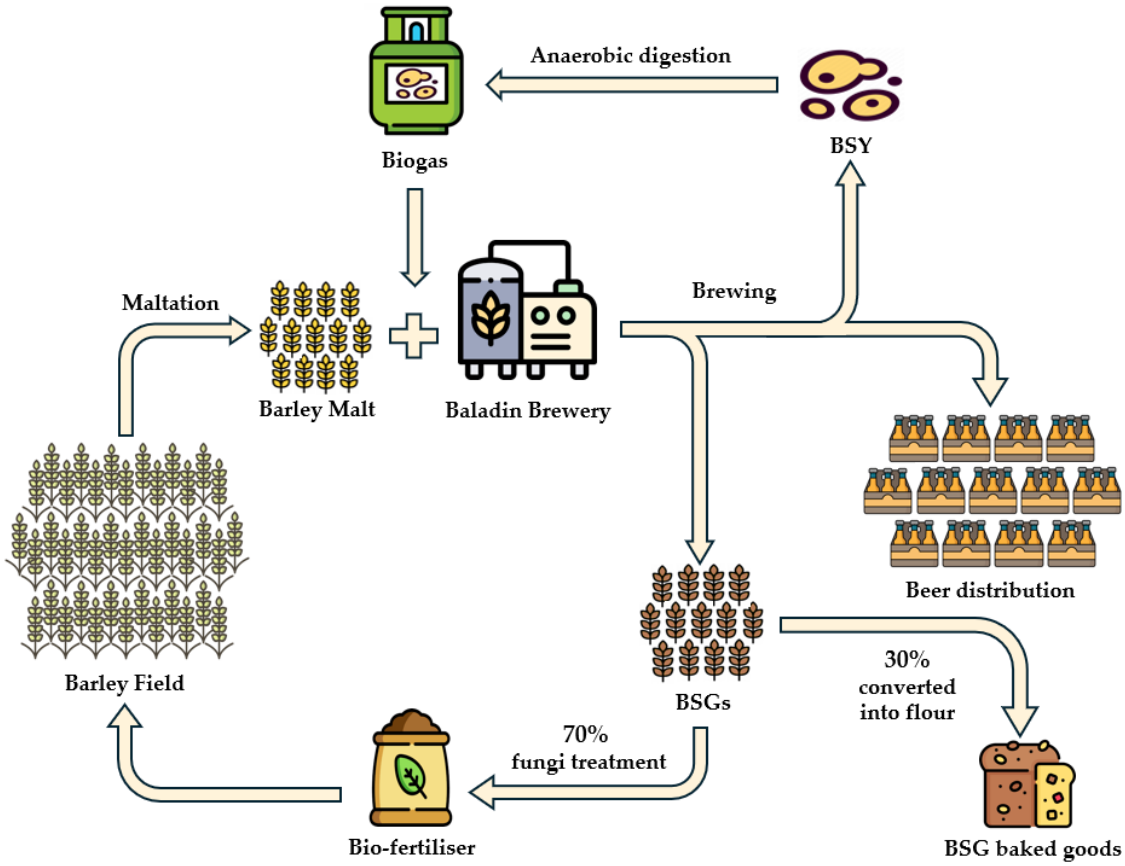


Figure 49: Schematic representation of the ideal circular brewery.

The other main point of focus of the Thesis after the exploitation of the brewery by-product was process intensifications with a specific focus on the extraction of volatiles for aromatisation purposes. Usually, this procedure is done via dry-hopping. This procedure consists of extended maceration, which is time-consuming and increases contamination risks since we introduce unsterile vegetal biomass.

The commercial success achieved by the beer aromatised with the EO extracted from cannabis inflorescences demonstrates the possible industrial application of this technology. Since it works on a non-pasteurized or microfiltered alcohol-free beverage, it can be easily applied to a classic beer with a much bigger market than its virgin version. This innovative procedure has been validated on its most challenging application, giving good insurance for broad applications. Not only because it is safer to introduce a new ingredient in an alcoholic beverage for microbiological stability reasons but also because the introduction of such lipophilic extract has been challenging, and we had to develop a specific technique to create a stable emulsion. However, this problem would be much easier to overcome if the solvent had some alcohol since the polarity of the solution would be more compatible with the essential oils. It is possible to firstly dissolve the essential oils in a volume 10 to 20 times higher than ethanol and then introduce it into the beer. The volatiles are so concentrated

that the final volume introduced to aromatise would not be significant for the total alcohol percentage of the product.

After the excellent market response collected on the virgin beer, we decided to implement the new technology in a regular beer, improving the dry-hopping procedure. Since hops inflorescence has many morphological similarities with cannabis, the extraction of the essential oil was operated with the same method described in **Table 47**. The volatiles recovered were used to aromatise an IPA style beer instead of traditional dry-hopping, and this beer was compared to the same IPA produced with standard dry hopping. To simplify, the beer that underwent the conventional procedure will be called DH (dry-hopped), while the innovative method will be called true-hopping, and therefore, the beer TH. The panel test unanimously said that the hops aroma intensity was significantly higher on the TH beer, proving higher extraction using MAHD than maceration.

We had to reduce the amount of extract introduced to 60-70% to get a balanced response from the panellist regarding the flavour intensity. However, the new tests showed that the aroma brought by the hops via DH or TH had some slight differences. Proving that the MAHD extract does not have a total superimposition with the volatiles extracted via DH. To overcome this new problem, we produced a beer with partial DH and TH—the first production phases until the fermentation phase was operated as usual. When the beer would usually receive its DH of 3 g/L, we introduced only 30% of it (0.9 g/L) and then before bottling, we mixed the beer with the extract recovered from 1.2 g/L, and we sent it to evaluation to the panellist. The result was very good; all the evaluators said that the two products were very similar and had difficulties discriminating them. Using this procedure, we could use only 2.1 g/L of hops, reducing its consumption by 30%. This reduction plays a significant role in total beer price; hops are beer's most expensive ingredient, and hoppy styles like I.P.A., hops cost could exceed 60% of the total raw material cost. So, such a significant reduction of hops consumed per litre produced significantly affects the producer's profit margins. This new methodology also has other positive effects, primarily the reduction of the recipe cost and the contamination risks, but not only. Typically, when the hops are introduced during dry-hopping, they tend to soak up ten times the beer's weight before depositing on the bottom of the fermentors with the yeast. This fact causes a net loss of product that gets removed along with the yeast slurry and hops (the deposit must be removed before bottling). Since the optimised procedure introduced only 0.9 g/L compared to 3 g/L, the beer loss during the beer purge dropped by 70%. Combined, those two aspects can significantly reduce production costs, especially for craft beers that are more prone to heavy dry-hopping procedures. The test made was so promising that the producer of the Ethos XL machine, Milestone srl, started an advertising campaign directly for craft brewers to enhance this new technology [144].

The Baladin Brewery decided to invest in this technology and wants to be the headliner of its application. During the next harvesting season, 50 kg of hop pellets will be treated with the ETHOS XL to extract the precious volatiles. The collected terpene will be used to aromatise the first commercial beer that used the true-hopping procedure, and we will evaluate the market response during the year. It is also important to assess the stability of the extracts. Since the hops are harvested once per year, the extract collected has to ensure correct stability, at least for this period. Otherwise, true hopping cannot follow the beer market's request that spikes from May to August. True hopping also comes with the help of craft breweries for the stocking problem of the hops; since this raw material is quite thermosensitive and prone to oxidation, it must be stored in a controlled sterile atmosphere without oxygen and refrigeration. Of course, this treatment is quite expensive compared to the other raw materials used and needs specific equipment. However, if a brewery decides to implement the true-hopping procedure, it would reduce the volume of stocked hops by 70%, adding only the refrigeration of the extracts that will occupy a small fraction of the original volume.

Climate change will probably be the biggest crisis humanity will have to face as a whole due to its numerous side effects that are difficult to anticipate and mitigate. One of the most discussed and studied problems is, of course, the shift of cultivars that tend to prefer every year terrain more north compared to decades ago. Hops cultivars can be found between 35° and 55° latitudes in both hemispheres. Climate change primarily affects farmers with fields closer to the equator, who report significant reductions in hop yield per hectare cultivated. Thanks to the considerable improvements made to the cultivation procedures, the farmers can still guarantee a high-quality product with the right balance of flavours. However, they report a statistical reduction in the quantity of inflorescence collected. This, of course, is a big problem for brewers who will inevitably see a steady increase in the hop cost. This problem cannot be resolved entirely by brewers who have no power to face or slow down global warming; however, exploiting at their full potential the inflorescence collected using the true-hopping procedure can mitigate this problem since fewer hops are needed per litre of beer produced.

After a whole year of testing this procedure on only a single beer, if we are satisfied with the final taste, we have the ambition to extend the true-hopping procedure to the whole beer produced by Baladin. The savings would be very significant for a craft brewery in this forecast. The Baladin brewery finished 2023 with 27’000 hL of beer produced, showing a stable increase yearly of around 8-15%. Even if we imagine that production will not increase in the near future, we underestimate the amount of hops used in dry-hopping, which is around 1.5 g/L. The full implementation of the dry-hopping procedure will reduce the quantity of hops consumed by around 1.2 tons per year. Since aromatic hops are the most expensive (20’000-40’000€ per tonne) and are used in dry-hopping, the shift to the true-

hopping procedure would save more than 25'000€ per year just on the raw material price. Let's consider reducing beer waste during purging. The savings increase even more, as shown in **Table 52**, where we made an educated prediction of the cost shifts between the two procedures.

Table 52. compares the different costs of implementing traditional dry-hopping to true-hopping.

Procedures	Raw material cost[a]	Purge waste[b]	Total process cost
Dry-hopping (1.5 g/L maceration)	Total consumption: 4.05 tonnes TOTAL COST: 81'000 €	hL of lost beer: 324 hL ECONOMIC LOSS: 97'200€	178.2 €
True-hopping (0.45 g/L maceration 0.6 g/L M.A.H.D. 1.05 g/L total)	Total consumption: 2.83 tonnes TOTAL COST: 56'700 €	hL of lost beer: 226.4 hL ECONOMIC LOSS: 67'920€	124.62 €

[a] *hops bought at 20'000 €/Tonne consumed to produce 27'000 hL*

[b] *For every kg of hops introduced, roughly 8 L of beer (3€ /L) are lost in the purge*

Even if all the calculations were made in defect, more than 50'000€ could be saved in a year without considering the storage and product stability simplifications. This process improvement can start a beer revolution in the craft world, and this is the perfect time for its implementation. The market trends show a substantial increase in the request from customers for beer focused on their hops flavour, and these styles of beer are the ones where true-hopping shows its best performances. Both in the economic and organoleptic aspects, since beer will generally have a dry-hopping around 5 to 10 g/L nearly five times, the margins are showcased in **Table 52**.

Different and innovative products were developed during these three years of research, and some are already on the market. To evaluate the whole project thoroughly, it is necessary to wait at least 12 months to see if the new line of true-hopped beer will be welcomed by customers, as happened with the Botanic beer. Unfortunately, the first craft beer produced using the essential oils will not be produced in the future. Due to a change in legislation implemented by the last government elected in October 2022, the essential oils derived from *Cannabis sativa* L. can only be commercialised using the pharmaceutical channel [145]. This change has been done because the EO derived from hemp is now considered a narcotic substance. Unfortunately, since the decree published does not express concentration limits, even if our aromatised beer has a not detectable concentration of CBD, it could still be considered non-compliant for trading. The Baladin Brewery has started an investigation with its lawyers to understand if there is some margin in our unique case. Since we are still waiting for a final response, we decided to stop its production, and we

started to try a new version that uses chamomile terpenes extracted via MAHD instead of the cannabis ones.

Even if the product developed during this project is currently out of the market, the overall evaluation is positive. All the work and time spent developing the Botanic beer will not be lost but directly implemented for the true-hopping procedure.

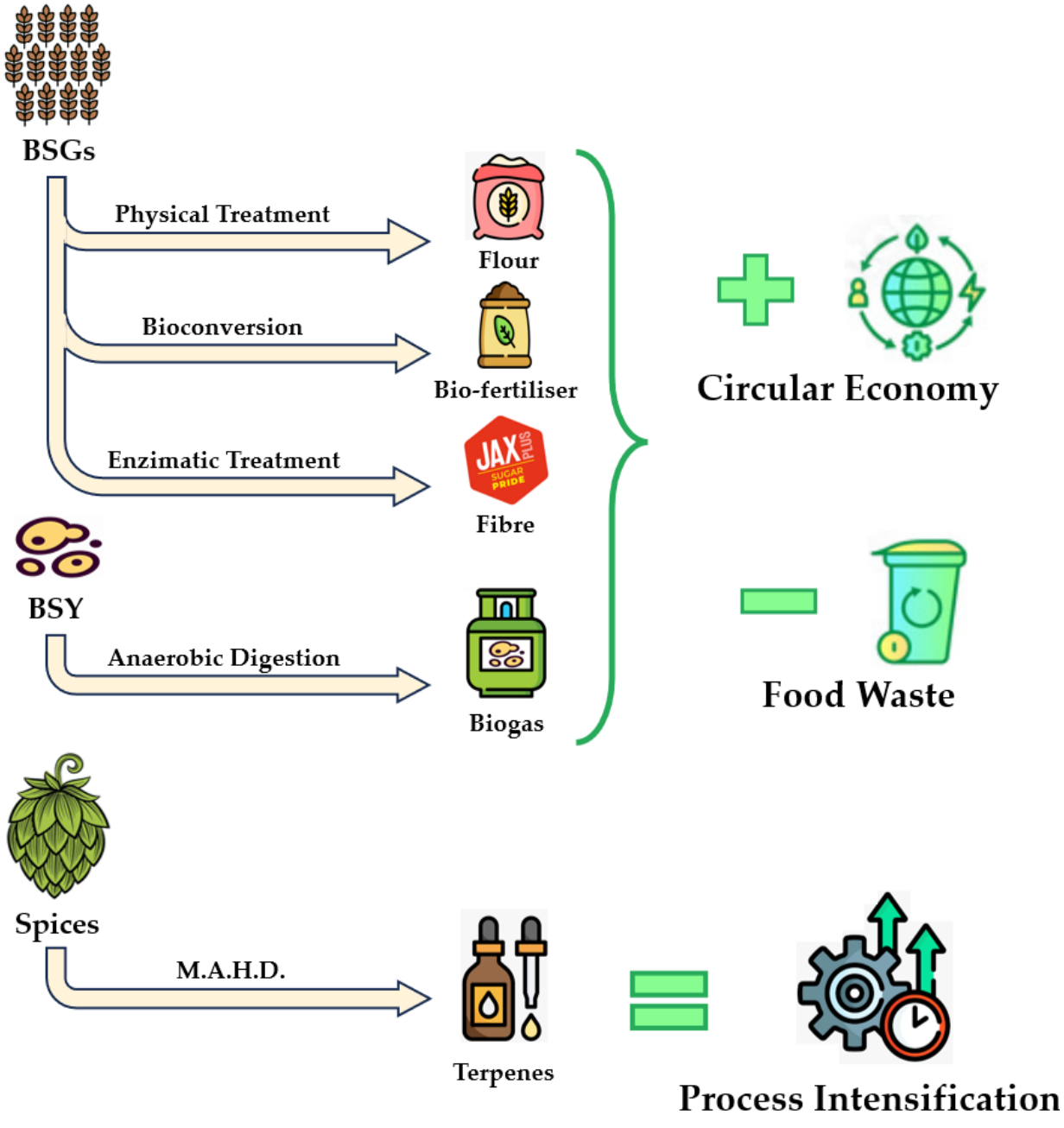


Figure 50. Schematic summary of the different technologies investigated and their industrial upside if implemented.

Figure 50 graphically summarises the main successes achieved on the different lines of research, enlightening the technology used and the up-side of the innovation. If all the innovative processes are implemented, the benefits will not be limited to reducing the environmental impact of beer production and lowering the LCA scores but will also create value. It is essential to merge the environmental and the economic aspects of this kind of innovation. If the benefits only have a positive environmental impact, it would be much more difficult for an entrepreneur to invest in these innovations since they would not directly impact but would only be appreciated as years of reduced pollution. The world politics are trying to support these practices by increasing taxes on wastes produced or allocating funds for sustainable innovations. Our project was even founded on national resources allocated to incentivise the development of collaborations between local industries promoting sustainable processes with a circular economy focus.

Ph.D. activities

Publications:

Publisher: Foods
Title: Microwave-Assisted Hydrodistillation Of Hop (Humulus lupu-lus L.) Terpenes. A Pilot Scale Study
Authors: L. Lamberti, G. Grillo, L. Gallina, D. Carnaroglio, F. Chemat and G. Cravotto

Publisher: Foods
Title: Industrial multiple-effect fractional condensation under vacuum for the recovery of hop terpene fractions in water
Authors: L. Lamberti, L. Boffa, G. Grillo, S. Concari, F. Cavani and G. Cravotto

Publisher: International Journal of Food Sciences and Nutrition
Title: The effects of a fibre-enriched bakery product on glucose, insulin values and appetite. A pilot randomised cross-over trial
Authors: V. Ponzo, D. Ojeda-Mercado, C. Finocchiaro, I. Goitre, E. Favaro, L. Lamberti, S. Bo

Publisher: Non-thermal Food Processing Operations
Title: Chapter Fourteen-Application of sonication in the food industry
Authors: F. Bucciol,L. Lamberti,G. Cravotto

Patent

Pending Title: Formation of a stable emulsion of essential oil for aromatization purposes in the beverage industry
Authors: Lamberti Lorenzo, Alessandro Ferrario

Congress Partecipations and Oral Presentations

21/05/2022 Festival of green and sustainable food industry.
07/09/2022 Winner of IFEAT scholarship and best young scientist award at ISEO2022 symposium
14/02/2023 Presentation at the BEER ATTRACTION of the Baladin Botanic Beer.
15/07/2023 Winner of IFEAT scholarship for the ISEO2023 symposium.
27/05/2024 Participation at European Brewing Congress 2024 at Lille

Supporting Material

Category	Sub-category	Compound
Monoterpenes	Acyclic	β-Mircene, β-ocimene
	Monocyclic	D-Limonene
	Bicyclic	*α*-Pinene, β-pinene
	Monoterpene alcohols	R- and S-Linalol, Gerianol, β-citronellol, *α*-terpineol, myrcenol, nerol, myrtenol
	Monoterpene epoxides	*cis*-Linalool oxide
	Monoterpene ketons	(E)-β-damascenone, β-ionone
Sesquiterpenes	Acyclic	β-Farnesene
	Monocyclic	*α*-Humulene
	Bicyclic	β-Caryophyllene, β-bergamotene, γ-muuroleene, β-eudesmene, δ-cadinene
	Sesq. alcohols	Humulol and humulenol II, nerolidol, δ-cadinol
	Monoepoxides	Humulene epoxide I, II, and III, caryophyllene oxide
Ketones		2-Nonanone, 2-undecanone, 2-dodecanone, 2-tridecanone
Isobutyrates		Octyl isobutyrate, geranyl isobutyrate, ethyl isobutyrate
Esters		Methyl octanoate, methyl 8-methylnonanoate, methyl (*E*)-4-decenoate, methyl geranate, geranyl propionate, methyl 3,6-dodecadienoate, ethyl 2-methylbutanoate, ethyl- 4-methylpentanoate, and ethyl 3-methylbutanoate

Table S1. Schematic overview of the main compounds recovered from the extraction of hops' EO [146] [147].

Volatile compounds HCH	vial 1					vial 2					vial 3					Mean	SD
	rt (min)	qual (%)	area	mg compound	ppm compound mg/kg aroma	rt (min)	qual (%)	area	mg compound	ppm compound mg/kg aroma	rt (min)	qual (%)	area	mg compound	ppm compound mg/kg aroma	ppm compound mg/kg aroma	
Toluene	***3.738***	***94***	***282022597***	***0.13005***		***3.607***	***94***	***295464665***	***0.1301***		***3.652***	***94***	***305732902***	***0.1301***			
β-Myrcene	10.602	96	17897703	0.00825	**16.51**	10.57	96	15768432.00	0.0069	**13.9**	10.606	96	14032455	0.0060	**11.9**	**14.1**	**2.3**
LINALOOL L	14.777	95	18746662	0.00864	**17.29**	14.768	96	18130064.00	0.0080	**16.0**	14.782	96	17609078	0.0075	**15.0**	**16.1**	**1.2**
Cyclopentasiloxane.											15.766	91	6758187	0.0029	**5.7**	**5.7**	**-**
GERANIOL	20.275	94	4367054	0.00201	**4.03**	20.284	93	2009120.00	0.0009	**1.8**						**2.9**	**1.6**
Methyl geranate						22.675	90	3268834.00	0.0014	**2.9**						**2.9**	**-**
α-Copaene	24.271	95	3787077	0.00175	3.49	24.271	96	3419945.00	0.0015	3.0	24.271	98	4102907	0.0017	3.5	**3.3**	**0.3**
trans-Caryophyllene	25.687	99	45622903	0.02104	**42.08**	25.687	99	45295221.00	0.0199	**39.9**	25.687	99	50227175	0.0214	**42.7**	**41.6**	**1.5**
α-Humulene	26.833	98	97658862	0.04503	**90.07**	26.838	98	96705046	0.0426	**85.1**	26.842	98	102214695	0.0435	**87.0**	**87.4**	**2.5**
α-Amorphene	27.440	98	6535544	0.00301	**6.03**	27.435	98	6241359.00	0.0027	**5.5**	27.435	99	7136906	0.0030	**6.1**	**5.9**	**0.3**
β-Selinene	27.889	99	5088165	0.00235	**4.69**	27.889	99	8604475	0.0038	**7.6**	27.889	99	8992058	0.0038	**7.6**	**6.6**	**1.7**
α-selinene	28.105	95	8760572	0.00404	**8.08**	28.101	99	11818810.00	0.0052	**10.4**	28.101	99	12811822	0.0054	**10.9**	**9.8**	**1.5**
γ. 2-cadinene	28.627	95	4941668	0.00228	**4.56**	28.622	96	4344489.00	0.0019	**3.8**	28.757	99	8940765	0.0038	**7.6**	**5.3**	**2.0**
δ-Cadinene	28.752	99	8329328	0.00384	**7.68**	28.757	99	8400058.00	0.0037	**7.4**						**7.5**	**0.2**
Isoledene	29.332	95	2427481	0.00112	**2.24**	29.341	95	2376833.00	0.0010	**2.1**	29.341	97	2334920	0.0010	**2.0**	**2.1**	**0.1**

Table S2. Data recovered from the HS-SPME/GC-MS of the three HCH samples analysed. Showing retention time (rt); percentage of quality recognition compared to the library (qual); total area of the peak (area) and the quantification of the compound.

Volatile compounds LCH	vial 1					vial 2					vial 3					Mean	SD
	rt (min)	qual (%)	area	mg compound	ppm compound mg/kg aroma	rt (min)	qual (%)	area	mg compound	ppm compound mg/kg aroma	rt (min)	qual (%)	area	mg compound	ppm compound mg/kg aroma	ppm compound mg/kg aroma	
Toluene	***3.603***	***94***	***125851422***	***0.13005***		***3.661***	***94***	***178310129***	***0.1301***		***3.670***	***94***	***187582777***	***0.1301***	***260.1***		
Isoamyl propionate						10.017	90	5183946	0.0038	7.6	10.004	83	5258217	0.0036	7.3	7.4	0.2
β-Myrcene	10.566	96	117449970	0.12137	242.74	10.606	96	227909151	0.1662	332.4	10.592	96	204551040	0.1418	283.6	286.3	44.9
Propanoic acid. 2-methylbutyl ester	11.590	90	2532871	0.00262	5.23	11.496	90	4640416	0.0034	6.8	11.482	82	4732323	0.0033	6.6	6.2	0.8
ISOPENTYL ISOBUTANOATE	11.478	90	11485547	0.01187	23.74	11.609	90	20094034	0.0147	29.3	11.595	90	19987126	0.0139	27.7	26.9	2.9
DL-Limonene	12.067	80	7524197	0.00778	15.55	12.076	95	13347945	0.0097	19.5	12.067	94	12703647	0.0088	17.6	17.5	2.0
Methyl 6-methyl heptanoate	14.287	86	3432014	0.00355	7.09	14.292	80	5293105	0.0039	7.7	14.287	90	4961994	0.0034	6.9	7.2	0.4
2-Nonanone						14.503	94	3317589	0.0024	4.8	14.490	94	3640918	0.0025	5.0	4.9	0.1
LINALOOL L	14.764	97	45420282	0.04694	93.87	14.778	97	58384743	0.0426	85.2	14.773	97	67005073	0.0465	92.9	90.6	4.8
Pentyl 3-methylbutanoate	15.070	90	4937096	0.00510	10.20	15.074	91	7789528	0.0057	11.4	15.070	90	7566151	0.0052	10.5	10.7	0.6
GERANIOL	20.216	95	14883825	0.01538	30.76	20.217	95	19649490	0.0143	28.7	20.216	95	19223700	0.0133	26.7	28.7	2.1
2-Undecanone	21.655	97	21366099	0.02208	44.16	21.655	96	29260959	0.0213	42.7	21.659	96	21699763	0.0150	30.1	39.0	7.7
4-Decenoic acid. methyl ester	22.145	96	18806925	0.01943	38.87	22.149	97	27527248	0.0201	40.2	22.149	93	20500308	0.0142	28.4	35.8	6.4
2.6-Octadienoic acid. 3.7-dimethyl-. methyl	22.608	83	51060706	0.05276	105.53						22.612	83	52505918	0.0364	72.8	89.2	23.1
Ylangene	24.046	99	18064048	0.01867	37.33	24.051	99	27657704	0.0202	40.3	24.046	99	19513184	0.0135	27.1	34.9	7.0
α-Copaene	24.271	99	60917611	0.06295	125.90	24.285	99	94949485	0.0693	138.5	24.275	99	67656576	0.0469	93.8	119.4	23.0
10.10-Dimethyl-4-acetyl-						25.211	81	4348477	0.0032	6.3						6.3	-
trans-Caryophyllene	25.781	99	511395410	0.52846	1056.91	25.826	99	765361199	0.5582	1116.4	25.795	99	579812125	0.4020	804.0	992.4	165.9
GERMACRENE-D/β cubebene	26.019	96	22390590	0.02314	46.28	26.042	98	36163181	0.0264	52.8	26.037	98	25249383	0.0175	35.0	44.7	9.0
α-Humulene	27.013	96	1040917012	1.07564	2151.29	27.076	96	1480849207	1.0801	2160.1	27.026	96	1121337457	0.7774	1554.8	1955.4	346.9
α-Amorphene	27.476	99	180650542	0.18668	373.35	27.503	99	279157207	0.2036	407.2	27.480	99	195299774	0.1354	270.8	350.5	71.0
α-Muurolene	27.588	98	20565565	0.02125	42.50	27.606	98	29758103	0.0217	43.4	27.593	97	20006582	0.0139	27.7	37.9	8.8
β-Selinene	27.925	99	161592346	0.16698	333.97	27.952	99	250306629	0.1826	365.1	27.930	99	173522769	0.1203	240.6	313.2	64.8
α-selinene	28.137	99	203872704	0.21067	421.35	28.164	98	307244066	0.2241	448.2	28.141	99	213435812	0.1480	295.9	388.5	81.3

α-Farnesene	28.375	98	9114635	0.00942	**18.84**	28.393	94	16759570	0.0122	**24.4**	28.379	91	10409172	0.0072	**14.4**	**19.2**	**5.0**
Geranyl propionate	28.501	90	10169803	0.01051	**21.02**	28.519	91	16617291	0.0121	**24.2**	28.510	87	9504737	0.0066	**13.2**	**19.5**	**5.7**
α-Amorphene	28.663	96	145057555	0.14990	**299.79**	28.685	97	220192619	0.1606	**321.2**	28.663	97	152201316	0.1055	**211.0**	**277.3**	**58.4**
δ-Cadinene	28.802	99	206141513	0.21302	**426.04**	28.838	99	307788919	0.2245	**449.0**	28.811	99	214530855	0.1487	**297.5**	**390.8**	**81.7**
1S.*cis*-CALAMENENE	28.923	97	67748892	0.07001	**140.02**	28.946	97	99361629	0.0725	**144.9**	28.932	97	68391834	0.0474	**94.8**	**126.6**	**27.6**
CADINA-1.4-DIENE	29.220	98	15014841	0.01552	**31.03**	29.238	98	25320702	0.0185	**36.9**	29.224	98	16639526	0.0115	**23.1**	**30.3**	**7.0**
Isoledene	29.355	97	99618757	0.10294	**205.88**	29.377	97	151252473	0.1103	**220.6**	29.364	97	102765033	0.0712	**142.5**	**189.7**	**41.5**
Selina-3.7(11)-diene	29.485	98	73879565	0.07634	**152.69**	29.508	98	110352665	0.0805	**161.0**	29.490	98	74363429	0.0516	**103.1**	**138.9**	**31.3**
1-Methyl-6-methylenebicyclo[3.2.0]heptane						29.800	91	5686293	0.0041	**8.3**						**8.3**	**-**
Caryophyllene oxide	30.699	95	7476032	0.00773	**15.45**	30.708	91	15253916	0.0111	**22.3**	30.699	95	12873199	0.0089	**17.8**	**18.5**	**3.4**
α-Caryophyllene	31.198	91	5387343	0.00557	**11.13**											**11.1**	**-**
caryophylla-4(12).8(13)-dien-5β-ol	32.299	93	3538508	0.00366	**7.31**											**7.3**	**-**
δ-gurjunene	32.820	81	13868928	0.01433	**28.66**											**28.7**	**-**
Ledene						32.825	90	16825783	0.0123	**24.5**						**24.5**	**-**
Eudesma-4(14).11-diene						33.252	94	10258788	0.0075	**15.0**						**15.0**	**-**

Table S3. Data recovered from the HS-SPME/GC-MS of the three LCH samples analysed. Showing retention time (rt); percentage of quality recognition compared to the library (qual); total area of the peak (area) and the quantification of the compound.

Volatile compounds PCH	vial 1					vial 2					vial 3					Mean	SD
	rt (min)	qual (%)	area	mg SI	ppm compound mg/kg aroma	rt (min)	qual (%)	area	mg SI	ppm compound mg/kg aroma	rt (min)	qual (%)	area	mg SI	ppm compound mg/kg aroma	ppm compound mg/kg aroma	
Toluene	**3.585**	**94**	**211387515**	**0.2601**		**3.684**	**94**	**240470482**	**0.2601**		**3.616**	**94**	**251940178**	**0.2601**			
2-Pentanol. propanoate						10.035	80	7190810	0.1301	13005.0						-	-
β-Myrcene	10.574	96	175422431	0.2158	21584.7	10.619	96	191450630	0.2071	20707.9	10.584	96	155164565	0.1602	16019.0	19437.2	2992.5
Propanoic acid. 2-methyl-. 3-methylbutyl ester						*11.505*	*72*	4956337	0.0054	536.1					0.0	268.0	379.1
Propanoic acid. 2-methyl-. 2-methylbutyl este	11.590	90	14407173	0.0177	1772.7	11.622	90	18250771	0.0197	1974.1	*11.595*	*90*	*15769983*	0.0163	1628.1	1791.6	173.8
Methyl 6-methyl heptanoate	14.269	90	14091482	0.0173	1733.9	14.292	83	14866670	0.0161	1608.0	14.278	86	13089501	0.0135	1351.3	1564.4	195.0
LINALOOL L						14.795	83	4770891	0.0052	516.0						516.0	-
n-Amyl isovalerate	15.065	91	7885067	0.0097	970.2	15.074	90	9031027	0.0098	976.8	15.074	90	6701615	0.0069	691.9	879.6	162.6
Octanoic acid. methyl ester	15.708	92	7678262	0.0094	944.8	15.708	87	6867418	0.0074	742.8						843.8	142.8
Methyl 6-methyloctanoate	18.135	91	12534752	0.0154	1542.3	18.140	91	14592128	0.0158	1578.3	18.140	91	12539444	0.0129	1294.6	1471.7	154.5
Nonanoic acid. methyl ester (CAS)	19.272	94	4897887	0.0060	602.7	19.281	97	4653744	0.0050	503.4	19.286	14	3930536	0.0041	405.8	503.9	98.4
2-Undecanone	20.414	87	3947911	0.0049	485.8	20.414	87	4416523	0.0048	477.7	20.419	87	4862243	0.0050	502.0	488.5	12.4
E-Citral	20.904	97	3952548	0.0049	486.3	20.895	97	6938441	0.0075	750.5	20.904	96	5304460	0.0055	547.6	594.8	138.3
2-Undecanone (CAS)	21.659	96	12847934	0.0158	1580.9	21.659	97	14715212	0.0159	1591.6	21.664	97	13285530	0.0137	1371.6	1514.7	124.1
4-Decenoic acid. methyl ester	22.145	99	48537805	0.0597	5972.3	22.145	99	44884682	0.0485	4854.9	22.145	96	40717819	0.0420	4203.7	5010.3	894.5
2.6-Octadienoic acid. 3.7-dimethyl-. methyl ester	22.612	90	27935438	0.0344	3437.3	22.612	95	24952793	0.0270	2699.0	22.612	83	24375623	0.0252	2516.5	2884.3	487.6
α-Ylangene	24.051	98	8285233	0.0102	1019.4	24.051	96	6665445	0.0072	721.0	24.046	99	10880214	0.0112	1123.3	954.6	208.9
α-Copaene	24.266	99	24379715	0.0300	2999.8	24.266	99	19682346	0.0213	2128.9	24.266	98	33409013	0.0345	3449.1	2859.3	671.2
trans-Caryophyllene	25.714	99	180130593	0.2216	22164.0	25.709	99	149912543	0.1621	16215.0	25.709	99	177695232	0.1835	18345.0	18908.0	3014.2
GERMACRENE-D	26.019	98	12854865	0.0158	1581.7	26.019	98	16156425	0.0175	1747.5	26.019	98	18863708	0.0195	1947.5	1758.9	183.1
α-Humulene	26.900	98	401765887	0.4943	49434.9	26.878	97	271877127	0.2941	29407.0	26.883	98	297016263	0.3066	30663.6	36501.9	11218.0
α-Amorphene	27.444	99	56294628	0.0693	6926.7	27.444	99	55278032	0.0598	5979.0	27.449	99	76318131	0.0788	7879.0	6928.3	950.0
Naphthalene. 1.2.4a.5.6.8a-hexahydro-4.7-dimethyl-1-(1-	27.557	98	5675128	0.0070	698.3	27.575	96	4816728	0.0052	521.0	27.566	96	7125162	0.0074	735.6	651.6	114.7
β-Selinene	27.889	99	29337219	0.0361	3609.8	27.894	99	29050396	0.0314	3142.2	27.894	99	40295008	0.0416	4160.0	3637.3	509.5

α-Cubebene	27.970	96	10214826	0.0126	**1256.9**	27.979	96	10320607	0.0112	**1116.3**	27.975	96	12106869	0.0125	**1249.9**	**1207.7**	**79.2**
α-selinene	28.101	99	58626027	0.0721	**7213.6**	28.101	99	31567365	0.0341	**3414.4**	28.105	98	68015246	0.0702	**7021.8**	**5883.3**	**2140.2**
α-Muurolene						28.163	99	19603627	0.0212	**2120.4**						**2120.4**	**-**
α-Fenchene	28.492	90	6546219	0.0081	**805.5**	28.487	92	6974692	0.0075	**754.4**						**779.9**	**36.1**
γ-Cadinene	28.627	97	53628510	0.0660	**6598.7**	28.626	97	55105469	0.0596	**5960.4**	28.631	97	73437057	0.0758	**7581.6**	**6713.5**	**816.7**
δ-Cadinene	28.766	99	63577998	0.0782	**7822.9**	28.757	99	20332093	0.0220	**2199.2**	28.761	99	21432650	0.0221	**2212.7**	**4078.3**	**3243.0**
1S.*cis*-CALAMENENE	28.896	97	16837791	0.0207	**2071.8**	28.896	97	12610036	0.0136	**1363.9**	28.896	97	16344637	0.0169	**1687.4**	**1707.7**	**354.4**
CADINA-1.4-DIENE	29.202	98	8526709	0.0105	**1049.2**	29.206	97	7731115	0.0084	**836.2**	29.202	91	10028507	0.0104	**1035.3**	**973.6**	**119.2**
α-cadinene	29.323	94	19517878	0.0240	**2401.6**	29.323	97	9824184	0.0106	**1062.6**	29.323	95	12562596	0.0130	**1296.9**	**1587.0**	**715.1**
(-)-Caryophyllene oxide						30.690	93	9647706	0.0104	**1043.5**	30.694	95	7515837	0.0078	**775.9**	**909.7**	**189.2**
E.E-α-FARNESENE						31.701	94	9140067	0.0099	**988.6**	31.697	86	6978020	0.0072	**720.4**	**854.5**	**189.7**
Alloaromadendrene	32.173	95	9071385	0.0112	**1116.2**				0.0000							**1116.2**	**-**
caryophylla-4(12).8(13)-dien-5β-ol						32.303	99	6756681	0.0073	**730.8**						**730.8**	**-**
δ-Cadinene	32.434	91	4161957	0.0051	**512.1**	32.434	94	6041919	0.0065	**653.5**	32.434	91	4854819	0.0050	**501.2**	**555.6**	**85.0**
α-Gurjunene	32.816	91	7248577	0.0089	**891.9**				0.0000							**891.9**	**-**
β-Panasinsene						32.820	91	11918780	0.0129	**1289.2**						**1289.2**	**-**

Table S4. Data recovered from the HS-SPME/GC-MS of the three PCH samples analysed. Showing retention time (rt); percentage of quality recognition compared to the library (qual); total area of the peak (area) and the quantification of the compound.

Volatile compounds HCT	vial 1					vial 2					vial 3					Mean	SD
	rt (min)	qual (%)	area	Compound (mg)	ppm compound mg/kg aroma	rt (min)	qual (%)	area	Compound (mg)	ppm compound mg/kg aroma	rt (min)	qual (%)	area	Compound (mg)	ppm compound mg/kg aroma	ppm compound mg/kg aroma	
Toluene	***3.612***	***94***	***250634484***	***0.1300500000***		***3.567***	***94***	***200184832***	***0.1301***		***3.729***	***94***	***233364006***	***0.1301***			
β-Myrcene	10.584	96	53922239	0.0279793390	**55.9587**	10.548	96	41640996	0.0271	**54.1**	10.579	96	48285536	0.0269	**53.8**	***54.6***	***1.2***
ISOBUTYL ISOPENTANOIC ACID ESTER	11.617	90	4064899	0.0021092074	**4.2184**	11.600	90	4290252	0.0028	**5.6**	11.622	86	4214940	0.0023	**4.7**	***4.8***	***0.7***
DL-Limonene	12.054	95	1285021	0.0006667757	**1.3336**	12.054	95	1197021	0.0008	**1.6**	12.054	95	1297021	0.0007	**1.4**	***1.4***	***0.1***
2-Nonanone	14.508	97	2241566	0.0011631107	**2.3262**	14.503	91	2011816.00	0.0013	**2.6**	14.508	95	2573127.00	0.0014	**2.9**	***2.6***	***0.3***
LINALOOL L	14.782	97	80377767	0.0417066655	**83.4133**	14.764	97	66063155.00	0.0429	**85.8**	14.782	97	95322150.00	0.0531	**106.2**	***91.8***	***12.5***
Cyclopentasiloxane. decamethyl-	15.762	91	1612310	0.0008366004	**1.6732**	15.753	91	5700819.00	0.0037	**7.4**						***4.5***	***4.1***
2-Decanone	18.230	93	938692	0.0004870714	**0.9741**	18.234	93	1490420.00	0.0010	**1.9**	18.243	90	2675456	0.0015	**3.0**	***2.0***	***1.0***
GERANIOL	20.239	93	4798767	0.0024899991	**4.9800**	20.243	93	5650073	0.0037	**7.3**	20.243	93	6220200.00	0.0035	**6.9**	***6.4***	***1.3***
2-Undecanone	21.659	97	23514347	0.0122011975	**24.4024**	21.655	97	28818367	0.0187	**37.4**	21.668	97	12140436.00	0.0068	**13.5**	***25.1***	***12.0***
methyl Z-4-decenoate	22.149	99	16927205	0.0087832407	**17.5665**	22.149	95	17600666.00	0.0114	**22.9**	22.154	98	10241959	0.0057	**11.4**	***17.3***	***5.7***
2.6-Octadienoic acid. 3.7-dimethyl-. methyl ester						22.612	87	84381162.00	0.0548	**109.6**	22.608	95	76832895	0.0428	**85.6**	***97.6***	***17.0***
α-Ylangene	24.046	97	1833375	0.0009513073	**1.9026**						24.051	94	2518447.00	0.0014	**2.8**	***2.4***	***0.6***
α-Copaene	24.271	99	6470917	0.0033576495	**6.7153**	24.051	96	9096174.00	0.0059	**11.8**	24.266	99	8836037.00	0.0049	**9.8**	***9.5***	***2.6***
trans-Caryophyllene	25.709	99	165623069	0.0859390128	**171.8780**	25.709	99	175387205.00	0.1139	**227.9**	25.718	99	216787468.00	0.1208	**241.6**	***213.8***	***36.9***
α-Longipinene	26.662	81	1475857	0.0007657973	**1.5316**											***1.5***	-
α-Humulene	26.883	98	311522348	0.1616436841	**323.2874**	26.883	98	336173718.00	0.2184	**436.8**	26.891	98	374688994.00	0.2088	**417.6**	***392.6***	***60.8***
α-Amorphene	27.431	99	18614481	0.0096587398	**19.3175**	27.436	99	24120058.00	0.0157	**31.3**	27.435	99	23156908.00	0.0129	**25.8**	***25.5***	***6.0***
α-Muurolene	27.566	95	2341705	0.0012150712	**2.4301**	27.56	99	2708054.00	0.0018	**3.5**	27.561	97	2867046.00	0.0016	**3.2**	***3.0***	***0.6***
β-Selinene	27.894	99	41718353	0.0216469487	**43.2939**						27.894	99	44523756.00	0.0248	**49.6**	***46.5***	***4.5***
α-selinene	28.096	99	41067386	0.0213091729	**42.6183**	28.096	99	48342428	0.0314	**62.8**	28.101	99	44460331	0.0248	**49.6**	***51.7***	***10.3***
α-Farnesene	28.357	98	9885095	0.0051292088	**10.2584**	28.352	99	13249387	0.0086	**17.2**	28.366	94	7892694	0.0044	**8.8**	***12.1***	***4.5***

Geranyl propionate	28.505	90	3414128	0.0017715333	**3.5431**	28.496	87	4722141	0.0031	**6.1**						***4.8***	***1.8***
γ-Cadinene	28.618	96	12442515	0.0064562109	**12.9124**	28.618	97	14431622	0.0094	**18.8**	28.618	97	15826798	0.0088	**17.6**	***16.4***	***3.1***
δ-Cadinene	28.757	98	20518665	0.0106467887	**21.2936**	28.753	99	23876929	0.0155	**31.0**	28.752	99	24158535	0.0135	**26.9**	***26.4***	***4.9***
1S.CIS-CALAMENENE	28.896	95	5964677	0.0030949701	**6.1899**	28.896	96	6870007	0.0045	**8.9**	28.896	97	6314125	0.0035	**7.0**	***7.4***	***1.4***
ADINA-1.4-DIENE	29.211	95	1702299	0.0008832942	**1.7666**	29.207	98	2307782	0.0015	**3.0**	29.206	93	1875049	0.0010	**2.1**	***2.3***	***0.6***
α-cadinene	29.328	95	2358230	0.0012236457	**2.4473**	29.328	97	3234705	0.0021	**4.2**	29.328	97	2660744	0.0015	**3.0**	***3.2***	***0.9***
α-CALACORENE	31.521	83	2778210	0.0014415662	**2.8831**	29.512	97	1467611	0.0010	**1.9**						***2.4***	***0.7***
Trans-γ-BISABOLENE						31.198	92	3337075	0.0022	**4.3**						***4.3***	***-***
3-Octyne. 5-methyl-											31.821	86	4068303	0.0023	**4.5**	***4.5***	***-***
7-Hexadecyn-1-ol						33.144	83	1866322	0.0012	**2.4**						***2.4***	***-***
Spiro[5.6]dodecane						33.324	90	1496771	0.0010	**1.9**						***1.9***	***-***

Table S5. Data recovered from the HS-SPME/GC-MS of the three HCT samples analysed. Showing retention time (rt); percentage of quality recognition compared to the library (qual); total area of the peak (area) and the quantification of the compound.

Volatile compounds LCT	vial 1					vial 2					vial 3					Mean	SD
	rt (min)	qual (%)	area	Compound (mg)`	ppm compound mg/kg aroma	rt (min)	qual (%)	area	Compound (mg)`	ppm compound mg/kg aroma	rt (min)	qual (%)	area	Compound (mg)	ppm compound mg/kg aroma	ppm compound mg/kg aroma	
Toluene	***3.553***	***94***	***191619736***	***0.1301***		***3.724***	***94***	***222157755***	***0.1301***		***3.585***	***94***	***204372693***	***0.1301***			
β-Myrcene	10.543	96	87659274	0.0595	119.0	10.579	96	90316911	0.0529		10.552	96	100309412	0.0638	127.7	117.5	11.0
Isobutyl isopentanoic acid ester ISOBUTYL ISOPENTANOIC ACID ESTER	11.581	90	10055595	0.0068	13.6	11.608	90	8705249	0.0051	10.2	11.585	90	10798443	0.0069	13.7	12.5	2.0
dl-Limonene	12.031	98	6006725	0.0041	8.2						12.04	97	6100988	0.0039	7.8	8.0	0.3
β-Phellandrene						12.125	96	4989908	0.0029	5.8						5.8	-
β-OCIMENE Y	12.741	96	3207075	0.0022	4.4						12.746	90	3330254	0.0021	4.2	4.3	0.1
2-Nonanone	14.481	97	4824210	0.0033	6.5	14.494	97	4722402	0.0028	5.5	14.485	97	5234858	0.0033	6.7	6.2	0.6
LINALOOL L	14.809	97	191123900	0.1297	259.4	14.831	97	228545346	0.1338	267.6	14.822	97	216488063	0.1378	275.5	267.5	8.0
BORNEOL L	17.425	90	3164748	0.0021	4.3	17.438	95	3625779	0.0021	4.2	17.43	90	3569743	0.0023	4.5	4.4	0.2
4-Terpineol						17.740	99	2527846	0.0015	3.0	17.74	97	2447329	0.0016	3.1	3.0	0.1
2-Decanone	18.194	93	2407275	0.0016	3.3	18.198	94	2215267	0.0013	2.6	18.189	97	2488409	0.0016	3.2	3.0	0.4
α-TERPINEOL	18.306	91	4685742	0.0032	6.4	18.310	91	5293229	0.0031	6.2	18.306	91	2488409	0.0016	3.2	5.2	1.8
Nerol	19.313	93	4943728	0.0034	6.7	19.317	96	5290019	0.0031	6.2	19.317	93	6024749	0.0038	7.7	6.9	0.7
GERANIOL	20.212	97	29374908	0.0199	39.9	20.216	95	37432781	0.0219	43.8	20.212	95	35874990	0.0228	45.7	43.1	3.0
2-Undecanone	21.097	97	25476226	0.0173	34.6	21.659	97	26944310	0.0158	31.5	21.655	97	26435490	0.0168	33.6	33.3	1.6
4-Decenoic acid. methyl ester	22.154	96	9203429	0.0062	12.5	22.158	97	8959084	0.0052	10.5	22.154	95	9244714	0.0059	11.8	11.6	1.0
2.6-Octadienoic acid. 3.7-dimethyl-. methyl ester	22.612	83	78632644	0.0534	106.7	22.617	95	76874870	0.0450	90.0	22.612	83	82360158	0.0524	104.8	100.5	9.2
α-Copaene	24.271	95	3406438	0.0023	4.6	24.266	99	4796853	0.0028	5.6	24.266	99	5284990	0.0034	6.7	5.7	1.1
trans-Caryophyllene	25.696	99	94979873	0.0645	128.9	25.705	99	130297434	0.0763	152.6	25.705	99	144641145	0.0920	184.1	155.2	27.7
α-Humulene	26.851	98	188877535	0.1282	256.4	26.873	99	241819629	0.1416	283.1	26.869	98	263873270	0.1679	335.8	291.8	40.4
α-Amorphene	27.435	99	6629811	0.0045	9.0	27.435	98	9800971	0.0057	11.5	27.431	99	10775104	0.0069	13.7	11.4	2.4
β-Selinene	27.885	99	20668421	0.0140	28.1	27.889	99	27414686	0.0160	32.1	27.894	99	30347689	0.0193	38.6	32.9	5.3
α-selinene	28.096	99	18954034	0.0129	25.7	28.101	99	25653497	0.0150	30.0	28.096	99	28829130	0.0183	36.7	30.8	5.5
α.-Farnesene	28.366	96	3301202	0.0022	4.5	28.370	98	3872023	0.0023	4.5	28.361	99	4507707	0.0029	5.7	4.9	0.7

Geranyl propionate						28.505	87	1695368	0.0010	**2.0**						**2.0**	-
δ-Cadinene	28.752	99	7264272	0.0049	**9.9**	28.752	99	10194378	0.0060	**11.9**	28.757	99	11190226	0.0071	**14.2**	**10.9**	**2.2**

Table S6. Data recovered from the HS-SPME/GC-MS of the three LCT samples analysed. Showing retention time (rt); percentage of quality recognition compared to the library (qual); total area of the peak (area) and the quantification of the compound.

Volatile compounds PCT	vial 1					vial 2					vial 3					Mean	SD
	rt (min)	qual (%)	area	Compound (mg)`	ppm compound mg/kg aroma	rt (min)	qual (%)	area	Compound (mg)`	ppm compound mg/kg aroma	rt (min)	qual (%)	area	Compound (mg)`	ppm compound mg/kg aroma	ppm compound mg/kg aroma	
Toluene	***3.715***	***94***	***270817180***	***0.2601***		***3.450***	***94***	***359153466***	***0.2601***		***3.706***	***94***	***343024554***	***0.2601***			
β-Myrcene	10.642	96	474950596	0.4562	45615.5	10.570	96	310062497	0.2245	22454.8	10.629	96	329971523	0.2502	25020.2	31030.2	12696.2
Heptane. 4-methyl-						11.465	72	3467450	0.0025	251.1						251.1	
ISOBUTYL ISOPENTANOIC ACID ESTER	11.595	90	15662653	0.0150	1504.3	11.568	90	15208655	0.0110	1101.4	11.604	90	14639539	0.0111	1110.1	1238.6	230.1
DL-Limonene	12.040	99	13299262	0.0128	1277.3	12.018	99	9118286	0.0066	660.3	12.053	99	8486572	0.0064	643.5	860.4	361.2
LINALOOL L	14.773	97	13888987	0.0133	1333.9	14.760	96	16339205	0.0118	1183.3	14.773	97	18721555	0.0142	1419.6	1312.3	119.6
Octanoic acid. methyl ester	15.690	94	6329451	0.0061	607.9	15.695	94	7286480	0.0053	527.7						567.8	56.7
2-Undecanone	21.655	96	32996212	0.0317	3169.0	21.655	97	39287302	0.0285	2845.2	21.655	97	42695496	0.0324	3237.4	3083.9	209.5
4-Decenoic acid. methyl ester	22.140	91	32183926	0.0309	3091.0	22.140	91	35341113	0.0256	2559.4	22.145	95	38834393	0.0294	2944.6	2865.0	274.6
2.6-Octadienoic acid. 3.7-dimethyl-. methyl ester	22.612	83	78854375	0.0757	7573.4	22.612	95	86971363	0.0630	6298.5	22.617	83	95822803	0.0727	7265.8	7045.9	665.3
α-Copaene	24.266	96	6309392	0.0061	606.0	24.267	96	4655690	0.0034	337.2	24.271	98	5041446	0.0038	382.3	441.8	144.0
trans-Caryophyllene	25.705	99	140930851	0.1354	13535.4	25.700	99	115058707	0.0833	8332.6	25.700	99	127998977	0.0971	9705.6	10524.5	2696.3
α-Humulene	26.869	97	245470593	0.2358	23575.6	26.860	98	216574256	0.1568	15684.4	26.869	98	239531102	0.1816	18162.6	19140.9	4035.6
α-Amorphene	27.431	99	10269331	0.0099	986.3	27.431	98	8437597	0.0061	611.1	27.435	98	10398835	0.0079	788.5	795.3	187.7
β-Selinene	27.894	99	34111900	0.0328	3276.2	27.890	99	27713202	0.0201	2007.0	27.894	99	31919142	0.0242	2420.3	2567.8	647.3
α-selinene	28.092	99	33905998	0.0326	3256.4	28.096	96	29833523	0.0216	2160.6	28.101	98	32903390	0.0249	2494.9	2637.3	561.6
α-Farnesene	28.352	99	7605074	0.0073	730.4	28.353	96	5035740	0.0036	364.7	28.352	96	7758084	0.0059	588.3	561.1	184.4
Geranyl propionate						28.492	87	3888620	0.0028	281.6	28.487	87	5215550	0.0040	395.5	338.5	80.5
γ-Cadinene	28.613	97	8233511	0.0079	790.8	28.622	97	6844792	0.0050	495.7	28.618	98	7430283	0.0056	563.4	616.6	154.6
δ-Cadinene	28.748	99	12118292	0.0116	1163.9	28.753	99	9035446	0.0065	654.3	28.753	99	11625278	0.0088	881.5	899.9	255.3

Table S7. Data recovered from the HS-SPME/GC-MS of the three PCT samples analysed. Showing retention time (rt); percentage of quality recognition compared to the library (qual); total area of the peak (area) and the quantification of the compound

Step	Time (min)	Power (W)
1	15	1800
2	75	1100

Table S 8: Standard Operating Procedure (SOP) standardized MAHD protocol.

A: Low Loading (LL)		
Step	**Time** (min)	**Power** (W)
1	Up to 100°C	4000
2	10	4000
3	60	3200

B: Full Loading (FL)		
Step	**Time**(min)	**Power**(W)
1	Up to 100°C	4000
2	30	4000
3	90	3200

Table S 9: ETHOS XL extraction protocols. A: half capacity or lower; B: more than half the capacity.

Protocol A			
Step	**Time** (min)	**Power** (W)	**Temperature** (°C)
1	10	1200	95
2	100	1200	95

Protocol B			
Step	**Time** (min)	**Power** (W)	**Temperature** (°C)
1	10	1800	99
2	100	1600	99

Table S 10: MAHD vacuum extraction protocol A and B.

°C/min.	Final temperature	Hold
0	35	2
5	65	0
2	85	0
10	130	0
2	150	0
10	300	0

Table S 11: GC temperature protocol.

Entry	Hops		L/S	Volatiles (mL)	Yield (ml_{VF}/kg)	Dry Yield ($mL_{VF}/kg_{dry\ matrix}$)	Type of evaluation
	Type	Weigh t (g)					
1	F	1200	0.5	6	5.00	16.67	SOP
2	D	400	1	3.7	9.25	10.51	
3	P	1000	3	3.3	3.30	3.75	
4	F	1200	0.25	*Burnt biomass, no recovery*			Moistening evaluation
5	F	1100	1	5.5	5.00	16.67	
6	F	1200	2	5.5	4.58	15.28	
7	F	1300	1	8.0	6.15	20.51	Mild MAHD
8	D	1000	1	7.5	7.50	8.52	
9	P	1300	1	3.2	2.46	2.80	
10	F	3000	1	15.9	5.30	17.67	
11	F	2500	0.5	13.0	5.20	17.33	Pilot (LL)
12	D	2000	1	20.7	10.35	11.76	
13	P	2000	1	11.0	5.50	6.25	
14	F	8200	0.5	36.0	4.39	14.63	Pilot (FL)
15	D	3820	1	50.0	13.09	14.87	
16	P	4000	1	38.0	9.50	10.80	
17	F	700	1	1.7	2.43	8.10	Vacuum MAHD
18	F	700	1	1.9	2.71	9.05	

Table S 12: MAHD screening summary. F: Fresh hops; D: Dried hops; P: Pelletized hops.

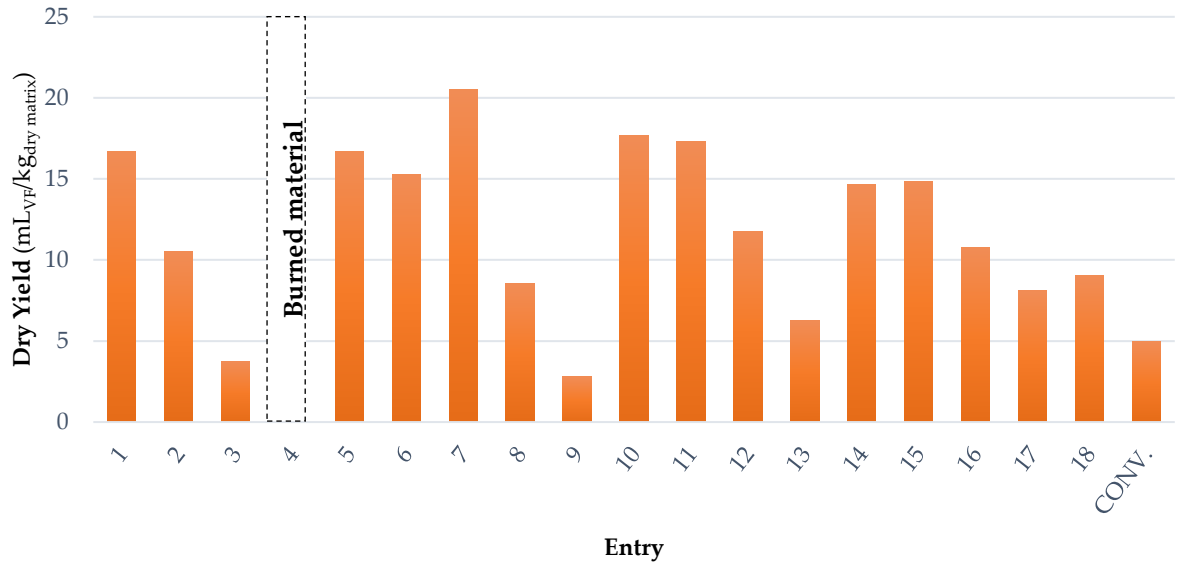


Figure S 1: Volatiles fraction extraction yields summary.

Entry	Volatile Fraction (mL)	Energy Consumption (kJ)	Energy Consumption (kJ/mL_{VF})
1	6	22770	3795
2	3.7	22770	6154
3	3.3	22770	6900
4	-	22770	-
5	5.5	22770	4140
6	5.5	22770	4140
7	8	29532	3692
8	7.5	29532	3938
9	3.2	29532	9229
10	15.9	97032	6103
11	13	40320	3102
12	10.5	40320	3840
13	11	40320	3665
14	36	65880	1830
15	50	65880	1318
16	38	65880	1734
17	1.7	22321	13130
18	1.9	24466	12877

Table S 13: Energy consumption evaluation. *Note*: In the energy consumption evaluation, both reactors and respective chiller has been taken in account.

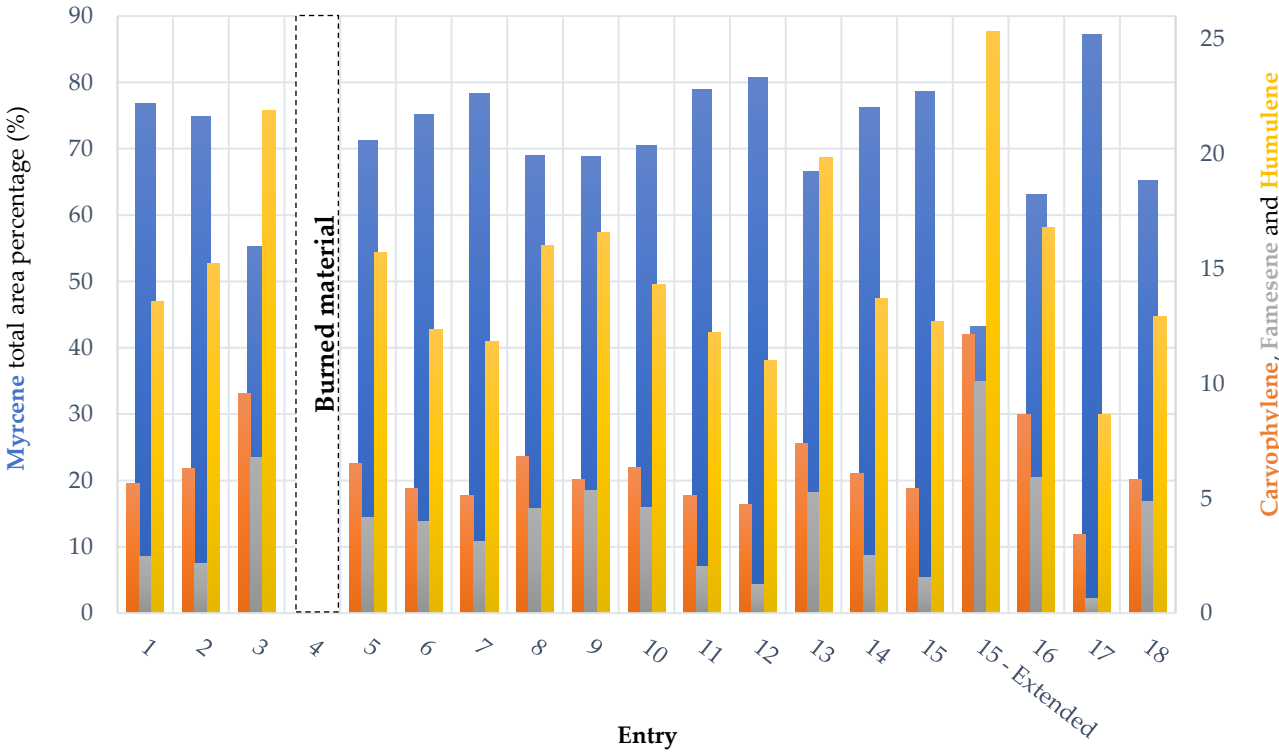


Figure S 2: Main terpenoids distribution across MAHD screening, GC-MS analysis.

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