EVALUATION OF BEER SENSORY QUALITY AND SHELF LIFE STABILITY USING MULTIVARIATE ANALYSIS TECHNIQUES

Ana Carolina De Lima E Silva

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Evaluation of Beer Sensory Quality and Shelf Life Stability Using Multivariate Analysis Techniques

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Ana Carolina de Lima e Silva

DOCTORAL THESIS

2024

ANA CAROLINA DE LIMA E SILVA

Evaluation of Beer Sensory Quality and Shelf Life Stability Using Multivariate Analysis Techniques

Doctoral Thesis

Supervised by Ph.D. Ricard Boqué Martí and Ph.D. Laura Aceña Muñoz

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Universitat Rovira i Virgili

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Tarragona, 2024

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CERTIFY,

that the Doctoral Thesis entitled "**Evaluation of Beer Sensory Quality and Shelf Life Stability Using Multivariate Analysis Techniques**", submitted by Ana Carolina de Lima e Silva to receive the degree of Doctor with International Mention by Universitat Rovira i Virgili has been carried out under our supervision in the Department of Analytical Chemistry and Organic Chemistry of this University, and all the results presented in this thesis were obtained in experiments conducted by the above mentioned student.

Tarragona, 9th January 2024,

Ph.D. Ricard Boqué Martí Ph.D. Laura Aceña Muñoz

This work constitutes a part of the research developed by the Chemometrics and Sensorics for Analytical Solutions (ChemoSens) research group of the Universitat Rovira i Virgili.

The results here presented have been obtained in the iSens laboratory at the Faculty of Oenology of Tarragona

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Generalitat de Catalunya
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"Have no fear of perfection; you'll never reach it."

Marie Curie.

A mis grandes Maestros, Kau Lima, Zé Geraldo,
Claudilene, Cláudia Lima, Ana Catarina y Sandro.

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Chapter 1
INTRODUCTION

1.1 A Brief History of Beer and Brewing

Beer is a fermented beverage made from water, yeast, malt, and hops, and it is considered one of the oldest beverages produced in the world. The first step that made possible the creation of beer occurred around 9,500 and 6,000 BC, when humans founded great settlements in the Fertile Crescent in the Middle East, along the Nile in Africa and the flood plains of Yangtze and Yellow Rivers in China, with a reliable supply of suitable grains [1].

The oldest writing in the world describing beer, the clay tablets, in Figure 1, dates back to 6,000 BC from the ancient region of Mesopotamia known as Sumeria [2–4]. It contains calculations of basic ingredients required to produce cereal products, such as different types of beer.

Figure 1: Archaic writing tablet from Mesopotamia [5].

These tablets described the recipes of beer brewed at that time in Babylonia. According to the literature [2], the grains used were barley, emmer wheat, or a mixture of both, and should be from the last harvest. The grains should not be husked or ground; they must be soaked in several changes of water and left in the sun to germinate. After germination,

the grain was stewed in the purest possible water and should be left to ferment. The mash might be cooked in pots over the fire or plunged into the vessel with the infusion.

Beer started to spread to ancient Egypt around 3,000 BC. The Egyptians believed that the god of agriculture Osiris, made a decoction of barley germinated with the sacred waters of the Nile, and forgot it out in the sun. When he returned, the mixture had fermented and when drunk it, it seemed so good to him that he let humankind benefit from it. It is said that this was the origin of beer [2]. For the Egyptians, the brewing process was a domestic chore delegated to the females. The Egyptians became famous brewers using scientific methods to produce beer and their exported beer called *zythos,* was very popular in Athens. It was only after Egypt was conquered by the Roman Empire that the Egyptian elite started to prefer wine to beer. However, beer remained the beverage of the Egyptian people.

According to Poelmans and Swinnen [3], the earliest indication of beer production in Europe dates from 3,000 BC and there is some uncertainty about whether Europeans discovered the fermentation process themselves or whether the technology used was based on knowledge from the Near East. Around 500 BC, the Greek civilization brewed beer in the way that they learnt of their ancestors. With the increase in the popularity of wine and the notion of wine as a more civilized drink, the Greeks started to drink wine instead of beer.

Beer was brought to the region of Gaul (France and Belgium), Spain and the east coast of the Adriatic through the Greek trade. From Illyria to Germania, beer spread fast and became very popular [2]. According to Nelson 2005 [6], there is no doubt that Celtic peoples in Europe were all avid beer drinkers, however, due to the spread of viticulture and the consumption of wine in Europe and the influence of the Greeks and especially of the Romans, beer was supplanted by wine as the upper-class beverage in most of these areas [3, 6].

Around the 8th century AD and with the spread of Christianity and large monasteries men took over the production task of brewing beer from women. Beer emerged as a commercial venture only in the 12th and 13th centuries [3]. Usually, the monasteries produced beer for their consumption, as well as to be given to pilgrims and to the poor, being the only institution where beers were manufactured on anything like a commercial scale. Around this century, the German monasteries innovated in beer production introducing hops in brewing to preserve their beer longer adding a balance between the sweet flavor of the malt and the bitterness of the hop. The use of hop that was initially forbidden in the British Isles was allowed after the Hundred Years' War between France and England (1337-1453) [3]. During Medieval times, beer was used for tithing, trading, payment and taxing.

In the 14th century, the emergence of commercial breweries changed the monasteries position in the brewing industry drastically. After the Black Death (1347-1352), the demand for beer increased since the awareness of water pollution. Since beer was made from boiled water (in which bacteria had been eliminated), a growing number of people started to prefer beer to water. Moreover, the increase in the number of merchants and consequently the increasing demand for lodging facilities, food, and drink which led to the emergence of 'inns' and 'taverns' contributing to the increase in beer demand. Together with the increase in beer consumption and the growth of the beer industry came tax regulations and rules that described beer production, ingredients, duration of the brewing process, beer prices, etc. In 1487, the law of Purity, which survived until 30 years ago, the so-called 'Reinheitsgebot' was introduced in Bavaria. This law stipulated that only barley, hops, and pure water could be used to produce beer [3].

As the beer became increasingly marketed, the competition between breweries increased contributing to the increase in taste and flavour quality. The Reformation, which took place in Europe in the 16th century, caused monasteries to lose their market share. In the countries that continued to be catholic, the monasteries received the advantage of being

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exempt from some taxes related to brewing production. In northern Europe, regions which turned to Protestantism, the catholic monasteries were eliminated, and with them, their beer production. The final element to complete the decline of the monasteries over the commercial breweries was the French Revolution in 1789, a period where many European monasteries were destroyed.

During early modern times, Spain and Portugal, followed by England, France and the Netherlands, made voyages to the 'New World' carrying beer with them on their ships. The brewing methods were introduced in some conquered territories. However, in some regions such as in North America and Latin America (actual Mexico) they found that the natives already produced some sort of beer made from fermented maize and sprouted kernels of maize, respectively. The globalization process brought to Europe other non-alcoholic competitors to beer such as tea, cocoa and coffee and wine became more affordable. In addition, distilled beverages, such as gin, rum, vodka and whisky, were increasingly produced and traded [3].

During the 18th and 19th centuries, several scientific discoveries had a direct impact on beer production. For instance, an increasing knowledge about yeast function and composition, which made possible to produce new types of beer and to better control the production process, the improvement of the steam engine, the invention of refrigerator, the glass bottles and new methods to seal beer bottles. The French scientist Louis Pasteur (1822-1895) was able to demonstrate that yeast consists of living cells responsible for the fermentation process and, in the 1860s, developed the so-called 'Pasteurization' method. Finally, other important discoveries in the 20th century were the invention of the metal cans and the 'lagering' process.

In conclusion, the important discoveries and innovations in the last two centuries had a major impact on the brewing industry. With increasing knowledge and technology it became possible to control the 'stability' of beer once it had been bottled.

Figure 2 shows beer production in the 19th and 20th centuries in Europe and the USA (1820-2000), in billion litres.

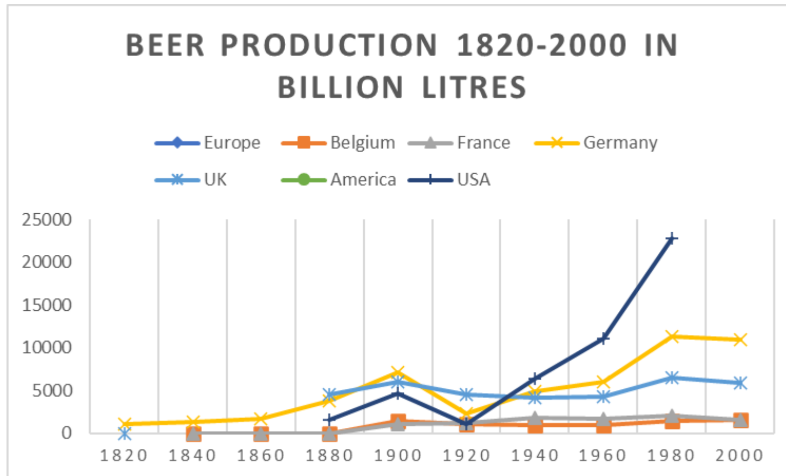

Figure 2: Beer production in Europe (Belgium, France, Germany, and the United Kingdom) and the USA in billion litres (1820-2000) [3].

Despite in the 19th century, beer production was characterized by strong and continuous growth, in 20th century is characterized by both growth and decline. During the World War I in Europe, beer production fell by around 70% mainly in the occupied regions as many workers had to move to the frontline. Moreover, grains were scarce and expensive, and many breweries had to close their business. World War II was not different and beer production declined dramatically again in the 1940s. Breweries used substitutes for normal brewing ingredients like several types of malt that had been flavoured/aromatized, beets (rich in sugar content), and several flavouring substances, such as coriander seed, chamomile blossom, and the skins of lemons and oranges.

In the USA, the impact of World War I was smaller than in Europe. A bigger impact on beer production in this country was caused by government regulation. A nationwide prohibition on alcohol in the USA from 1919 to 1933 made many American breweries close down. However, according to Poelmans and Swinnen [3], a few years after the

prohibition was suspended, beer production increased to the level before the prohibition years.

A major structural change in beer consumption both in Europe and the USA started in the 1980s when alternative alcoholic drinks, in particular wine, became more readily available in traditional beer-drinking countries. During the last 20 years of the 20th century, an increased number of breweries started looking abroad with the intent of expanding their market, establishing new firms, and engaging in 'licensing deals' with other breweries that started brewing their products. At the beginning of the 1990s, people started to show an interest in the 'older' beer styles such as porter, pale ales and brown cask ales, stout and bitters and this trend led to the movement of microbreweries. With a small size and reduced brewing capacity than the existing breweries, microbreweries started to produce beer to supply the new demand of consumers, as can be seen in Table 1.

Table 1: Number of active breweries 1950-2020 [6, 7].

COUNTRY	1950	1960	1970	1980	1990	2000	2005	2010	2015	2020
Europe										
Belgium	663	414	232	143	126	113	115	123	199	408
Germany		2216	1778	1366	1232	1289	1276	1342	1388	1536
UK	567	358	220	142	279	500	570	828	1424	1810
Spain	0	0	0	0	0	12	9	19	352	392
America										
USA	358	173	65	43	270	1491	1601	1766	4803	8905

The end of the 20th century and the beginning of the 21st century represents a moment of transition in the structure of production and consumption around the world. On one hand, the merge of the breweries led to an increasing concentration of the market in the hands of new companies. On the other hand, the proliferation of microbreweries diminished that pressure and favoured diversification and experimentation.

Although this microbrewery movement first started in the USA, it spread around the world, including many traditional beer-consuming countries, thus complementing its diversity of beers produced nowadays.

1.2. *Brewing Material, Process and Beer Styles*

Comprehending the brewing process and the key factors influencing product stability is essential to ensure the quality of the product. In general, the brewing process can be divided into nine steps such as malting, milling, mashing, boiling, fermentation, maturation, packaging and warehouse and distribution [8, 9], as can be seen in Figure 3.

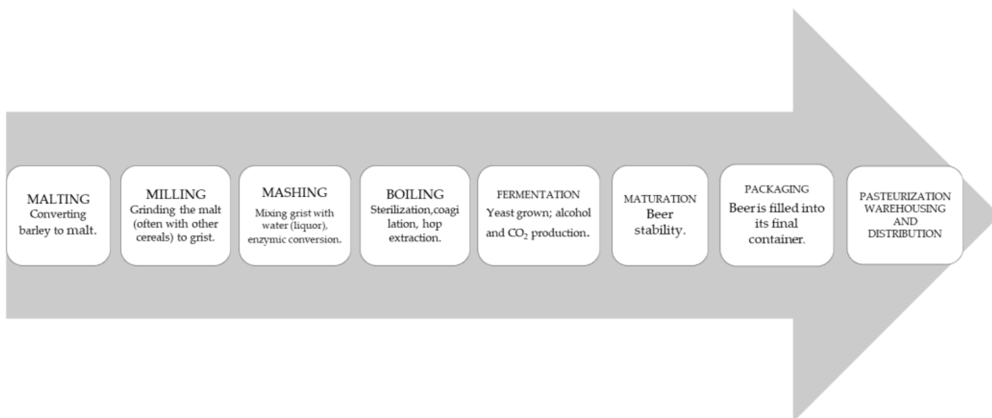

Figure 3: Main steps in the brewing process including the most important effects produced.

The first step in the brewing process is malting, where the raw barley is converted into a product with increased enzyme levels and modified physical and chemical properties via controlled steeping, germination and kilning processes [8, 9]. The next step consists on grinding the malted barley for mashing. The grinding characteristics will depend on the type of equipment used by the brewer and could be a coarser grind by roll mills or a much finer grist produced by a hammer mill [8, 9]. After grinding, the barley malt is mixed with hot water to produce a soluble malt extract, rich in small sugars such as maltose and glucose. In the boiling phase, hops are added to the wort. In general, the main objective of this phase is wort sterilization and extraction of bitter and aroma compounds. The next step is fermentation, where the sugars present in the wort are converted into alcohol and carbon dioxide, and many volatile compounds are generated. Now, beer is maturated for the removal of undesirable compounds. The filtration process is applied in some types of beers to help the stability of the product. After maturation and/or filtration (depending on the type of beer) beer is packaged. In

general, large breweries apply heat treatment to beer to improve and extend product shelf life before sending it to the warehouse. Finally, beer is distributed.

1.2.1. Malting

Barley is the most used cereal malted for beer due to its high enzymatic ability to convert starch into fermentable sugars needed by the yeast during the fermentation process. Apart from barley, other cereals such as oats, maize, rice, rye, sorghum and wheat are malted to be used as adjuncts in the maceration process or to produce specialty beers. During the malting process, barley (i) is steeped to increase the water contents, to stimulate the respiration in the embryo and to hydrate the starch reserves in the endosperm [8, 10]; (ii) is germinated to produce enzymes and to process part of the high molecular weight, insoluble protein into smaller fragments (peptides and amino acids) [10]; (iii) is kilned to prevent further enzyme activity, to remove undesirable 'grainy' flavours and to enable the formation of more attractive malty and biscuity flavours, and also to develop colour characteristics through the Maillard reactions [11].

1.2.2. Mashing

The first step in the mashing stage is grinding the malt in a mill. A roller mill or a hammer mill can be used. The former keeps the husk largely intact so that it can serve as a filtration aid later in lauter tun processing. The hammer mill produces fine grist as flour required for mash filter separation. The combination of hammer milling and mash filtration produces more extract than the roller milling and the lauter tun method. Then, the malt grist (and adjuncts) is mixed with hot water for the malt enzymes to convert the various cereal components into fermentable sugars and other nutrients [9, 10]. After mashing, the wort is separated from the solid parts to clarify it and obtain the highest extract content of the solid part, and then it is sent to the boiling stage.

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1.2.3. Boiling

After separation, the wort is transferred to the kettle for boiling. In this step liquid adjuncts and hops can be added at various points during boiling. The purpose of this stage is wort sterilization, extraction of bitter and aroma compounds from hops, trub formation and subsequent removal, flavour and colour formation, concentration of the wort and removal of undesirable volatiles by evaporation [8]. After clarification, the wort is cooled and aerated before sending it to the fermentation vessel.

1.2.4. Fermentation

The aerated and cooled wort is transformed into beer through the fermentation process. During this stage, the sugars present in the wort are converted into alcohol and carbon dioxide, and also many aroma compounds are generated. The proper fermentation temperature is typically 8 to 15 °C and 14 to 25 °C and the yeast strains used are *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* for lager and ale beers, respectively. Yeast strain can have an important influence on the method chosen to separate the yeast from the beer. Powdery strains are best removed by centrifugation or filtration, and flocculent strains are more efficiently separated by sedimentation [8].

1.2.5. Maturation

After the fermentation process, the beer is considered as 'green' beer. The maturation process aids to reduce the levels of undesirable aroma compounds produced during fermentation. The purpose of this step is to clarify the product, prevent oxidation during storage and contribute to microbiological stability. During this stage, the concentration of carbon dioxide (CO_2) is adjusted to a specified concentration. This adjustment can be performed by injecting CO_2 or by a secondary fermentation. Before packaging, beer needs to be clarified to obtain a brilliant and clear product. The clarification must remove some yeast, colloidal particles of protein-polyphenol complexes, and other insoluble materials [9, 12]. Generally, four clarification techniques are used by breweries: (i) sedimentation; (ii) use of finings (iii) centrifugation, and (iv) filtration.

1.2.6. Packaging

Beer packaging needs meet some specific requirements, such as: (i) be able to withstand the pressure generated when a carbonated liquid is heated, either during the pasteurization process or during transport, (ii) offer light protection to avoid beer degradation, and (iii) be impermeable to gases. Beer can be packaged in kegs, cans and bottles, and the material used for this packaging can be glass, steel and plastic. A major concern in this phase is oxygen uptake and contamination if the beer has not been pasteurized. Packaging is of enormous importance to the brewing industry, since sometimes beer is transported over long distances and stored without temperature control, which can spoil the product [13].

1.2.7. Pasteurization

Pasteurization is a heat treatment applied to foods and drinks to sterilize the product by eliminating the spoilage organisms. Beer does not contain any pathogens and the common spoilage organisms present are usually residual pitching yeast, wild yeast, lactic acid bacteria, acetic acid bacteria, and cocci. A pasteurization unit (PU), for beer, is defined as keeping a beer at 60 °C for 1 minute. In practice, pasteurization is divided into two categories:

(i) Flash pasteurization, always used for kegs, bottles and cans. This method involves using a plate heat exchanger to rapidly heat the beer to a temperature of about 70 °C, holding it at this temperature for a few seconds, and then cooling it again to leave it ready for packaging. Brewers rarely use less than 10 PU, being 20 to 50 the most common.

(ii) Tunnel pasteurization, used in bottles and cans. This method involves heating the package with hot water spray to a temperature of 60 °C, holding this temperature during a certain period of time and then cooling it down. The time scale is up to one hour and the number of PUs is usually 10 in this method [9].

1.2.8. Beer Styles

Diversity on beer styles stem from a range of different factors such as raw materials, brewing techniques, fermentation processes, alcohol content, hop characteristics (bitterness and aroma), colour and clarity, carbonation and mouthfeel. Some of the main styles commonly recognized include [14]:

1. Lager: a type of beer that is fermented and conditioned at low temperature, resulting in a clean, crisp taste. Varieties include Pilsner, Helles and Bock.
2. Pale Ale: a hoppy with a pale colour, often featuring fruity and floral notes.
3. Indian Pale Ale (IPA): known for its strong hop flavours and aromas, with variations like American IPA, New England IPA, and Double IPA.
4. Stout: a dark, rich beer with flavours of roasted malt, chocolate and coffee. Varieties include Dry Stout, Sweet Stout and Imperial Stout.
5. Wheat Beer: brewed with a significant proportion of wheat, these beers often have a light and refreshing character. Examples include Hefeweizen and Witbier.
6. Porter: similar to Stout, but generally lighter and featuring a range of flavours as chocolate, caramel and toffee.
7. Sour Beer: brewed to intentionally have a sour taste, often achieved through wild yeast strains, or added fruit. Styles include Berliner Wise, Gose and Lambic.
8. Belgian Ale: a diverse category that includes Belgian Dubbel, Tripel and Quadrupel, with fruity and spicy yeast characteristics.
9. Amber Ale: a beer with a medium to dark amber colour, showcasing a balance of malt sweetness and hop bitterness.
10. Barleywine: a strong ale with a high alcohol content, often featuring rich, malty flavours.

1.3. Contaminants in Beer

Beer is microbiologically stable due to its low pH (around 4.0), which inhibits most microorganisms; the high concentration of alcohol, which is toxic to many microorganisms; the antiseptic action of hops, which is bacteriostatic for many bacteria; and the low amount of residual nutrients that remain available as carbon sources, thus inhibiting microbial growth. Despite these limiting factors some spoilage microorganisms such as yeast and bacteria can flourish in beer depending on storage conditions and the availability of oxygen. Although none of these microorganisms are pathogenic, they have an influence on the organoleptic quality parameters of the final product [9]. Table 2 shows the microbiological spoilage organisms along the brewing process.

Table 2: Spoilage microorganisms present in the beer process [9, 27, 28].

STAGE OF PROCESS	MICROORGANISM	POSSIBLE EFFECTS
Mashing	Lactic acid bacteria tolerant to heat treatment	Nitrogenous compounds development
Cooled wort	*Obesumbacterium proteus*, Enterobacteria	Off-flavours and nitrogenous compounds
Pitching yeast	Wild yeasts, *O. proteus*, *Lactobacillus*, *Pediococcus*, acetic acid bacteria	Abnormal fermentation, off-flavours, diacetyl production
Fermentation	Wild yeasts, *Lactobacillus*, *Pediococcus*	Abnormal fermentation, off-flavours, diacetyl production
Maturation/storage/ bright/packaged beer	Wild yeasts, *Lactobacillus*, *Pediococcus*, *Zymomonas*, *Pectinatus*	Sour taste and off-flavours with sulfury aromas, diacetyl production, haze formation and turbid beers
Cask-conditioned beer	Acetic acid, wild yeasts, *Lactobacillus*, *Pediococcus*, *Zymomonas*	Acid beers, haze formation, off-flavours and aromas

1.3.1. Wild Yeast

Wild yeasts are those yeasts that have not been deliberately used to carry out the fermentation and that are not under full control during the brewing process. Rogue

strains of *Saccharomyces cerevisiae* are the main form of wild yeast contamination in beer. These spoil beer through ester or phenolic off-flavor production, formation of haze sediment, or superattenuation, leading to over-carbonation and diminished body [27]. *Brettanomyces* yeasts (teleomorph *Dekkera)* including *Brettanomyces bruxellensis, Brettanomyces custersii,* and *Brettanomyces anomalus,* are serious contaminants of most beers, spoiling them through the production of highly volatile phenolic compounds [27]. The major genera and types encountered in breweries are *Debaryomyces, Dekkera, Issatchenkia, Pichia, Saccharomyces, Torulaspora, Williopsis, Zygosaccharomyces, Hanseniaspora, Saccharomycodes* and *Schizosaccharomyces* [9, 27]. Generally, wild yeasts are competing with the culture yeast for nutrients, but some can even kill sensitive culture yeast.

1.3.2. Moulds

Some airborne microorganisms colonize barley in the field. The most commonly dominant species are *Alternaria* and *Cladosporium* [9]. *Fusarium ssp.*represents a major problem since this species is responsible for the synthesis of highly toxic mycotoxins [27, 29]. Malt typically has *Eurotium (Aspergillus)* species including *Aspergillus fumigatus, Rhizopus* species, and *Penicillia* [9]. These microorganisms can cause significant losses to breweries since they influence malt quality by reducing its efficiency, thus directly affecting beer quality [30].

1.3.3. Bacteria

1.3.3.1. Gram-positive Bacteria

These bacteria possess a thick cell wall composed almost entirely of a polysaccharide-like material, which complexes with the crystal violet/iodine dye used in the Gram stain test, characterizing it as gram-positive [9]. The only group of gram-positive bacteria capable of causing a significant threat to beer are lactic acid bacteria (LAB). LAB are fermentative bacteria that prefer an anaerobic environment in which some LAB conduct

homofermentative and other heterofermentative catabolism of sugars, producing lactic acid, and lactate, acetate, and carbon dioxide respectively as end-products [9]. LAB are divided into several genera, being *Lactobacillus* (heterofermentative bacterium) and *Pediococcus* (homofermentative bacterium) the most important to the brewers. The most commonly reported contaminants of finished beer are *Lactobacillus brevis* and *Pediococcus damnosus* [27]. These beer-spoilage LAB are strongly resistant to hop-bitter acids. LAB spoil beer through acidification, haze formation and/or diacetyl production, giving an intense aroma of artificial butter [27, 31].

1.3.3.2. Gram-negative Bacteria

These bacteria contain more lipids in their cell walls and do not complex the Gram stain as strongly as the Gram-positive one. Gram-negative bacteria are not so sensitive to hop α-acids and their growth is not totally inhibited by these compounds [9]. Aerobic acetic acid bacteria (AAB) are not a big problem for production in modern breweries, because their activity is negligible since exposure to oxygen can be avoided [27]. They are a more prevalent threat in barrel-aged beers. The AAB spoil beer through the oxidation of ethanol to acetic acid, effectively transforming beer into vinegar. They include *Acetobacter aceti*, *Acetobacter pastorianus* and *Glucanobacter oxydans* [27]. Nowadays, a lower level of dissolved oxygen present in beer leads to new anaerobic threats such as *Pectinatus*, *Megasphera*, *Selenomonas* and *Zymophilus.* These new contaminants can spoil beer through haze formation, overwhelming production of propionic acid, acetic acid, hydrogen sulfide, and mercaptans, and inhibition of yeast growth and alcohol production [9, 27].

1.3.4. Biogenic Amines

Biogenic amines and polyamines produce serious consequences related to microbial contamination of beer, representing a danger to the health of sensitive individuals. They cause reactions similar to allergies, migraine and/or toxic reactions with monoamine

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oxidase inhibitor drugs [27]. They are produced during fermentation but can also be produced by microbes present in barley, malt, wort and hops.

1.4. Volatile Compounds in Beer

Beer is a complex mixture produced from basic raw materials such as water, barley, hops and yeast, containing a wide range of different chemical compounds that may react and interact at all stages of the brewing process. The distinctive aroma and characteristic flavour associated with the different beer styles is mainly determined by its volatile fraction. Over 1,000 different chemical substances have been identified in beer and some of those compounds play an important role in beer flavour characteristics and are described as 'volatile compounds' [8, 15, 16]. They can be derived from (i) raw materials (barley and hops), (ii) from roasting malt and boiling wort, (iii) as by-products of yeast metabolism, (iv) from contaminant microorganisms, and finally (v) from the effects of oxygen, sunlight and vibration during product storage and transportation [17, 18]. The volatile fraction is composed of chemically diverse compounds, such as higher alcohols, esters, carbonyl compounds, acids, terpenoids, volatile phenolic compounds or sulphur-containing compounds [16, 17]. Table 3 summarizes the main volatile compounds present in beer.

Table 3: Main volatile compounds present in beer and their respective olfactory thresholds in this matrix.

COMPOUND [19,20]	**FLAVOR** [17,19,20]	**THRESHOLD (mg/L)** [17,19,21]
	HIGHER ALCOHOLS	
Propan-1-ol	Alcohol	800
2-methyl propanol	Alcohol	200
2-methyl butanol	Alcohol, banana, medicinal, solvent	65
3-methyl butanol	Alcohol, banana, sweetish, aromatic	70
2-phenyl ethanol	Roses, sweetish, perfumed	125
	ESTERS	
Ethyl acetate	Solvent, fruity, sweetish	30
Isoamyl acetate	Banana, honey, apple, solvent, ester	1.2
2-phenylethyl acetate	Roses, honey, apple, sweetish	3.8
Ethyl hexanoate	Sour apple	0.21
Ethyl octanoate	Sour apple	0.9

CARBONYL COMPOUNDS		
Acetaldehyde	Green apple, fruity	25
2,3-butanedione	Butter-scotch	0.15
2,3-pentanedione	Toffee-like	0.9
Furfural	Caramel, bready, cooked meat	150
5-hydroxymethylfurfural	Bready, caramel	1.0
Trans-2-butenal	Apple, almond	0.00011
2-methylpropanal	Banana, melon	1.0
Hexanal	Bitter, vinous	0.35
2-methylbutanal	Grainy, varnish, fruity	1.25
3-methylthiopropanal	Cooked potatoes, worty	0.25
3-methyl butanal	Malty, chocolate, almond, cherry	0.60
2-phenylacetaldehyde	Hyacinth, flowery, roses	1.60
Benzaldehyde	Almond, cherry, stone	2.0
ACIDS		
Acetic acid	Sour	175
Butanoic acid	Buttery, fruity, rancid, cheese	2.2
Hexanoic acid *(Caproic acid)*	Sweet	8.0
Octanoic acid *(Caprylic acid)*	Sweet, cheese, fatty, oily, waxy	14
Decanoic acid *(Capric acid)*	Rancid, fat	10
Lauric acid	Metal	6.1
3-methylbutanoic acid	Cheesy,sweaty	1.5
2-phenylacetic acid	Honey-like, sweet	2.5
PHENOLIC COMPOUNDS		
4-vinylguaiacol	Herbal, cloves, spicy	0.68
4-vinylphenol	Almond, shell	0.15
4-ethylphenol	Smoky, phenolic, spice	0.01
4-propenylguaiacol	Flower	0.04
4-ethylguaiacol	Spice, clove	
TERPENOIDS		
β-myrcene	Green, resinous	0.013
α-terpeniol	Woody	2.0
β-citronellol	Rose, citrusy, fruity	
Linalool	Flower, lavander	0.08
Geraniol	Sweet, citrus, floral	
D-limonene	Citrus, mint, orange	
β-caryophyllene	Woody, spice	
SULPHUR COMPOUNDS		
Dimethyl sulphide	Sweetcorn, tin tomatoes	0.03
Hydrogen sulfide	Sulfidic, rotten eggs	0.008
Sulfur dioxide	Sulfidic, burnt match	25
Methionol	Raw potatoes	
Diethyl sulfide	Garlic, burnt rubber	0.0004
3-methyl-2-burnt-1-thiol	Skunk, leek-like, lightstruck	0.00001

1.4.1. Higher Alcohols

The major alcohols found in alcoholic beverages are *n*-propanol, 2-methylpropan-1-ol, 3-methylbutan-1-ol and the aromatic alcohols *β*-phenylethanol and benzyl alcohol. These compounds have both positive and negative impacts on aroma and flavour. Higher alcohols provide beer with many odours such as alcoholic, fruity, solvent-like and rose-like or floral, depending on the concentration and type of alcohol. Above 300 mg/L in beer, higher alcohols can lead to a strong, pungent smell and taste, whereas lower concentrations provide desirable characteristics [15]. In quantitative terms, the most important higher alcohol for flavour in beer is isoamyl alcohol, which is capable to influence beer drinkability. Higher alcohols are formed as by-products of amino acid synthesis during yeast metabolism. Higher alcohols are also involved in the biochemical pathways leading to the synthesis of esters.

1.4.2. Esters

Esters are one of the most volatile compounds in beer and are produced during alcoholic fermentation, due to yeast metabolism. When present in moderate amounts, they add a pleasant character to the beer aroma and contribute to the full-bodied characteristics of the product. On the contrary, in excess, they give an undesirable fruity aroma to beer [15]. It is generally recognized that their formation is mediated by co-enzyme A and their production is regulated by the yeast strain, temperature, hydrostatic pressure, wort composition, sugar type and concentration, type and amount of yeast-assimilable nitrogen, aeration, and unsaturated fatty acids [22, 23].

1.4.3. Carbonyl Compounds

1.4.3.1 Aldehydes

Aldehydes formed during wort preparation come from processes such as Maillard reactions and lipid oxidation and as a function of the pathways for higher alcohol

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formation during fermentation. Aldehydes play an important role in beer flavour instability since their concentration coincides with the appearance and intensity of aged or stale beer aroma, for instance, cardboard (trans-2-nonenal), cooked potato-like (methional) and honey-like (2-phenylacetaldehyde) [22, 24]. The main aldehyde to consider in beer is acetaldehyde because of its importance as an intermediate in the formation of ethanol and acetate [20].

1.4.3.2 Ketones

Among the few ketones present in beer, vicinal diketones (VDK) such as diacetyl and 2,3-pentanedione are the most important in terms of their impact on beer flavour due to their low flavour threshold. In general, VDKs are considered off-flavours in most beers. They are produced by yeast in presence of oxygen and enzymatically consumed in the absence of oxygen.

1.4.4. Acids

Volatile acids in beer are composed of organic acids and exert a significant effect on the sour and/or caprylic odour of beer if present in high concentration [22]. The acids originate from the wort and yeast autolysis and metabolism.

1.4.5. Phenolic Compounds

A wide structural variety of phenolic compounds are present in the volatile matrix of beer and contribute to its aroma. These compounds are simple phenols such as benzoic and cinnamic acid derivatives, coumarins, catechins, di-, tri- and oligomeric proanthocyanidins, (prenylated) chalcones and flavonoids [19]. They are derived from malt or hops during the mash and brewing process [25]. Some of these compounds have little impact on finished beer, while others contribute with positive like pleasant vanillin, clove-like spicy or smoky odour. Besides, some phenolic compounds have a negative effect in beer leading to an unpleasant 'phenolic' smell. Their levels in beer are generally low 500 – 1,000 mg/L [16, 18, 20]. In general, phenolic flavour is undesirable in most

types of beer, but it is considered positive or even essential in certain specialty beers such as lambic and wheat beers.

1.4.6. Terpenoids

Hops are crucial to beer flavour as they contribute to the fruity, citrus-like and floral aroma attributed to the terpenes and terpenoid compounds. Some of the terpenes are carried out from the hops, whereas others such as terpenoid esters and terpene alcohols are produced or released from other monoterpene alcohols and glycoside precursors during yeast fermentation [22, 26].

1.4.7. Sulphur Compounds

Many sulphur-containing compounds found in beer derive from raw materials such as malt and hops, and some are produced through yeast metabolism [21]. Most of the volatile sulphur compounds in beer cause off-flavours such as rotten egg-like, cabbage-like, onion-like, and garlic-like. On the contrary, some of these compounds have a positive impact on beer flavour by accentuating fruitiness. These compounds are typically present at low levels (µg/L-mg/L); however, they have an extremely low olfactory threshold.

1.5. Quality Analysis

To achieve quality in the final product it is very important to understand and control the brewing process. Traditionally, the brewing industry applies classical methods of analysis to control the process and to analyse the quality of the final product. There are several sets of methods with some differences depending on their origin. The standard methods of The Institute of Brewing and Distilling (IBD) were originally developed in England for the analysis of ale-style beer; the methods of The European Brewery Convention (EBC) are based on the analysis of lager-style beers; and The American Society of Brewing Chemists (ASBC) has the point of view of North American brewers, which produce lager and ale beer styles [28].

In general, the quality examination of the finished product covers three different aspects such as microbiological examination, the analysis of chemical and physicochemical properties and the sensory analysis.

1.5.1. Microbiological Examination

Regarding microbiological analyses, there are essentially two approaches: (i) the traditional one, which consists of the cultivation of yeasts and bacteria in a culture of suitable media followed by the identification of the spoilage organism if necessary, and (ii) the modern rapid approaches for microbial detection such as the Polymerase Chain Reaction (PCR), which have minimal reliance on prior culture [9].

Since not all microorganisms that enter the wort or beer are spoilage organisms, the microbiological analysis must distinguish three groups with regard to these types of organisms: (i) harmless accompanying microorganisms (mould spores and many species of bacteria and yeast); (ii) potentially damaging microorganisms (can only multiply in finished beer with high oxygen content, high pH and low hop bitterness); and (iii) beer spoilage organisms (they are immune to low oxygen content and low pH). Samples should be collected on the production line, according to a plan established by

the brewery, at as many points as possible. Samples must be taken carefully to avoid external contamination. Then, the sample material is added under a sterile condition to shallow Petri dishes, which contain a nutrient medium, and incubated at 37 °C in an incubating cabinet. The colonies soon become visible and can be analyzed under the microscope. Rapid detection methods such as PCR are required in the yeast and filter cellar as well as before filling. With this method, it is possible to recognize false findings and detect and determine different beer spoilage *Lactobacillus* strains, such as *Pediococcus*, *Megasphera* and *Pectinatus*, within a few hours [32]. The rapid detection method should be coupled with continuous sampling (bypass in membrane filtration) after filtration or before filling to detect all foreign microbes. Swabs should be taken at intervals, particularly in the bottle cellar, from exposed sites and then incubated for three days aerobically at 25 – 28 °C. Figure 4 shows a combined examination plan for the safe detection of trace contaminations.

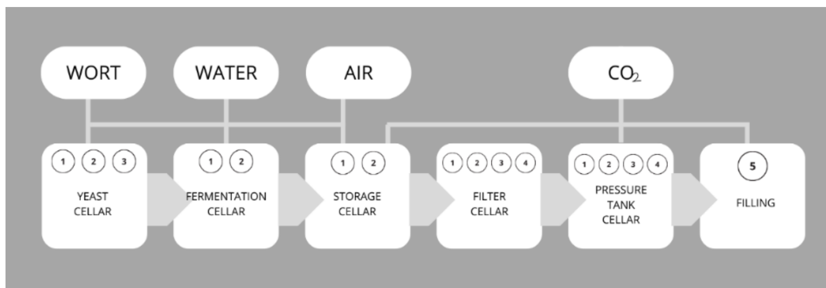

Figure 4: Combined examination plan for the safe detection of trace contaminations [32]. (1) Step by step control, (2) special samples at weak point, (3) rapid detection by means of PCR analysis, (4) by-pass membrane filtration, (5) swabs.

A stability sample is taken from every filled batch and stored in an incubator at a constant temperature (26 °C). The sample should not present any sediment or turbidity in the minimum shelf life period.

1.5.2. Chemical and Physicochemical Analysis

Several parameters must be considered to produce a good beer with consistently high quality. These mainly involve the analysis of the original gravity to determine the extract and alcohol content, the measurement of colour, pH determination, measurement of the bitterness units, measurement of the CO_2 content, and determination of the colloidal stability, among others.

1.5.2.1. Original Gravity

This analysis is supposed to provide information about the constituents of beer. The extract content of the wort determined before fermentation is the original gravity or original wort extract. The fermentable extract is split into almost equal parts of alcohol and CO_2 during fermentation. After fermentation, the unfermentable extract (Apparent Extract - AE) can be measured using a hydrometer. To obtain the real extract (RE) content the alcohol must be removed by distillation. To determine the original gravity it is necessary to ascertain the alcohol content and real extract content, which is performed by means of a distillation or a refraction analysis. Nowadays, some instruments are replacing the traditional analysis because they allow many samples to be analysed quickly with high level of reproducibility. For instance, by means of Near Infrared (NIR) spectroscopy, a selective alcohol measurement can be performed quickly and with great accuracy [32].

1.5.2.2. Colour

Colour measurements are usually performed visually by comparison with standardized colour discs. Colour can also be determined by visible-spectrophotometry to avoid the subjective influence of the human eye. In this case, the wavelength used is 430 nm and the measurement is compared against a factor on the European Brewery Convention (EBC) scale according to the following formula [33]:

$$C = A_{430} \cdot f \cdot 25$$

where A_{430} is the absorbance at 430 nm measured in a 10 mm pathlength cell and f is the dilution factor.

1.5.2.3. pH

Measuring the pH of beer is very important because of its influence on enzymatic processes and the behaviour of the microorganisms. pH is measured with a glass electrode. The optimal pH values are 5.0 to 5.2 for wort and 4.2 to 4.3 for beer [32].

1.5.2.4. Oxygen Content

This is one of the most important control analyses in the brewing process, since high oxygen values have a detrimental effect on the quality of beer and its flavour stability. The measure of the air in the headspace of the bottle can be performed using the underwater funnel method, by collecting the air in the neck of the bottle with a funnel filled with gas and then slowly passed through a liquid column of sodium hydroxide solution, where CO_2 is absorbed as $NaHCO_3$. The residual gas – air, consisting of nitrogen and oxygen, is collected in a burette and the volume is read off as 'air in the bottleneck'. The results are always given in mL air/bottle or can [32].

More important than the oxygen in the neck of the bottle or can is the total oxygen content, which consists of both the oxygen dissolved in the beer and the contained in the bottleneck. This measurement is performed by first shaking the bottle according to defined rules and then directly measuring the total oxygen electro-chemically and determined using a table value [32].

1.5.2.5. Foam Stability

Two methods are used to measure foam stability: (i) the Ross and Clark method and (ii) the NIBEM foam stability method. The results of the two methods are not directly comparable [32].

(i) Ross and Clark method: a defined foam volume is produced in beer and the measure used is the average lifetime of the foam bubbles, which is determined from the collapse time of the foam and the ratio of the collapsed foam to the foam still present. The higher the value, the more stable the foam. Foam values are between 90 and 140 seconds and, on average, about 120 seconds.

(ii) NIBEM method: the time is measured within which the surface of the foam head is lowered 10, 20 or 30 mm. The measurement is made by a movable electrode system with long needles. The time measured is in seconds and the evaluation scale is: values below 220 s are very bad; values between 260 and 280 s are good; and values above 300 s are very good.

1.5.2.6. Carbon Dioxide

The content of dissolved CO_2 in beer is an important quality parameter to produce good acidity in beer. Contents of CO_2 in beer are typically 0.45 to 0.60% by weight in the case of bottom-fermented (lager) beers, and 0.40 to 1.00% by weight in the case of top-fermented (ale) beers [32]. The two basic methods used to measure CO_2 are manometric and titrimetric. The most common is the manometric method, where the pressure in the thoroughly shaken bottle is measured by a manometer. As a result of this and the measured temperature, it is possible to calculate the CO_2 content [32].

1.5.2.7. Bitterness

Bitterness greatly affects the taste of beer, hence the importance of measuring it. The determination of the bitterness unit is spectrophotometrically performed using a European Brewing Convention (EBC) or American Society of Brewing Chemists (ASBC) method. The EBC method can be applied directly to filtered beers and, in case of turbid beers, the samples must be clarified by centrifugation. The principle of this method is that the bitter substances are extracted from acidified beer with iso-octane, centrifuged and then the absorbance of the iso-octane layer is measured in a 10 mm path length cuvette at 275 nm [32, 33]. Bitterness is calculated as:

$$\text{Bitterness (IBU)} = 50 \cdot A_{275}$$

where A_{275} is the absorbance at 275 nm measured against a reference of pure iso-octane.

1.5.2.8. Haze stability

Clear and bright beers lose their brilliance during their shelf life, becoming hazy. It is important to know if the beer will be stable for a long time or if it will soon form a haze. The most important method to detect the tendency of a beer to form a haze is the forcing test [34]. In this test, at least 5 bottles are immersed alternately for 24 hours in warm water at 40 °C (untreated beer) or 60 °C (stabilized beer) and then immersed in cool water at 0 °C until an increase in haze of 2 EBC formazin units or a light opalescence is observed [33, 34]. By multiplying the stability determined, expressed in warm days, with a conversion factor, the approximate time during which the beer will remain haze-free is obtained. In addition, precipitation methods are used to determine the tendency of a beer to form haze, such as the precipitation of proteins or phenols with reactive substances or the determination of tannins by means of the alcohol cooling test [12, 32].

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1.5.3. Sensory Analysis

Sensory analysis includes a set of techniques for accurate measurement of human organoleptic response to foods and minimizes the potentially biasing effects of brand identity and other influences on consumer perception. Sensory evaluation has been defined as a scientific method used to evoke, measure, analyse and interpret those responses to products as perceived through the senses of sight, smell, touch, taste and hearing [12].

Four factors govern any measurement in sensory analysis: (i) precise definition of the problem; (ii) test design; (iii) instrumentation; (iv) interpretation of results [33]. Generally, three types of sensory tests are commonly used, each with a different goal, (i) discrimination test, (ii) descriptive test, and (iii) affective test. Only the two sensory tests that were carried out in this Doctoral Thesis will be described in more detail in each topic.

1.5.3.1. Discrimination Test

This simple test attempts to answer whether any perceptible difference exists between two types of products. The analysis is usually based on the statistics of frequencies and proportions. From the test results, we infer differences based on the proportions of persons who are able to choose a test product correctly from among a set of similar or control products. The discrimination test is divided into the triangle test, the duo-trio test, and the paired comparison test. The triangle test, the one applied in this Doctoral Thesis, is used to assess the presence of noticeable sensory distinctions between two products. This test involves presenting assessors with three coded samples, two of which are identical. Assessors are then required to identify the sample that differs. Employing a forced choice methodology, assessors must make a guess if they cannot identify a difference. The number of correct responses is counted and compared with statistical significance tables [29].

1.5.3.2. Descriptive Test

This test provides a detailed profile of the sensory attributes of a product, as well as a qualitative measurement of the intensity of each attribute. The sensory assessment is conducted by subjects who have been specially trained, to obtain a comprehensive analysis of the four key qualitative sensory descriptors (appearance, aroma, flavour and taste). The descriptive test can be used to characterise and describe product standards, to examine influencing product properties due to modified raw materials or changes in the formulation or the process, and to register modified influence factors in the framework of production, warehouse or along shelf life.

In the case of beers, taste and aroma are the main characteristics considered by consumers. Unfortunately, some flavour and aroma attributes, such as the cleanness and fineness of its bitterness and the tingle component of its taste, cannot be measured analytically [12]. To assess these quality parameters, beer must be tasted by tasters. These tasters must be previously selected from the brewery workforce available and then trained. Tasting requires people who can detect very small differences in flavour and aroma. It is also important to know how consumers will perceive individual products, so it is necessary to conduct consumer preference tests with the general untrained public.

Tasting must be performed in a tasting room with controlled temperature, light and position of tasters to avoid biased results. The beer should be poured without foam into a dark-coloured 200 mL tasting glass and tasted at a temperature between 8 and 14 °C. It is important to include older samples in this test in order to objectively determine when flavour deterioration can be detected [32]. An example of the scheme for tasting samples is the German taste scheme used for the DLG quality test analysis (Table 4), which comprises a laboratory analysis and the verdict of a jury composed of trained tasters.

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Table 4: Scheme of the DLG sensory quality analyses [32].

Feature examined	Points awardable	Description
Aroma	5	Perfect
	4	Still perfectly acceptable
	3	Slight odour faults (diacetyl, oxidized)
	2	Pronounced odour faults (very estery)
	1	Great odour faults (musty, yeasty)
Taste quality	5	Perfect
	4	Still perfectly acceptable
	3	Slight taste faults (diacetyl, oxidized)
	2	Pronounce taste faults (very estery)
	1	Great taste faults (musty, metallic, ligh-struck)
Body	5	Full bodied, well rounded
	4	Full bodied
	3	Not very full bodied
	2	Not well rounded
	1	Empty, watery
Liveliness	5	Desirably lively
	4	Lively
	3	Not very lively
	2	Flat
	1	Very flat
Bitterness quality	5	Excellent
	4	Good
	3	Somewhat lingering
	2	Lingering
	1	Very lingering

1.6. Instrumental Techniques

1.6.1. Gas Chromatography

Gas chromatographic analysis is basically used for the analysis of volatile compounds and it is defined as the separation of a mixture into its individual components based on the differential distribution of the components between a stationary phase and a mobile phase [35]. Some non-volatile compounds need derivatization to be analysed by gas chromatography (GC).

Gas chromatographic instruments include an inlet and a detector, which are used to introduce the sample into the capillary column where the separation takes place, and to convert the chemical signal into an electronic signal as analytes elute from the column, respectively [35]. To ensure precise and accurate injections for a large number of samples, most chromatographic instruments are equipped with an autosampler and a computer used to control the instrument and record the detector responses.

The separation mechanism for GC is typically based on the partitioning of the analytes between the gas phase and a liquid phase coated on the column surface (Gas-Liquid Chromatography - GLC). The mobile phase is usually an inert gas such as helium, hydrogen or nitrogen, and the flow is controlled with pressure and/or flow controllers. By heating the column in the GC oven, analytes volatilize at temperatures and pressures corresponding to their vapour pressures. As a result, analytes are separated based on their vapour pressures as well as their chemical/physical interactions with the stationary phase.

Gas-liquid separations based on the distribution of the analytes between the gas phase and a liquid stationary phase are the most common for GC separations. The column stationary phase can vary in composition and polarity, ranging from highly nonpolar (polydimethylsiloxane) to highly polar (polyethylene glycol) phases. Even though alcohols and aldehydes predominate in the volatile composition of alcoholic beverages

and are more optimally separated using a more polar stationary phase, there are many exceptions and for complex mixtures it can be difficult to optimize the separation for all components in the mixture [35]. In addition to the stationary phase, it is possible to select the column length (lengths of 10-60 m), column diameter (inner diameter range from 0.2 to 0.53 mm), and the amount of liquid phase (liquid phase thicknesses range from 0.1 to 5 μm).

In GC analysis, the carrier gas flows continuously to push the components in the injected sample through the column so that they can be separated and eluted from the column outlet. The flow rate of the mobile phase is very important since it may affect the efficiency of the column and the retention of the components. In capillary columns, there are four basic types of injection techniques such as isothermal (hot) split and splitless, on-column, and programmed temperature vaporization (PTV). In splitless injection, the sample is introduced into the heated liner and brought into the gas phase. The main purpose of this type of injection is to introduce the entire injected sample into the column and use it for determination. Following sample transfer to the column, the column oven temperature is increased, and thereafter the analytes will subsequently be separated in the column. The splitter valve is opened when the whole sample has been transferred to the column to wipe out the remains of the sample before the next injection. Then, the carrier gas and the sample pass through a detector. This device generates an electrical signal which gives rise to a chromatogram for each sample for qualitative/quantitative data collection and analysis.

Regarding GC detectors, common universal detectors are the flame ionization detector (FID) and the mass spectrometric (MS) detector. The FID detector responds to all organic analytes and it is widely used for analysis of alcoholic beverages. The MS detector provides structural information [35–37] and will be explained in more detail in the next section. Selective detectors such as the electron capture detector (ECD) for halogens, the

nitrogen-phosphorous specific detector (NPD), and the sulphur-specific detector (FPD) can also be used [38].

1.6.2. Gas Chromatography-Mass Spectrometry

Once the chromatographic separation takes place in the capillary column, both the carrier gas and the volatile compounds reach the MS detector, where analytes are ionized. The mass spectrometer consists of an ionization unit, a mass analyser or mass/charge (*m/z*) separation unit, and an ion detector. When combined with GC, the most common ionization technique is electronic impact (EI). With EI, ionization occurs in a source that is at a very low pressure and the predominant molecules in the source chamber are the molecules of the analyte. In the source, analytes are bombarded with high energy electrons (70 eV), lose an electron, become positively charged, and are accelerated to the mass analyser. The molecules will also break down into smaller fragments based on the molecular structure of the initial molecule and following predefined fragmentation patterns, giving rise to a mass spectrum. The output signal of the detector is a chromatogram that can be used for qualitative or/and quantitative analysis [37,38].

The analysis of the chromatogram aims to identify the compounds by comparing spectral properties with information in databases such as the NIST (National Institute of Standards and Technology) mass spectral database, or by comparing retention times and spectra of standards. As retention times vary with the instrument, column conditions and temperature, a system of identified relative retention times called Kovats Index (KI) or Retention Index (RI), was developed. KI calculates the retention of an analyte relative to that of a series of alkane hydrocarbons (or alkyl ethyl esters). Tentative identification can be obtained by matching the KI of an unknown with that in the database. Quantification in GC is based on the peak area or peak height of integrated peaks in the chromatogram. The use of internal standards can also aid the quantification; in this case, one or more internal standards are added to each sample before sample preparation

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and/or before the injection. The internal standard should be selected to match as closely as possible the chemical and physical properties of the analytes of interest, but not be present in the samples.

1.6.3. Headspace Solid-Phase Microextraction

The sample preparation for GC analysis involves techniques that preferentially isolate volatile substances and prevent the presence of ionic or high molecular weight species in the mixture injected into the GC. All sample preparation methods involve the transition of analytes between phases. The ability to accomplish this step is defined by the chemical equilibrium, which determines the amount of analyte that may be transferred from the original phase to the final phase, and is expressed as the recovery or the amount that is extracted. The solid-phase microextraction (SPME) technique uses a polymeric phase that is coated onto the surface of either a 1-2 cm long fused silica capillary that is attached to the end of a microsyringe plunger and can be stored within the syringe barrel [37]. The most used fibre is the one with a nonpolar polydimethylsiloxane (PDMS) coating, but there are different types of extraction phases: polyacrylate (PA), polyethylene glycol (PEG), divinylbenzene (DVB) and/or carboxen (CAR) to ensure the extraction of all possible analytes. SPME sampling can occur in either the headspace (HS) or the liquid phase. The technique of headspace extraction is used when the analysis involves extraction of a vapour phase in equilibrium with a liquid or solid phase [37]. In all cases, analytes are retained by partition into the polymeric phase from the liquid or vapour phase of the sample and the amount of the sorption will depend on the analyte, the chemistry of the polymeric phase, the temperature, the sampling time, and the partition coefficients for the analyte between the liquid, vapour, and polymer phases. Following exposure, the fibre is retracted into the syringe needle and transferred to GC for desorption under splitless injection conditions. The splitless time, inlet temperature, and initial column conditions must be optimized to ensure complete analyte desorption from the fibre.

1.6.4. Electronic Nose

In food research and food industry, electronic noses are used to predict shelf life and to detect the freshness of food products. The system was designed to mimic the human olfactory system and it is capable to analyse, recognize and examine volatile compounds from samples. A classic e-nose system is composed of two parts:

(i) an array of gas sensors that detects the volatile compounds from the sample [39]. The sensors are classified according to their detection principles into conductive sensors, optical sensors, surface acoustic wave sensors, gas-sensitive field effect transistors, and quartz microbalance sensors;

(ii) a signal processing system, which processes the information obtained using chemometric algorithms for classification and/or quantification purposes [40].

The sampling system that allows introducing samples into the sensor array involve mechanisms like pumps or airflow systems to ensure a controlled and consistent delivery of the sample to the sensors. The common samplers are classified into static headspace or dynamic headspace [41].

In this Doctoral Thesis a special e-nose configuration has been used (Figure 5). Regarding sample preparation, the sample of interest was placed in a sealed vial for a certain time, at a given temperature and under continuous magnetic stirring, to allow the volatile substances from the sample in the vial to reach an equilibrium between the liquid and the vapour phases. Then, a SPME fibre was exposed to the vial headspace to retain the volatile fraction. This system increases the sensitivity of the analysis, unlike the classic configuration. After that, the volatile fraction retained in the fibre was desorbed in the injector port of the GC and transferred, without chromatographic separation, to the MS detector, with the aim of obtaining a global fingerprint (mass spectrum) of the whole sample.

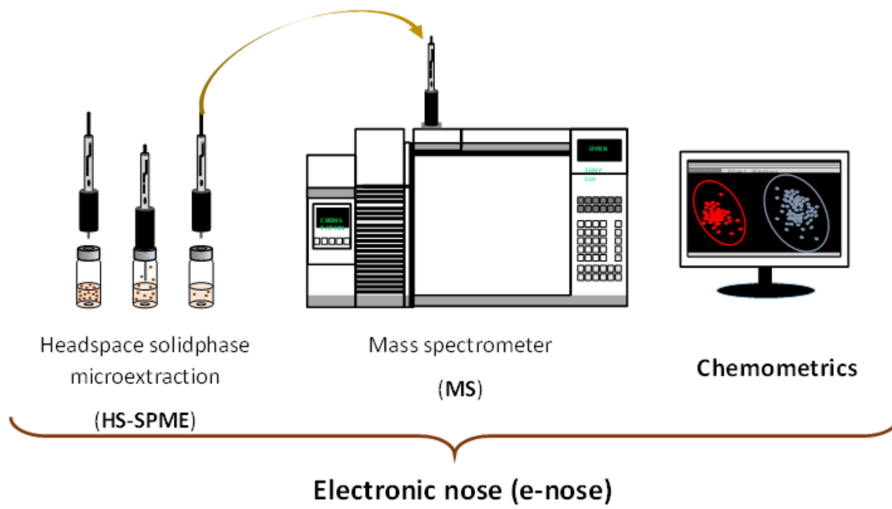

Figure 5: Configuration of an HS-SPME-MS-based e-nose.

Finally, after collecting the data, the total mass spectra obtained can be analysed, typically using a commercial software, and useful information can be extracted from the data using chemometric techniques.

1.7. Multivariate Data Analysis

The Knowledge Centre for Food Fraud and Quality of the European Commission describes food quality as 'a complex and multidimensional concept, which is influenced by a wide range of situational and contextual factors' [42]. Sensory quality is not different and involves instrumental and sensory data analysis. The amount of data collected is usually very large, and to process this volume of data it is necessary to use multivariate analysis. The use of Multivariate Data Analysis (MVDA) approaches allows the researcher to obtain an integrated view of food quality and a deeper understanding of the process and the product. MVDA (also known as chemometrics) can be defined as a tool to extract useful information from large data sets using mathematical and statistical methods. MVDA covers a wide range of data analysis principles and can be used for different purposes, such as exploration, classification or prediction. A summary of the main chemometric tools used during the development of this Doctoral Thesis is detailed below.

1.7.1. Preprocessing

Adequate data preprocessing is very important before data modelling because of the impact of this step on the data analysis itself. Data preprocessing removes undesired and irrelevant variation in the data set, which is not related to the chemical problem of interest. Some examples of preprocessing techniques are mean-centering, autoscaling, baseline and scattering correction, and time alignment. In this Doctoral Thesis autoscaling was applied to the data obtained from sensory and instrumental analyses to ensure comparability among variables with different scales. When autoscaling, each variable is first column centered (each value in a column is subtracted from the average value of that column) and then standardized (each value in a column is divided by the standard deviation of that column). The resulting data have zero mean and a standard deviation of one.

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1.7.2. Unsupervised Methods

Typically, the first chemometric approach to get a preliminary view of the data is Exploratory Data Analysis (EDA), also known as unsupervised pattern recognition. The prevalent EDA technique used in the sensory analysis of beer is Principal Component Analysis (PCA). PCA is a dimensionality reduction technique that facilitates the interpretation of the data set by converting the original variables into a reduced set of new variables, called latent variables or principal components (PC). Each PC is a linear combination of the original variables and is orthogonal to each other. The first principal component (PC1) retains the maximum of the original data variance and subsequent components hold the maximum of the remaining variance. The information provided by PCA is basically summarized in two plots, score and loading plots, which show the projections of the samples and the variables, respectively, on the new PC space [43]. Score plots allow finding groups or trends in the data, and also identifying potential outlier samples. Loading plots allow identifying the most important variables for each PC and finding correlations between variables and samples.

1.7.3. Supervised Methods

Supervised methods take into account the information captured by the X-variables (the independent predictor variables) and the Y-variables (the dependent variables). The Y-variables can be continuous or categorical. Among the supervised techniques are Partial Least Squares Regression (PLSR) and Partial Least Squares Discriminant Analysis (PLS-DA).

- Partial Least Squares Regression – PLSR: is a statistical technique used for predictive modeling and analysis, especially in situations where there are high-dimensional datasets or multicollinearity issues. PLSR is particularly beneficial when dealing with situations where the number of predictors (X-variables) is large compared to the number of observations or when these predictors are

highly correlated. PLSR begins with a dataset containing two sets of variables: the X-variables (independent variables or predictors), typically representing analytical data, and the Y-variables (dependent variables or responses), which denote the characteristic or parameter of interest. The PLSR algorithm decomposes both the **X** and **Y** matrices into a series of latent variables (LVs). These latent variables are constructed as linear combinations of the original X-variables and are designed to capture the maximum covariance between the **X** and **Y** matrices. The PLSR model relates the X-variables to the Y-variables, considering the covariance structure captured by the latent variables. The calibration aims to establish a relationship that can be used to predict the Y-variables for new observations based on their X-variables. Once the PLSR model is calibrated, it can be employed to predict the Y-variables for new sets of X-variables. PLSR often incorporates cross-validation techniques to assess the model performance and ensure its generalizability. This involves splitting the dataset into training and testing sets, iteratively fitting the model, and evaluating its predictive accuracy on unseen data [44].

- Partial Least Squares Discriminant Analysis – PLS-DA: is an extension of the Partial Least Squares Regression (PLSR) method, specifically tailored for classification problems. While PLSR is used for regression tasks where the goal is to predict a continuous variable, PLS-DA is employed when the outcome variable is categorical and involves classification into distinct classes or groups. Similar to PLSR, PLS-DA begins with a dataset containing two sets of variables: the X-variables (independent variables or predictors) and the categorical Y-variables (class labels). The X-variables typically represent analytical measurements or features, while the Y-variables define the classes or groups to which the observations belong. Typically, in PLS-DA the **Y** matrix contains dummy values that represent the classes/categories. If a sample belongs to a class, the dummy value is equal to one. In contrast, the dummy

value is zero if that sample it does not belong to that class PLS-DA. Like PLSR, decomposes the **X** and **Y** matrices into a series of latent variables (LVs). These latent variables are constructed as linear combinations of the original X-variables and are designed to capture the maximum covariance between the X-variables and the class information in the Y-variables. PLS-DA builds a model that maximizes the separation between different classes in the X-space. This is achieved by identifying the directions in the X-space that explain the most variance while also considering the class information contained in the Y-variables. The goal is to find latent variables that discriminate between different groups effectively. The calibration process optimizes the discrimination between classes and results in a model that can be used to classify new observations into the predefined groups. Once the PLS-DA model is calibrated, it can be employed to predict the class labels of new sets of X-variables. The algorithm assigns observations to the class that is most appropriate based on their X-variable values. Similar to PLSR, PLS-DA often incorporates cross-validation techniques to assess the model classification performance. This involves splitting the dataset into training and testing sets, iteratively fitting the model, and evaluating its ability to correctly classify unseen data [44].

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ARTICLE 1: AN OVERVIEW OF THE APPLICATION OF MULTIVARIATE DATA ANALYSIS TO THE EVALUATION OF BEER SENSORY QUALITY AND SHELF LIFE STABILITY - REVIEW

Article 1: An overview of the application of multivariate analysis to the evaluation of beer sensory quality and shelf life stability – Review

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Abstract

Achieving beer quality and stability remains the main challenge for the brewing industry. Despite all the technologies available, to obtain a high-quality product, it is important to know and control every step of the beer production process. Since the process has an impact on the quality and stability of the final product, it is important to create mechanisms that help manage and monitor the beer production and aging processes. Multivariate statistical techniques (chemometrics) can be a very useful tool for this purpose, as they facilitate the extraction and interpretation of information from brewing datasets by managing the connections between different types of data with multiple variables. In addition, chemometrics could help to better understand the process and the quality of the product during its shelf life. This review discusses the basis of beer quality and stability and focuses on how chemometrics can be used to monitor and manage the beer quality parameters during the beer production and aging processes.

Keywords: sensory quality, brewing process, aging, multivariate analysis

1. Introduction

Alcohol consumption has historically held an important role in social engagement and bonding for many cultures. In Europe, one of the largest alcoholic markets in the world, beer, wine, and spirits are the dominant beverages [1]. In 2021, the main beer producers in Europe: Germany, Poland, Spain, United Kingdom, Netherlands, France, Czech Republic, Romania, and Italy were responsible for producing 75% of the total volume of beer in the continent [2].

Most of the market concerning the production of beer in Europe is dominated by a few multinational companies, although in the last decade, there has been an increase in the number of independent craft breweries in countries with no historical links with the tradition of beer crafting, such as Greece, Italy, Portugal and Spain [3, 4]. Nowadays, Europe has more than 11,000 active breweries, with around 73% of them being microbreweries [3, 4]. The result is that this market has experienced notable changes in the behaviour of consumers, who are more aware and demanding in terms of their desired quality and consumption habits [5].

As opposed to industrial beers, craft beers are produced on a small scale by independent craft breweries whose focus is on product innovation, emphasizing flavor, variety of styles, and techniques committed to the sensory quality of the final product [5]. Despite the differences between industrial and craft beers, the basics of the process remain the same, including water, malted barley, hops, and yeast, and producing beer via alcoholic fermentation.

Different factors such as appearance, aroma, taste, and texture define the organoleptic quality of the final product. During the shelf life of the product, the stability of these quality factors can be influenced by several parameters, including raw material, microbial activity, light, oxygen, and temperature [6–8]. Therefore, it is important to

control both the raw materials and the process variables to achieve the best sensory quality of the beer and ensure its stability, especially in the case of unpasteurized beer.

Among the different sensory properties of beer, aroma is the most studied [9]. However, most of these studies focus on reviewing the application of multivariate analysis during the aging process, so there is a lack of information on the aroma evolution during the beer production process and also on the evolution of other sensory parameters. That is why this review focuses on the sensory quality of beer but links it to the different brewing stages. In addition, it reviews and describes the use of chemometric methodologies that allow better understanding and monitor different quality parameters during beer production and shelf life. The review is therefore divided into two parts: (*i*) Beer Production Process, focusing on the three main steps that directly influence the final quality of the product: malting, boiling, and fermentation; (*ii*) Analysis of beer quality and stability by means of different chemometric tools, which focuses on exploratory analysis, classification techniques and multivariate calibration.

2. Beer Production Process

The beer production process can be divided into five steps: malting, mashing, boiling, fermentation, and maturation. It starts with malting the barley via controlled steeping, germination and kilning, transforming it into a product that is much more friable, with active and increased enzyme levels and with different chemical and physical properties [10].

The next step is to grind the malted barley for mashing. The grinding characteristics (coarse or fine) will depend on the type of equipment used by the brewer. Mashu tuns or lauter tuns use a coarser grind produced by roll mills, but mash filters can use much finer grist produced by a hammer mill [10]. During mashing, the ground malt—and cereal adjuncts, if used—are mixed with hot water and the enzymes present in the cereal will degrade the proteins in small molecules and the starch into small sugars such as maltose and glucose, producing a soluble malt extract, wort. Before being sent to boiling,

the wort is separated from the solid part. For the classic infusion mashing, this separation takes place in the mash tun. For the other separation, such as decoction mashing and Double mashing the mash separation is usually carried out using other equipment such as a lauter tun or a mash filter [10]. This filtration process aims to brighten the wort and obtain the maximum amount of extract from the solid residue.

After filtration, the wort is boiled, and the hops are added to the kettle. The main objectives of this step are wort sterilization, extraction of bitter and aroma compounds from hops, coagulation of the excess of proteins and tannins to form trub that can be removed later, colour and flavour formation, removal of undesirable volatiles via evaporation, and concentration of the wort by evaporation of water [11, 12].

Before sending the wort to the fermentation vessel, it must be aerated and cooled until it reaches the appropriate fermentation temperature, which is typically 8 to 15 °C for lager and 14 to 25 °C for ale beers. During fermentation, yeast is mainly responsible for converting sugars into alcohol and carbon dioxide, but many aroma compounds are also generated. A great fermentation performance demands control of many key variables, such as yeast amount and viability, oxygen input, nutritional wort ability, pH, temperature, and agitation [6, 12]. The key to fermentation efficiency and, for definition, of many sensory characteristics that contribute to the quality of the final beer, is the yeast.

At this point in the brewing process, the wort has already been transformed into beer, known as '*green beer*', and most of the yeast is removed from the vessel, giving a path to maturation. The function of the remaining yeast during maturation is to produce higher carbon dioxide amounts and chemical removal of undesirable compounds. The main objective of this step is to initiate beer clarification and prevent oxidation of the product during the storage by maintaining beer in a reduced state [12]. To contribute to the microbiological stability and help with the clarification process, some types of beer are filtered.

Despite the importance of each step in the beer production process, in this review we will focus on the main three of them: malting, boiling, and fermentation. These steps have important contributions to the quality and stability of the final product, such as improvement of color, transparency, bitterness, and foam properties; wort sterilization and protection; and alcohol, carbon dioxide, and desirable flavors formation.

2.1. Malting

Barley (*Hordeum vulgare* L.) is the main cereal used worldwide for malting due to its high enzymatic ability to convert starch into fermentable sugars [13]. Nevertheless, wheat (*Triticum aestivum* L.) and sorghum (*Sorghum vulgare* L.) are also malted in great quantities, followed by rye (*Secale cereale* L.), oat (*Avena sativum* L.), rice (*Oryza Sativa* L.), and millet (*various* spp.), used in small amounts [14–16].

Barley for brewing must meet a series of quality requirements, such as germinative capacity, suitable protein and water contents, sorting (size of kernels), and absence of kernel abnormities and infestation [17, 18]. Its composition includes starch, protein, cell wall polysaccharides, and a small amount of fat and minerals. Protein and starch are among the barley components essential for malt and beer quality. According to Jaeger *et al*. [19] the content of protein in the barley grain is between 8 and 30% of its total mass. Although such a high content of protein is desirable for feeding applications, a lower level is desirable when dealing with malting barley, being 10 to 12% the ideal content of protein. On the contrary, a high level of starch in the barley grain for malting is desirable. Starch is a complex sugar constituted of glucose molecules and its content in a barley grain can range from 45.7% to 70%, which is a suitable amount for malting as, according to Błazewicz *et al*. [20], barley grain for this purpose should contain 52–67% starch.

The development and retention of foam in beer depends on the ratio of malt-derived proteins with specific molecular weights, metal cations from malt or water, and natural stabilizers such as polysaccharides derived from malt and iso-α-acids from hops [21, 22].

Moreover, fatty acids and basic amino acids derived from malt also affect foam formation and retention [21].

The malting process aims to activate enzymes to promote the barley to provide the optimal levels of saccharides, proteins, free amino nitrogen (FAN), and enzymes to ensure the organoleptic quality of the final product [23]. To initiate the malting process, barley is immersed in the steep water. This phase is called steeping and is designed to increase the moisture level of the grain around 42–47%, to initiate germination and to develop and facilitate the transport and action of the gibberellic acid, an important hormone in the production of enzymes needed by the seed for growth [14, 24, 25]. The temperature of the water during this phase should be kept between 12–18 °C, to avoid acceleration of the microbial growth or damage to the grain [14, 17].

According to Brigs *et al*. [14] an excess of microbes in the steep water is undesirable because they compete with the grain for oxygen and reduce both the percentage and vigor of germination. Furthermore, some microbes could produce mycotoxins such as deoxynivalenol (DON), which damage the yeast and/or are toxic to human beings; others could produce plant growth regulators (including gibberellins), which can inhibit or stimulate the malting process, and some others could produce some agents that cause gushing (over-foam) [14, 26]. On the contrary, the controlled microbial activity in the grain results in the production of some hydrolytic enzymes that may improve malt performance during mashing [10, 17].

When the grain starts the germination process, it is transferred to the germination vessel. [14]. The main goals of this step are the control of the breakdown of cell walls and matrix proteins, production of an optimal level of hydrolytic enzymes, hydrolyzation of certain barley reserves, such as protein, to form free amino nitrogen (FAN), and production of well-modified and balanced green malt for kilning [17].

The physical modifications that occur in the grain during germination are a breakdown of β-glucans and pentosans, followed by the partial degradation of the protein within

the cells and breakdown of some of the starch granules [14]. In a greatly modified malt, 90% of β-glucan is broken down. The whole breakdown process is limited by the availability of water. The inhomogeneity of malt modification can cause unexpected problems during brewing, such as slow wort separation, slow beer filtration, and sometimes it can cause haze development. Generally, at this stage, the germination is finished by kilning.

The kilning stage of the malting process inactivates many microorganisms and stabilizes the grain, allowing long-term storage as it reduces the moisture content of the undried green malt from about 42–48% to 3–6% [27]. The main goals of this stage are to finish the modification process and the growth of the plant to reduce moisture to levels suitable for grain storage, and to develop colour and flavour characteristics through the Maillard reactions and, in some cases, caramelization and pyrolysis reactions [17, 28].

The kilning or roasting process determines the quality of the produced malt and its classification as base or specialty. Base malts provide extracts that are used by the yeast to produce alcohol and flavour compounds, and specialty malts mainly provide colour and flavour compounds, adding complexity and diversity to a beer. Table 1 shows the malt type, colour description, and general characteristics of the base and specialty malts.

Table 1. Malt type, color, and general characteristics [10, 18].

Malt Types	Color SRM [1]	Color Description	Organoleptic Characteristics
Base Malts			
Pilsner	1.2–2	Very pale	Little green, with the smell and taste of fresh wort
Pale	1.6–2.8	Light coloured	Deeper malt aroma than Pilsner
Pale Ale	2.7–3.8	Darker than standards pale malts	Not excessively pronounced malt aroma, with notes of biscuit or toast
Vienna Malt	2.5–4.0	Imparts a rich orange colour to beer	Slightly toasty and nutty
Melanoidin Malt	17–25		Sweet honey-like flavour
Munich	3–20	Covers a broad range of colours	Malty profile

Specialty Malts			
Caramel Malts			
Special Glassy (Carapils)	1–12		Add body and impart sweetness to beer
Caramel/Crystal	10–200	Can imply significant colour differences depending on the method of manufacture	Can imply significant aroma differences depending on the method of manufacture.
Roasted Malts			
Biscuit	20–30		Bread crust, nutty, and toasted aromas. Dry finish.
Amber	20–36		Nutty, biscuit, toffee taste.
Brown	40–150	Darker than Amber.	Nutty, biscuit, toffee taste.
Chocolate	350–500	Dark colour.	Treacle and chocolate aromas. Present dray and ashy aspects.
Black	435–550		Bitter, dry, and burnt aromas.
Roasted	300–650		Smoky, coffee, chocolate, and roast aromas.

[1]—The Standard Reference Method, abbreviated SRM, is the color system used by brewers to specify finished beer and malt color.

Specialty malts are subject to higher kilning and roast temperatures, which define their colours and flavours. As a result of this process, they lose their enzymatic activity, so they are used in small amounts compared to the base malts, which retain their enzymatic activity [29].

2.2. Boiling

The main purpose of wort boiling is the evaporation of water and unwanted volatile compounds, isomerization of humulones, fixation of wort composition by inactivation of enzymes, sterilization of wort, and removal of proteins. However, although the boiling stage can positively contribute to the formation of colour during beer storage, it should be carefully monitored, as it can lead to the formation of a non-biological haze due to the oxidation of polyphenols derived from malt and hop vegetative matter [28].

Humulus lupulus, commonly known as hop, is used in the brewing process during the boiling phase to enhance the quality and stability of beer and to introduce the characteristic hoppy aromas and bitter taste, which leads to desirable flavour for

consumers [30, 31]. The most important biochemical markers that differentiate hop varieties are hop acids, hop oils, and polyphenols. The hop acids include the so-called α-acids (humulone, cohumulone, and adhumulone) and, β-acids (lupulone, colupulone, and adlupulone) [30, 32].

The α-acids are tasteless; however, upon boiling in the wort, they are isomerized to the bitter-tasting iso-α-acids or isohumulones [32]. During boiling, only 50% of the α-acids are isomerized and less than 25% of their bittering potential is preserved in beer [33]. This occurs because of the restricted solubility of the α-acids in beer and the slightly acid wort (pH 5–5.5) [31]. On the other hand, the volatile fraction contained in the hop oil (0.5–3% in hops), together with the non-volatile fraction present in the hop polyphenols (3–6%) contribute to a full mouthfeel sensation during beer tasting [31, 34]. Therefore, hops play a major role in product quality; in addition, they improve flavour stability, as hop polyphenols have antioxidant capabilities, and their other compounds are effective at masking the development of stale flavours [21]. In order to stabilize the foam head, some breweries use processed hop advanced products, such as reduced iso-α-acids, which enhance foam stability to a greater extent than iso-α-acids [35].

2.3. Fermentation

Fermentation is the process in which fermentable sugars are transformed into alcohol, carbon dioxide, and many other compounds by the *Saccharomyces* yeast. To ensure a high-quality product, an effective brewing fermentation is required, as most of the flavour-active compounds of a beer are produced at this stage of the process.

Although all strains of *Saccharomyces* produce ethanol as an end-product of fermentation, the production of most aroma-active compounds is strictly dependent on the yeast strain chosen for the fermentation and has a great impact on the beer flavour [6, 30, 36].

In beer production, the yeast strains used as starter cultures are divided into two groups: ales and lager yeasts. *Saccharomyces cerevisiae* strains belong to the group of ale yeasts,

and require a high range of temperature (14–25 °C), and are often referred to as top-fermenting yeasts because in open fermenters, they rise to the surface of the vessel facilitating their collection [6, 30]. On the contrary, lager strains *Saccharomyces pastorianus* are more complex organism than ale yeast, run the fermentation at cool temperatures (8–15 °C), and are known as bottom-fermenting yeasts, because they sediment at the bottom of the vessel at the end of fermentation.

The key elements produced by yeast, which will determine the final quality of the product, are vicinal diketones (VDKs), esters, and higher alcohols. While esters and higher alcohols could be considered pleasant and desirable volatile constituents in beer, depending on their concentration level, VDKs are frequently considered off-flavors [36].

During yeast metabolism, higher alcohols are formed as by-products of amino acid synthesis or catabolism [30, 36]. At optimal levels, higher alcohols help with the drinkability and give the beer a desirable and pleasant aroma. On the contrary, above 300 mg/L, these compounds can lead to a strong, pungent smell and taste [30].

Esters are one of the most volatile compounds in beer and have a great impact on aroma. In moderate quantities, they add a pleasant and full-bodied character to the aroma [37]. However, in excess, they give an overly fruity aroma, which is considered undesirable by most consumers.

Finally, oxygen should be also considered, as it plays an essential role in the brewing process, especially during fermentation, where it is required by all yeast cells to support the synthesis of sterols and unsaturated fatty acid components of the cell membranes [14, 38]. Oxygen is also required for lipid synthesis, which is necessary to maintain the integrity and function of the plasma membrane and, consequently, the cell replication [39]. On the contrary, an excess of oxygen might damage cell components, contribute to cellular aging, and finally lead to cell death [38,39]. Thus, to obtain a high-quality product, it is necessary to achieve optimum oxygen levels.

3. Analysis of Beer Quality and Stability Using Chemometric Tools

Despite all the technologies available, obtaining a high-quality product is a hard task, as explained above. To achieve this purpose, a brewer must control several parameters in all phases of the process, as described before, ensuring the quality of the final product. In addition, product stability is also essential, as when beer leaves the brewery it is subject to the conditions that distributors and consumers may impose and which can lead to a rapid degradation of quality [6, 8, 40].

Managing and monitoring beer production and aging processes requires both knowledge and techniques to interpret and extract information from datasets with multiple variables [9]. Multivariate statistical techniques, also known as chemometric techniques, are commonly used in food science to help better understand and manage the connections between different types of data. During the beer production process and shelf life of a beer, chemometrics can be a very useful tool for monitoring the quality and stability of the product.

To monitor beer quality during the beer production process, the most important chemical measurements are those related to sensory perceptions that are routinely measured in a brewery, such as flavor, color, haze, foam, and mouthfeel [41]. On the other hand, monitoring the stability of the product during its shelf life is a hard task due to the complexity of the aging process. During beer aging, many oxidative and non-oxidative reactions occur, in which new molecules may be formed and some existing molecules may increase in concentration or degrade, thus changing the sensory profile of the product [40, 42–44]. In addition, the chemical and sensorial aspects of the aged beer depend on the beer style and its characteristics (ethanol content, pH, raw materials, and ingredients), the brewing process (temperature and time), and its handling process after packaging (exposition to light, temperature, and vibration) [8, 37, 40].

Many instrumental techniques can be employed to monitor both the beer production process and the aging of the samples, such as nuclear magnetic resonance (NMR) [45,

46], near-infrared spectroscopy (NIR) [47,48], electronic-nose (e-nose) [49,50], liquid chromatography–mass spectrometry (HPLC-MS) [13,51], gas chromatography–mass spectrometry (GC-MS) [52,53], and gas chromatography with olfactometric detection (GC-O) [54,55]. In addition to these instrumental techniques, sensory panels can also be performed to better understand the product [56]. In this case, chemometrics can be applied to help explain the relationship between product composition and sensory properties and between sensory properties and instrumental measurements [41]. Table 2 summarizes the scientific literature related to chemometric techniques applied to beer quality and stability over the last 10 years. Other references related to the chemometric techniques used in this article can be found in the References section.

Table 2. Scientific literature (2012–2021) correlated to the chemometric techniques applied to beer quality and stability.

Aim of the Study	Year	Analytical Techniques	Chemometric Techniques	Reference
Proposal of a methodology fast non-destructive metabolomic characterization of beer exploring the compositional profile of the product	2021	NMR spectroscopy	PCA MCR (Multivariate Curve resolution)	[57]
Evaluation of the factors that influence the perception of the intensity of palate fullness and selected descriptors of mouthfeel in fresh lager beer	2021	Physical chemical parameters Macromolecular characterization Sensory panel	HCA PLS	[58]
Understand the changes during the drying process to optimize the process, improving the process performance and the quality of the product	2020	Hyperspectral imaging	PLSR	[34]
Metabolomic profiling of beers to discriminate craft and industrial products	2020	NMR spectroscopy	PCA PLS-DA	[59]

Build and test a model capable of estimating the quality of beer	2019	Sensory panel	The model was created using Curve Fitting Toolbox in Matlab	[40]
Differentiate Brazilian lager beers by styles employing NMR spectroscopy combined with chemometric approach	2019	H NMR	PCA PLSDA SIMCA	[45]
Characterize the craft beers to differentiate them from the other competing and lower-quality products	2019	GC-MS	PLS-DA LDA	[60]
Multivariate analysis as a tool to discriminate and characterize differences in barrel diverse-aged beers using volatile fingerprinting	2019	GC-MS	PCA	[61]
Understand if there would be metabolite differences among six commercial barley sources and if this difference is reflected in the chemistry and in the sensory attributes of beer	2018	UHPLC-MS HILIC-MS GC-MS ICP-MS Sensory analysis	PCA	[23]
Compounds behaviour in natural and forced aging—recommendations as to how prediction by forced aging should be used	2018	GC-O GC-MS Sensory panel	PCA	[62]
Beer volatile terpenic compounds	2018	HSPME-MS GC x GC-TOF-MS	HCA	[63]
Traceability, quality control, and food adulteration	2018	Mir spectroscopy coupled with attenuated total reflectance (ATR)	PCA PLS-DA	[64]
Method optimization for volatile aroma profiling of beer.	2017	GC x GC-TOF-MS	PCA HCA	[65]
Characterization of brewing process—“Processomics”	2016	Electro spray ionization-Mass Spectrometry (ESI-MS)	PLS-DA	[12]

Differentiation between beers according to their price market	2016	Paper spray mass spectrometry (PS-MS)	PLS-DA	[66]
Create mathematical models that can be used during the measurement of beer shelf life	2016	Physical chemical parameters Haze	PLSR-PR PLSR-RSM	[67]
Developing accelerated model to evaluate brewing techniques that affect flavor stability using metabolomics on non-volatile compounds in beer	2016	UPLC-MS	PCA	[7]
Study volatile profiles and characterize odor-active compounds of brewing barley in order to determine the variability of the aroma composition among different brewing barley cultivars	2015	GC-MS	PCA Hierarchical Clustering	[52]
Propose a methodology for determining the start of the period of time in which beer fresh features start to change	2015	GC-MS	PCA	[53]
Using data fusion to establish a model to classify Chinese lager beer according to the manufacturer	2015	Fluorescence/UV/Visible spectroscopies	PCA LDA	[68]
Monitoring the aging process in alcoholic and non-alcoholic beers	2014	NIR	PCA KNN LDA StepLDA GA SELECT	[47]
Investigate the volatile metabolomic profile of raw materials used in beer	2014	HS-SPME GC-qMS	PCA SLDA	[69]
Determine the effectiveness of incorporating an oxygen sensor into lager beer	2013	Optic oxygen sensors Sensory panel	PLSR	[56]

bottles and predicting the sensory quality of the beer with respect to the oxidation and staling				
Clarify the aroma compounds affecting the various hop aroma characteristics, using beer prepared with different hop varieties	2013	GC x GC-TOF-MS	PCA	[70]
Development of a method for retrospective determination of temperature conditions to which beer had been exposed	2013	GC-MS Sensory panel	MLR ANN	[71]

3.1. Exploratory Analysis

The chemometric techniques applied to monitor and control the quality of beer production and aging processes cover a wide range of applications, which include exploratory data analysis (EDA), pattern recognition (or classification), and multivariate calibration. The EDA techniques, also known as unsupervised pattern recognition, are usually the first approach used to visualize the data, as they reduce the dimensionality of complex datasets and produce a graphical representation that is easy to understand and interpret. The procedure consists of grouping data based on their similarities, with no prior assumptions about class membership. In food science, the most popular EDA techniques used are principal component analysis (PCA) and hierarchical cluster analysis (HCA) [9].

An example of the application of EDA in the beer production process is related to the characterization of raw materials. Inui *et al*. [70] used PCA to characterize the volatile compounds responsible for the differences in hop aroma characteristics in beer. PCA was able to indicate the positional relationship among six hop aroma characteristics and five hopped beers. Furthermore, the authors could conclude that understanding the relationship between instrumental data and organoleptic evaluation using PCA is

effective and reliable for determining the key aroma compounds from numerous unknown components. Dong *et al*. [52], applied PCA to evaluate the variability of volatile aroma compounds among barley cultivars. The results obtained via PCA analysis showed that the aroma characteristics of brewing barley cultivars from different countries were quite different, while those from the same country were similar, especially the Chinese domestic barley cultivars. In another work, Bettenhausen *et al*. [23] used PCA to study if there were sensorial differences among six different beers produced from six different malt sources. A descriptive sensory analysis was performed on 45 attributes at 0, 4, and 8 weeks of storage, revealing flavor differences at 8 weeks and thus showing the importance of the malt source in beer flavor stability.

Another example using both EDA techniques, PCA and HCA, was shown by Rendall *et al*. [53], where the authors analyzed the evolution of the volatile fraction of Portuguese beers over a period of one year under standard shelf storage conditions using gas chromatography coupled with mass spectrometry (GC-MS). A total of 39 lager beers from the same production batch, kept at room temperature for 12 months, were analyzed. The chemometric analysis conducted focused on detecting the early onset of meaningful changes in chemical composition, and then on the analysis and characterization of the evolution of groups of compounds. The chemometric analysis revealed that the chemical composition of the beer presented a statistically significant deviation after 7 months, although the deviation trend had its onset during the sixth month. Furthermore, the authors concluded that there is no single resulting compound that can be identified as a unique aging marker, but rather two sets of compounds acting in a synergistic or antagonist way to produce significant changes in fresh beer flavor.

To propose a methodology aiming at fast non-destructive metabolomic characterization of a beer, exploring its compositional profile, and highlighting potential trends or peculiar samples, Cavallini *et al*. [57], combined NMR spectroscopy and chemometrics. In this study, one hundred pale beers from different brands were analyzed. PCA was used for exploratory purposes, on both the full spectrum and features datasets and

Multivariate Curve Resolution (MCR) was used for extracting the chemical features from the NMR spectra, which allowed a reduced dataset of resolved relative concentrations to be obtained. This approach using NMR spectroscopy and chemometrics offered clear information about beer composition, providing valuable information about beer characterization that proved to be very useful to the producers in terms of both quality control and innovation.

Coelho *et al*. [61] studied the beer aging process in wood barrels previously used to age Port wines. The volatile GC-MS fingerprints of unaged beers and beers aged in different times and conditions were analyzed using PCA. Samples showed groups depending on the aging time, which in turn was positively correlated with the presence of a higher number of volatile compounds. Differences in volatile composition were also found between the barrel aged beers and the unaged beer, thus showing that reutilized barrels may have an impact on aged beer production.

3.2. Classification Techniques

Pattern recognition methods or classification techniques are supervised methods that aim to recognize patterns in the data and classify observations by assigning a new sample into a category or class [9]. There are two different approaches in classification: discrimination and class modelling. When a discriminant approach is used, the focus is put on the difference between classes, and a sample is always assigned to a given class. For a two-class problem (classes A and B), a sample will be always assigned to A or to B. Examples of discriminant techniques are *k*-nearest neighbours (*k*NN), Artificial Neural Networks (ANN), Linear Discriminant Analysis (LDA), or Partial Least Squares Discriminant Analysis (PLS-DA). On the other hand, class-modelling methods focus on the similarities among samples from the same class rather than the differences between classes. In class modelling, classes are modelled individually and independently, and a sample can be assigned to a given class, to more than one class, or to none of the classes. For a two-class problem (A and B), a sample could be assigned to A, to B, to A and B, or

to neither A nor B. A typical example of a class-modelling technique is SIMCA (Soft Independent Modelling of Class Analogies). In beer science, classification methods are extensively applied to discriminate between geographical origins, to assess brand authenticity or beer style, to assess raw materials, and also to discriminate between fresh and aged beer [9, 41, 66]. The choice of a particular technique will depend on the nature of the problem at hand; that is, whether it is discriminant or class modelling (i.e., PLS-DA *vs*. SIMCA), whether it is linear or non-linear (i.e., LDA *vs*. ANN), or depending on the number and degree of correlation of the measured variables (i.e., LDA *vs*. PLS-DA).

Classification techniques can be applied in the beer production process in order to verify the authenticity of the product based on the raw material or the production process. Silva *et al*. [45] built PLS-DA models to distinguish lager beers based on the type of raw materials employed in the brewing process. The authors used NMR spectroscopy combined with chemometrics to discriminate lager beer samples according to their style and the raw material information provided on the label. The authors concluded that the approach adopted could be very useful when applied to a suitable set of samples. The models obtained had a prediction power higher than 90%, considering the raw material employed in the brewing processes. Vivian *et al*. [12] used PLS-DA to characterize markers of key production stages of the brewing process of a Brazilian craft brewery using electrospray ionization (ESI) high-resolution mass spectrometry (HRMS). The authors concluded that their approach allows a quick assessment of the process status before it is finished without the subjectiveness of sensorial analysis, thus preventing higher production costs, ensuring quality, and helping with the control of desirable features, such as flavour, foam stability, and drinkability. Gianetti *et al*. [60] evaluated the flavour profile from craft beers (unfiltered and unpasteurized) and industrial beers. The aim was to characterize craft beers to differentiate them from industrial mass-produced beers. PLS-DA was used to classify beers according to their different production methods. The results showed a good classification, both in calibration

(96.2%) and cross-validation (94.2%), enabling a good separation between beer categories with high prediction accuracy (96.23%).

Several studies involving pattern recognition methods to monitor the aging process have been carried out. Linear techniques such as PCA and LDA were applied by Ghasemi-Varnamkhasti *et al*. [50] to characterize the change of aroma of alcoholic and non-alcoholic beers from the same brand during the aging process by using metal oxide semiconductor-based electronic nose. The results did not reveal clear discrimination among alcoholic aged beers, showing more stability of such types of beer compared with non-alcoholic aged beers. Rodrigues *et al*. [46] applied PCA and PLS-DA to NMR spectra to monitor the chemical changes occurring in a lager beer exposed to forced aging. Inspection of PLS-DA loadings and peak integration enabled the changing compounds to be identified and revealed the importance of well-known aging markers, as well as other relevant compounds.

Understanding and controlling the aging process of a beer remains a hard task for brewers. In a study by Ghasemi-Varnamkhasti *et al*. [47], the potential of NIR spectroscopy for the qualitative analysis of different types of beer during the aging process was measured. PCA, KNN, LDA, Stepwise LDA, Genetic Algorithms (GA) and Gram–Schmidt supervised orthogonalization (SELECT) were employed to characterize the aging phases as well as beer types. The results demonstrated that the computational tools were capable of discriminating and classifying the aged beers, showing high classification accuracies for all aging treatments.

Classification techniques have also been applied for authentication purposes. Tan *et al*. [68] discriminated Chinese lager beers produced by different manufacturers, with good accuracy. They used a data fusion approach by combining fluorescence, UV and visible spectroscopies. LDA and PCA-LDA (LDA applied on the scores of a previous PCA on the data) were applied, showing a much better classification accuracy (79–87%) when compared with the classification models on the individual instrumental techniques (42–

70%). Gordon *et al*. [64] used Mid-infrared (MIR) spectroscopy coupled with attenuated total reflectance (ATR) to classify different beer types (ale *vs*. lager and commercial *vs*. craft beer). PLS-DA was used to analyze and to discriminate the beer samples based on their infrared spectra. Correct classification rates of 100% were achieved in all cases, showing the capability of MIR spectroscopy combined with PLS-DA to classify beer samples according to style and production. Furthermore, dissolved gases in the beer products were shown not to interfere as overlapping artefacts in the analysis. The benefits of using MIR-ATR for rapid and detailed analysis coupled with multivariate analysis can be considered a valuable tool for researchers and brewers interested in quality control, traceability, and food adulteration.

3.3. Multivariate Calibration

Multivariate calibration aims to create a mathematical model to predict properties of interest from instrumental measurements. Modelling can help control and optimize process performances, which are very useful to the beer production process. Moreover, to better understand the aging process, multivariate calibration can link chemical and sensory data [41]. The most commonly used multivariate calibration techniques are multiple linear regression (MLR), principal component regression (PCR), and partial least squares (PLS) regression. These calibration models need a thorough validation before they can be used to reliably predict system or product properties [9].

An example of the use of multivariate calibration was shown in Sturm *et al*. [34]. In this study, the authors investigated the dynamics in the drying behaviour and quality development of hops using visual and environmental sensors combined with chemometrics. To better understand the dynamics of the drying process, a full array of visual sensors was integrated into a pilot scale drying system to investigate the colour changes, Hop Storage Index, α acids and β acids, product and air temperature, and air humidity, throughout the drying process with different bulk weights and drying temperatures. PLSR was applied in combination with spectroscopy and hyperspectral

imagining. The results showed that, besides bulk weight and temperature, harvesting conditions and specific air mass flow have a significant influence on both drying time and colour changes of hops during drying in identical conditions.

Gagula *et al*. [67] created mathematical models using two partial least squares regression methods: polynomial regression (PLSR-PR) and response surface method (PLSR-RSM) to describe changes in beer properties during storage based on three measured properties: colour, bitterness, and haze values. The samples used were lager beers packed in glass bottles and polyethylene terephthalate (PET) bottles and samples of malt beer in glass bottles. The authors concluded that PLSR-RSM models were more accurate when describing property changes for the lager and malt beer in glass bottles, while PLSR-PR was better for the lager beer in PET bottles. By comparing the samples, the models showed that beer packaging in PET bottles showed larger changes than lager beer in a glass bottle during the storage period. In contrast, both lager beer and malt beer showed great changes in different periods of storage.

Partial Least Squares (PLS) regression was used by Krebs *et al*. [58] to create a model to predict the palate fullness intensity in beers. In this article, the authors reported a chemometric analysis of 41 lager beers based on the evaluation of the analytical data of beer composition, palate fullness, and mouthfeel. Ethanol, original gravity, dynamic viscosity, nitrogen and β-glucan were analyzed. The macromolecular profile of the samples was analyzed and a sensory characterization was performed by certified panellists. The authors concluded that palate fullness and mouthfeel are key factors that determine the quality of lager beer and consumers' acceptance, and that the prediction model can be used for a targeted design of palate fullness by weighting the influence factors. Calibration models can also be used to predict the organoleptic quality of the beer during the aging process. Hemp *et al*. [56] used an optical oxygen sensor to assess the level of residual oxygen in the headspace of bottled beers by monitoring the product over time before and after pasteurization. A sensory panel was also used to determine the effect of the residual oxygen on the sensory quality of the product. PLS-R was used

to process the sensory data obtained by the 26 panellists. The results showed that the higher the oxygen level prior to pasteurization, the more negative the attributes associated with the sensory quality of the beer, especially those related to beer staling.

Another example of multivariate calibration was used to create a method for the retrospective determination of temperature based on the determination of carbonyl compounds determined by GC-MS. Čejka *et al*. [71] used three approaches: regression graph, multiple linear regression (MLR), and neural networks, to calculate the storage temperature of samples. In this study, 11 samples from the eight major Czech breweries were stored for 6 months at 0, 8, 20, and 30 °C. The MLR calculation used only 2-furfural as representative indicator of aging. The exponential dependency of 2-furfuralwith storage was converted to a linear dependency using a logarithmic transformation and a regression equation was created, with months and ln $_c$(furfural) as the input variables and storage temperature as the output variable. The uncertainty of the final predictions was 5 °C.

4. Conclusions

Despite being extensively studied, beer quality is still a challenge for the beer industry. Maintaining the quality and stability of the beer during its shelf life requires monitoring the entire beer production process from the field to the final consumer. Chemometric techniques can help us to better understand both the beer production and the aging processes, by extracting useful information from large and complex brewing datasets. Further studies involving a comprehensive view of beer stability, combining analytical techniques and chemometric solutions, could help us to better understand the process and make corrections in real time, avoiding losses, saving time, and guaranteeing a higher-quality product.

In conclusion, unsupervised and supervised chemometric methods are powerful tools that have proved to be quite useful for monitoring the beer production and aging processes. Unsupervised exploratory methods allowed us to obtain the first insights

about the data, and supervised approaches (discriminant analysis and multivariate calibration) revealed the underlying correlations between sensory and chemical changes in beer during the process and shelf life of the product.

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Chapter 2
HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

Beer is a fermented beverage produced from water, malted barley, hops and yeast. To achieve quality and to maintain the stability of the product during its shelf life, it is necessary to understand and control all the parameters involved in the brewing process, from the raw material to the storage conditions. Multivariate analysis is commonly used in food science to help understand and manage the connections between the different types of data. Therefore, it is necessary to recognize the main stages of the brewing process that have important contributions to quality and stability and to review which chemometric techniques are used in the literature to better understand and monitor the different quality parameters during production and shelf life of beer.

Beer quality and shelf life are generally determined by its appearance, aroma, taste and texture, and are conditioned by its microbiological, colloidal and flavour stability. During the ageing process, the sensory quality attributes of a beer change due to changes in its chemical composition. The ageing process depends on the conditions of the brewing process and it is greatly affected by the type of packaging used, as well as storage conditions such as time, temperature and light. Under optimal storage conditions of temperature and light, in a natural ageing process, the sensory changes in the volatile matrix of the beer are similar regardless of the type of container used, aluminium cans or glass bottles.

With the increase in beer export rate, shelf life problems have become an important issue for breweries. However, some factors in beer composition such as alcohol content, low pH, low content of nutrients and reduced oxygen content make it an inherently stable product. To achieve microbial stability and to guarantee product quality during its shelf life, breweries must adopt measures such as microfiltration and/or pasteurization. Both conservation processes have advantages and disadvantages regarding sensory quality parameters, but the efficiency of the process should not be related just to the sensory characteristics but also to the microbiological and colloidal stability.

2.2. Objectives

2.1. General Objectives

The general objective of this Doctoral Thesis has been to further study, using multivariate analysis tools, beer quality and stability during its shelf life to understand and monitor the main parameters that influence them. To understand the quality and stability of beer, the evaluation of many factors, both internal and external to the brewing process is necessary, as well as the analysis of many physicochemical, microbiological, sensory and instrumental parameters. Multivariate analysis was applied to facilitate the extraction, connection, and interpretation of the acquired data. Based on this main objective, four specific objectives were outlined:

2.2. Specific Objectives

1. Review and describe the use of chemometric methodologies that allow the understanding and monitoring of different quality parameters during the production and shelf life of a beer.

2. Monitor the evolution of the aroma profile and determine the main alterations that occurred in beer samples during their shelf life according to the type of container, aluminium can or glass bottle, using the HS-SPME/GC-MS technique and chemometrics.

3. Create a prediction model of beer shelf life by comparing the changes that occurred during one year in the volatile matrix of beer in optimal storage conditions, using the HS-SPME-MS e-nose technique and chemometrics.

4. Assess the efficiency of microfiltration and pasteurization processes during the shelf life of beer, correlating the main parameters that influence sensory quality and product stability, such as microbiological, colloidal and flavour stability, by multivariate analysis.

Chapter 3

ARTICLE 2: MONITORING THE EVOLUTION OF THE AROMA PROFILE OF LAGER BEER IN ALUMINIUM CANS AND GLASS BOTTLES DURING THE NATURAL AGEING PROCESS BY MEANS OF HS-SPME/GC-MS AND MULTIVARIATE ANALYSIS

Article 2: Monitoring the evolution of the aroma profile of lager beer in aluminium cans and glass bottles during the natural ageing process by means of HS-SPME/GC-MS and multivariate analysis

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Abstract

Headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME/GC-MS), sensory evaluation, and multivariate analysis were applied to monitor and compare the evolution of the aromatic profile of a lager beer in different types of containers (aluminium cans and glass bottles) during the natural ageing process. Samples were aged naturally for a year in the absence of light with a controlled temperature of around 14 ◦C +/- 0.5 °C. The sensory evaluation applied was a blind olfactometric triangle test between canned and bottled samples at different periods of aging: fresh, 6 months, and 11 months. The sensory evaluation showed that the panellists were able to differentiate between samples, except for the fresh samples from the brewery. A total of 34 volatile compounds were identified using the HS-SPME/GC-MS technique for both packaging types in this experiment. The application of multivariate analysis to the GC-MS data showed that the samples could not be differentiated according to the type of packaging but could be differentiated by the ageing time. The results showed that the combination of sensory, HS-SPME-GCMS, and multivariate analysis seemed to be a valuable tool for monitoring and identifying possible changes in the aroma profile of a Beer during its shelf life. Furthermore, the results showed that storing beer under optimal conditions helped preserve its quality during its shelf life, regardless of the type of packaging (aluminium can and glass bottle).

Keywords: aluminium can; glass bottle; natural ageing; sensory analysis; packaging; HS-SPME/GC-MS; multivariate analysis

1. Introduction

The freshness of a beer is a key factor in determining its quality and is directly associated with the contents of aromatic compounds in the final product. From the moment beer is packaged, its desirable freshness decreases, while the undesirable aromas characteristic of ageing may increase in concentration and perception [1]. This decline in product quality during its shelf life depends on the storage conditions, which in turn are affected by several factors. Beer ageing is considered one of the most important issues challenging the brewing industry. Ageing occurs during product storage and involves many chemical reactions that can cause changes in the chemical composition of the beer, thus altering its aroma profile [2].

Primarily, positive aromas, such as fruity and floral aromas, tend to decrease in intensity during the storage period, and aromas such as catty, black-currant, or cardboard along with other aromas such as sweet, caramel, honey, bread, earth, straw, wood, and sherry can arise [3, 4].

The ageing process is a phenomenon that depends on many factors, such as raw materials and processing conditions, and is greatly affected by the type of packaging used, as well as the storage conditions, such as time, temperature, and light. Appropriate packaging can help delay or reduce these changes in the product during its shelf life, by slowing down the product deterioration, retaining the beneficial effects of processing, extending shelf life, and maintaining the quality and safety of the final product [5, 6]. Lorencová *et al*. [3] evaluated selected physicochemical parameters and organoleptic properties of Czech-type lager beer during a 12-month storage period, concerning the applied type of packaging: glass bottle, aluminium can, polyethylene terephthalate (PET) bottle, and stainless steel beer keg. The study showed that, generally, the type of packaging significantly influenced the physicochemical and organoleptic properties of the examined samples. The authors concluded that the aluminium can and the stainless steel keg were evaluated as the most suitable types of packaging for beer storage and

PET the least suitable. The same results were found by Gagula *et al*. [7] in their study about the influence of packaging material on the volatile compounds of beer.

To monitor the evolution of aroma compounds in beer during its shelf life, natural and forced ageing processes can be used as ageing methods. The forced ageing process is a pervasive but discriminatory method for accelerating the process that occurs during the natural ageing of a beer. Different forcing regimes, involving changes in certain parameters, such as the temperature, time, impact of light, oxygen content, alteration in pH values, and mechanical action (e.g., vibration), can be applied [4]. However, some results from the literature [4, 8] indicate that forced ageing alters the aroma profile of beer, unlike natural ageing.

Lehnhardt *et al*. [8] used sensory and analytical approaches to assess the prediction power of a forced ageing method. To carry out their study, a Pilsen and a Lager beer were stored for up to 17 months at 20 °C (natural ageing) and 40 °C for up to 9 days (forced ageing) and analyzed by gas chromatography olfactometry (GC-O), gas chromatography mass spectrometry (GC-MS), and sensory analysis with a trained panel. The authors demonstrated that the forced ageing method led to the development of cardboard and bready notes, whereas natural ageing led to fruity and berry notes. According to Saison *et al*. [9], the flavor of an aged beer varies significantly depending on the conditions to which it is subjected. In their study, the authors applied different temperature-time profiles, oxidative conditions, and varied pH and ethanol concentrations of the samples. The samples were analyzed by gas chromatography and sensory analysis. The authors concluded that the ageing process was accelerated when samples were submitted to higher temperatures, to oxidative conditions and, in a lesser degree, to a lower pH. On the other hand, changes in the flavor profile could be observed between samples exposed to different temperatures and oxidative conditions.

Considering consumers' expectations and knowing that their preferences differ, the type of beer packaging varies significantly between countries. In European countries, for example, the preference is for bottles in the first place, and in second place aluminium

cans. Thus, to better understand the issues challenging the brewing industry, such as the ageing process, it is necessary to understand the changes that occur in the volatile matrix of a beer during its shelf life in both types of containers. Therefore, considering the chemical properties of the volatile compounds involved, proper isolation and concentration of the compounds, with an adequate identification with gas chromatography coupled to mass spectrometry (HS-SPME/GC-MS) seems to be the best technique to monitor the evolution of the aroma profile in beer during its natural ageing. Regarding the determination of volatile compounds causing changes during beer natural ageing, Lenhardt *et al*. [1] compared the results from the different established analytical methods commonly used for that type of analysis, such as headspace solid-phase microextraction (HS-SPME), solvent-assisted flavour evaporation (SAFE), and steam distillation (SD). The article discussed the effect of these methods on flavour stability assessment. The comparison was conducted for four different commercial pale lager beers at different stages of ageing at 20 °C (fresh, 5 months, and 10 months). The results showed that ageing-related changes in pale lager beer presented altered profiles and behaviour with SD compared to the non-invasive HS-SPME, due to heat intake. Based on the results presented in the comparison between the analytical methods, the authors indicated that the most gentle and non-invasive method was the best option to apply for analysis. Several reviews and papers are available on the aroma profile of beer; however, there are not many studies on the influence of different packaging types on the aroma profile evolution during natural ageing. In this context, this work aimed to monitor the evolution of the aroma profile of beer packaged in aluminium cans and glass bottles, stored in the absence of light at 14 °C +/- 0.5 °C for a period of 11 months, correlating the sensory data with instrumental data obtained from HS SPME/GC-MS. We also aimed to determine the main alterations that occurred in the volatile profile of the samples, which allowed classifying the beer according to the container, aluminium can or glass bottle, and the time of storage.

2. Results

2.1. GC-MS

The GC-MS analysis of beer samples led to the identification of 34 volatile compounds from different chemical families, namely esters, alcohols, acids, ketones, aldehydes, monoterpenes, and phenols. A set of 11 major volatiles were detected in the lager beers: octanoic acid, decanoic acid, caproic acid, 2-phenylethyl alcohol, 2-methyl-1-butanol, 3-methyl-1-butanol, β-phenethyl acetate, isoamyl acetate, ethyl octanoate, ethyl decanoate, and ethyl hexanoate. Table 1 shows the 34 compounds identified using aluminium cans and glass bottles.

Table 1. GC-MS Compound identification during beer ageing under optimal storage conditions.

Number	**CAS Number**	**Retention Index* [10]**	**Compound Name**	**Odor Impression [10]**	**Threshold [11]**	**Class Compound**
01	108-10-1	969	Methyl isobutyl ketone	—	240 to 640 ppb	Ketone
02	110-19-0	1014	Isobutyl acetate	Fruit, apple, banana	65 to 880 ppb	Ester
03	105-54-4	1024	Ethyl butyrate	Apple	0.1 to 18 ppb	Ester
04	71-23-8	1039	Propanol	Alcohol, pungent	5.7 to 40 ppm	Alcohol
05	108-64-5	1055	Ethyl 3-methylbutanoate	Fruit	NA	Ester
06	123-86-4	1058	Butyl acetate	Pear	10 to 500 ppb	Ester
07	78-83-1	1104	Isobutanol	Wine, solvent, bitter	360 ppb to 3.3 ppm	Alcohol
08	123-92-2	1123	Isoamyl acetate	Banana	2 to 43 ppb	Ester
09	539-82-2	1130	Ethyl valerate	Yeast, fruit	1.5 to 5 ppb	Ester
10	5989-27-5	1172	D-limonene	Citrus, mint	NA	Monoterpene
11	137-32-6	1209	2 -methyl-1-butanol	Malt	0.14 mg/L	Alcohol
12	123-51-3	1213	3-methyl-1-butanol	Whiskey, malt, burnt	250 ppb to 4.1 ppm	Alcohol
13	123-66-0	1222	Ethyl hexanoate	Apple peel, fruit	0.3 to 5 ppb	Ester
14	142-92-7	1247	Hexyl acetate	Fruit, herb	2 to 480 ppb	Ester
15	110-93-0	1320	6-methyl-5-hepten-2-one	Herb, butter, resin	50 ppb	ketone
16	111-27-3	1342	Hexanol	Resin, flower, green	200 ppb to 2.5 ppm	Alcohol
17	106-32-1	1422	Ethyl octanoate	Fruit, fat	5 to 92 ppb	Ester
18	98-01-1	1446	Furfural	Caramel, bready, cooked meat	280 ppb to 8 ppm	Aldehyde
19	628-99-9	1521	2-nonanol	Cucumber	52 to 82 ppb	Higher alcohol
20	78-70-6	1544	Linalool	Flower, lavender	4 to 10 ppb	Monoterpene alcohol
21	111-87-5	1557	Octanol	Chemical, metal, burnt	42 to 480 ppb	Alcohol
22	513-85-9	1602	2,3-butanediol	Fruit, onion	NA	Alcohol
23	110-38-3	1629	Ethyl decanoate	Grape	8 to 12 ppb	Ester
24	503-74-2	1660	Isovaleric acid	Sweet, acid, rancid	190 ppb to 2.8 ppm	Acid
25	505-10-2	1730	Methionol	Sweet, potato	0.2 ppb	Alkyl sulfide
26	106-22-9	1748	Citronellol	Rose	11 ppb to 2.2 ppm	Monoterpene

27	103-45-7	1815	β-phenethyl acetate;	Rose, honey, tobacco	3.8 ppm	Ester
28	106-33-2	1827	Ethyl laurate	Leaf	NA	Ester
29	142-62-1	1830	Hexanoic acid	Sweet	93 ppb to 10 ppm	Fatty Acid
30	2021-28-5	1902	Ethyl dihydrocinnamate	Flower	17 to 40 ppb	Ester
31	60-12-8	1918	2-phenylethyl alcohol	Honey, spice, rose, lilac	0.015 ppb to 3.5 ppm	Alcohol
32	124-07-2	2035	Octanoic acid	Sweet, cheese	910 ppb to 19 ppm	Fatty Acid
33	7786-61-0	2203	p-vinylguaiacol	Clove, curry	0.75 to 3 ppb	Phenol
34	334-48-5	2229	Decanoic acid	Rancid, fat	2.2 to 10 ppm	Fatty acid

* Retention index for a polar column. NA: not available.

2.2. Sensory Analysis

The sensory analysis performed was a blind triangle olfactometric test of difference [12], where the untrained assessors were allowed to use only the olfactory sense (nose) to distinguish between samples. The main objective of this test was to differentiate the samples by focusing only on their volatile compounds. A total of 227 individual triangle tests were conducted. To avoid odour saturation of the panellists, only two sessions were held per day. The results were interpreted and analyzed according to the European Brewery Convention Analytica of Sensory Analysis (13.7) [12]. First, triangle tests were performed of beers packaged in aluminium cans and glass bottles, at different periods of their shelf life (fresh from the brewery, fresh from the supermarket, 6 months, and 11 month-aged), as shown in Table 2.

Table 2. Results from the Triangle Olfactometric Test I with $\alpha \leq 0.05$.

Triangle Test	Trials [a]	Successes [b]	p-Value	Minimum Number of Correct Responses	Results
Triangle Test I: Aluminium Can and Glass Bottle (Fresh, 6 and 11 months)					
1	28	12	0.1911	15	ND
2	30	16	0.0188	15	D
3	26	14	0.0247	14	D
4	30	18	0.0025	15	D

Triangle Test I: 1 = Fresh aluminium can and fresh glass bottle from brewery; **2** = Fresh aluminium can and fresh glass bottle from supermarket; **3** = 6-month aged aluminium can and 6-month aged glass bottle from brewery; **4** = 11-month aged aluminium can and 11-month aged glass bottle from brewery. a: Number of participants; b: Number of correct responses; ND = No difference between samples; D = Samples are different.

The results from the sensory analysis performed on fresh beer from the brewery (Triangle Test 1) showed that panellists were not able to differentiate between the samples, with only 12 correct responses. The panellists were indeed able to differentiate between beers packaged in aluminium cans and glass bottles in the triangle test performed with samples bought in the supermarket (Triangle Test 2). Triangle Tests 3 and 4, with 26 and 30 trials, respectively, performed for beers aged 6 and 11 months packaged in aluminium cans and glass bottles, showed that the panellists were able to differentiate between the samples. These results show that, even under optimal conditions of light and temperature, beer can present differences from the sixth month of ageing. A PCA of the data obtained from the chromatographic analysis revealed that aluminium cans (AC) and glass bottles (GB) could not be distinguished from each other, as shown in Figure 1A.

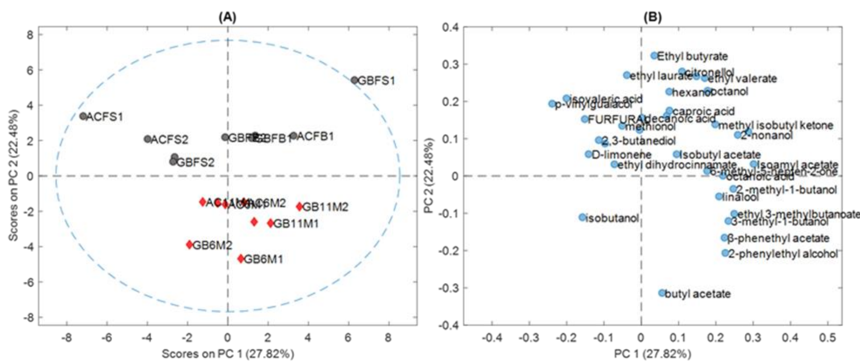

Figure 1. (**A**) Score plot of PCA analysis; (**B**) Loading plot of PCA analysis. ACFB−aluminium can fresh brewery; GBFB−glass bottle fresh brewery; ACFS−aluminium can fresh supermarket; GBFS−glass bottle fresh supermarket; AC6M−aluminium can 6 months aged brewery; GB6M−glass bottle 6 months aged brewery; AC11M−aluminium can 11 months aged brewery; GB11M−glass bottle 11 months aged brewery. Grey circles indicate fresh beers, red triangles aged beers and blue circles names of volatile compounds identified.

In Figure 1A, no difference can be seen between the groups of samples (canned or bottled samples). Therefore, we decided to perform triangle tests between the fresh and aged beers in the same container, either aluminium cans or glass bottles. The results are shown in Table 3.

Table 3. Results from the Triangle Olfactometric Test between Fresh and Aged beers with $\alpha \leq 0.05$.

Triangle Test II: Fresh and Aged Beer without Differentiate Types of Packaging (6 Months and 11 Months)					
Triangle Test	**Trials** [a]	**Successes** [b]	***p*-Value**	**Minimum Number of Correct Responses**	**Results**
5A	26	14	0.0247	14	D
5B	28	12	0.1911	15	ND
6A	30	16	0.0188	15	D
6B	29	17	0.0045	15	D

Triangle Test II: **5A** = Aluminium can fresh and aluminium can aged for 6 months; **5B** = Glass bottle fresh and glass bottle aged for 6 months; **6A** = Aluminium can fresh and aluminium can aged for 11 months; **6B** = Glass bottle fresh and glass bottle aged for 11 months. a: Number of participants; b: Number of correct responses; ND = No difference between samples; D = Samples are different.

The second group of triangle olfactometric tests (Triangle Tests II) was carried out with samples of fresh beer and aged beer (6 and 11 months), in the same type of containers (aluminium can fresh *vs*. aluminium can aged, and glass bottle fresh *vs*. glass bottle aged). For all tests except test 5B, which was performed between fresh and 6-month aged bottled samples, the panellists were able to distinguish fresh from aged beer samples with a 95% level of confidence. These results show that if beer is naturally aged in the absence of light and with a controlled temperature, bottles seem to be the type of container that olfactometrically best preserves the product up to 6 months of ageing.

2.3. *Chemometric Analysis*

The first step in the chemometric analysis was to apply principal component analysis (PCA) to the chromatographic data. The first four PCs explained 73% of the total variance of the model. For a preliminary visualization of the data, we decided to show the score plot of the first two PCs, since they explained 50% of the total variance in the data. Figure 1A,B shows the score and loading plots of the PCA analysis for the first two principal components (PCs).

Although the sample size (18 beers) was certainly not large, at least on an exploratory level, some trends were detected. As mentioned above, it was observed that no differences existed between the aluminium cans and glass bottles, although some grouping appeared related to the ageing time. In Figure 1A, the group of fresh beers from the brewery and supermarket show an opposite correlation in PC1 between both types of samples; while the supermarket fresh samples had a negative score on PC1, the brewery samples had a positive one. PC2 explained the difference between the fresh and aged beers. No outlier samples were detected; however, some of the samples had a higher influence on the PCA model. This can be seen in Figure S1 (Supplementary Materials), were the Hotelling T2 *vs* Q residual plots are shown for the PCA models with 1, 2, 3, and 4 PCs. GBFS1, ACFS1, and GBFS2 were the most influential samples. GBFS1 and ACFS1 had a higher leverage (T2 value) than the rest of the samples; that is, they appeared at the extreme of all PCA models. However, for the model with four PCs, they were within the limits. Instead, GBFS2 lies in the center of the model (low T2 value) but has a higher residual; that is, part of the chromatogram (peak areas) of GBFS2 was different from the rest of samples and was not modelled by PCA. By inspecting the original data matrix, we could observe that GBFS2 had a much lower peak area for octanoic acid. Figure 1B shows the PCA loading plot. It can be observed that ethyl butyrate (3) and butyl acetate (6) were the variables with most weight on PC2 and showed an opposite sign, indicating that they were negatively correlated. Ethyl butyrate (3), which did not present a significant variation during ageing, had a positive loading in PC2, suggesting that it was positively correlated to fresh samples. Butyl acetate (6), which showed an increase in the peak area during ageing, had a negative loading in PC2, suggesting that it was positively correlated to aged samples.

After the preliminary PCA analysis, we applied partial least squares discriminant analysis (PLS-DA) to the data, to try to discriminate beers depending on the type of container (can/bottle) and the ageing time (fresh/aged). As the number of samples in the training was not set very high, the PLS-DA models built were validated using the leave-

one-out cross-validation technique, and the optimal number of latent variables was determined based on the percentage of correctly classified samples for the cross-validation set, as shown in Table 4.

Table 4. Results of the PLS-DA models can *vs*. bottle and fresh *vs*. aged beer.

Model	n° LVs	% Accuracy	% Sensitivity	% Specificity
Can *vs* bottle	4	56	67	50
Fresh *vs* aged	4	100	100	100

Figure 2 shows the score and loading plots for the first two factors of the PLS-DA model fresh *vs* aged samples. In the score plot, it can be observed that the difference between the fresh and aged samples was even more evident than in the score plot of the PCA model (Figure 1A). According to the loading plot, the compounds that helped to characterize samples as fresh were isoamyl acetate (8), ethyl hexanoate (13), hexanoic acid (29), and decanoic acid (34). For the aged samples, the compounds that stood out were 2-methyl-1-butanol (11), 3-methyl-1-butanol (12), linalool (20), β-phenethyl acetate (27), and 2 phenylethyl alcohol (31).

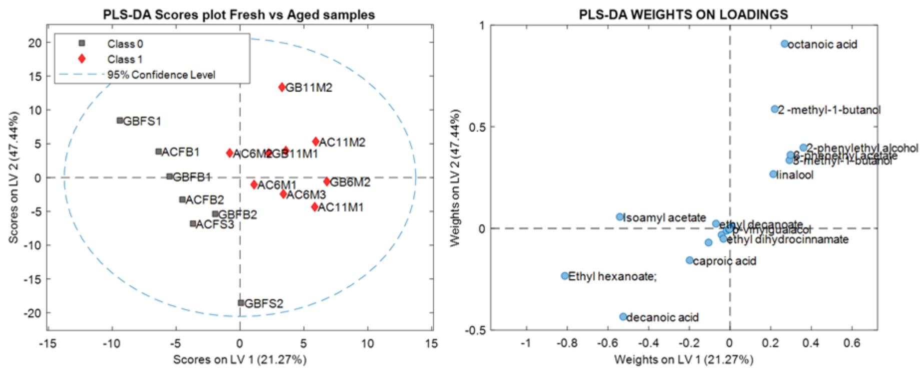

Figure 2. Score and loading plots for the first two factors of the PLS-DA model fresh *vs*. aged. Grey circles indicate fresh beers, red triangles aged beers and blue circles names of volatile compounds identified.

Finally, partial least squares regression (PLSR) was applied to build a model to predict the ageing time of a beer. For this, a regression model using the training set was built between the **X**-matrix (chromatographic peak areas) and a **y**-vector containing the

months of ageing of the beers. Four levels of ageing were used: 0 months (fresh beer from the brewery), 1 month (fresh beer from the supermarket), and 6 and 11 months (aged beers). The model, as for PLS-DA, was leave-one-out cross-validated. In this case, the optimal number of LVs of the model was determined based on the minimum value of the prediction error for the crossvalidation set and expressed as the root mean square error of cross validation (RMSECV). Figure 3 shows a plot of the predicted *vs*. actual values for the validation set and some parameters of the model. The RMSECV value found was around 1.1 months, for a model with seven LVs. This is the average error one could expect when predicting the ageing time of a beer.

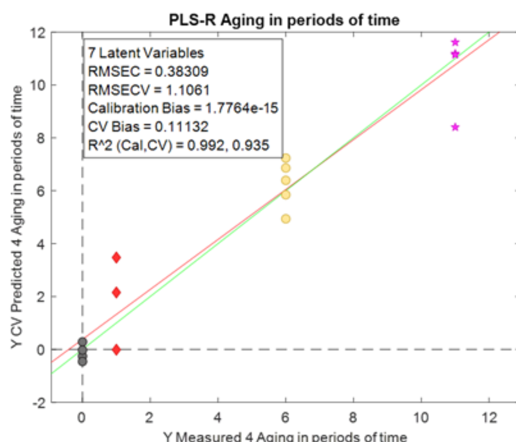

Figure 3. Plot of predicted *vs* actual values for the validation set.

3. Discussion

During ageing, the beer samples analyzed showed a slight variation in the abundance of some fruity, floral, and sweet aroma compounds. 6-methyl-5-hepten-2-one (15), furfural (18), and β-phenethyl acetate (27) showed an increase in the peak area, while isoamyl acetate (8), linalool (20), ethyl hexanoate (13), and ethyl octanoate (17) showed a decrease in the peak area. For some authors, instead of the formation of new compounds, the changes in aged beer were more related to the variation of the molecules already present in fresh beer, as we observed in the naturally aged samples [13-15]. During the natural ageing process, some esters, such as ethyl butyrate (3), ethyl valerate

(9), hexyl acetate (14), and ethyl ester octanoic acid (17), showed a decrease in the peak area in both types of container. This decrease occurred due to the ester hydrolysis during ageing [16]. On the contrary, other esters, such as ethyl 3-methylbutanoate (5), isoamyl acetate (8), ethyl hexanoate (13), and ethyl decanoate (23), showed a slight difference between both types of container during the 11 months of storage.

In the study by Vanderhaegen *et al.* [16], the levels of ethyl 3-methylbutanoate increased in all beers analyzed during the ageing process, due to the reaction of 3-methylbutyric (acid resulting from the degradation of hop bitter compounds) with alcohol. In the same study, isoamyl acetate and ethyl hexanoate showed a decrease from levels below their threshold, diminishing the fruity aroma and consequently decreasing the intensity of the 'back-ground' flavor and increasing the perception of eventual stale aromas. Regarding the sensory analysis, the results of sensory Test 1 between the fresh samples in aluminium cans and glass bottles from the brewery showed that, in the freshest conditions, the panellists were not able to distinguish the samples. Since these samples were transported under the same conditions, kept at a controlled temperature and in the absence of light, and analyzed as soon as possible, to maintain freshness, they should have shown no differences, as observed in the results from test 1 in the sensory analysis. On the subject of the test performed with fresh samples from the supermarket, Triangle Test 2, the panellists were able to differentiate between aluminium cans and glass bottles. These results could be explained by the different storage conditions imposed on these samples compared to the fresh samples from the brewery. The fresh samples from the brewery were delivered directly from the brewery after packaging and were kept in darkness and at a controlled temperature of 14 °C +/- 0.5 °C. The samples purchased from the supermarket had been packaged approximately one month previously and stored under supermarket conditions of light and temperature. Many studies have proven that an increase in the storage temperature generally has a negative impact on beer quality and stability, due to the degradation of iso- α -acids and the deterioration of aroma (an increase of staling compounds, beer colour, and haze formation) [17-19].

According to Paternoster *et al.* [20], since high temperatures can increase the energy that enables ageing reactions, exposing beer to different temperatures can lead to the formation or degradation of metabolites, which can activate or deactivate various ageing reactions, leading to different sensory attributes. Additionally, the exposure to light could contribute to accelerating the deterioration rate of the glass bottled samples. Moreover, by inspecting the chromatogram data, we could observe that the peak area of furfural (18) in the fresh beers purchased in the supermarket was higher than that of the fresh samples from the brewery. For some authors, furfural can be considered a heat indicator during the ageing process [9, 21]. In addition, according to Saison *et al*. [22], despite its low threshold, furfural has been used as an ageing indicator, due to its close correlation with sensory scores of flavor staling. If the supermarket samples were exposed to variations in temperature during transportation or storage, this could have caused a change in the volatile profile of the samples that would allow the panellists to differentiate these samples from the fresh samples from the brewery.

The sensory tests applied to samples aged for 6 and 11 months, Triangle Tests 3 and 4, showed that, for the panellists, these samples were olfactometrically different. In the study performed by Lorencovà *et al*. [3] with forced aging, the results of the sensory analysis between different types of packaging showed that the samples stored in aluminium cans were the best evaluated by the panel of expert assessors. According to the authors, this type of packaging is capable of retaining all organoleptic characteristics, and showing a weaker fading and a slight increase in bitterness after 10 months of storage. This could be explained by the oxygen contained in the beer, both the oxygen dissolved in the liquid and the oxygen present in the headspace. According to studies related to the oxygen content of beer, it decreases during transportation, which could be the result of oxidative reactions, which contribute to the deterioration of the aroma quality during storage [13, 23, 24]. Unlike what is discussed in the previous paragraph, the results obtained from the Triangle tests II, carried out with samples in the same type of container, showed that, for naturally aged beers under optimal conditions of light and

temperature, bottles seem to be a better container than a cans, since the panellists were not able to detect a difference between fresh and 6 month aged bottled samples, which was not the case with samples packed in aluminium cans.

According to Onaliran *et al*. (2017) [25] and Saison *et al*. (2018) [9], during storage, the sensory quality of the product tends to deteriorate significantly over time, at a rate that depends on the beer composition and storage conditions. As mentioned previously, the time of storage, temperature, oxygen, and light are important contributors to the degradation of aroma quality. Even under optimal storage conditions, beer quality deteriorates significantly as the product approaches the expiration date, regardless of the type of container in which it is packed.

Furthermore, when analyzing and comparing the GC-MS data of the beers contained in aluminium cans and glass bottles, and their evolution during ageing using chemometric tools, the variation shown in the peak areas of certain compounds listed above was not sufficient to instrumentally determine that the samples were different based on the type of packaging. Figures 1A and 2 show that the samples were divided in two main groups: fresh and aged. This could be verified by the results of the PLS-DA in Table 4, which better classified the samples as fresh and aged.

The aroma compounds responsible for the classification of the samples into fresh and aged were: isoamyl acetate (banana), ethyl hexanoate (apple peel, fruit), caproic acid (sweet) and decanoic acid (rancid, fat); and, 2-methyl-1-butanol (malt), 3-methyl-1-butanol (malt), linalool (flower, lavender), β-phenethyl acetate (rose, tobacco, honey), and 2-phenylethyl alcohol (honey, spice, lilac). During the ageing process beer tends to show a decrease in bitterness, which is partly explained by the sensory masking effect produced by an increasing sweet aroma [26]. Additionally, positive fruity/estery aromas that come from compounds such as isoamyl acetate tend to decrease in intensity. Finally, the PLS-R model built was capable of predicting the ageing period of lager beer (fresh, 1 month, 6 months, and 11 months aged), with an error of 1.1 month.

4. Materials and Methods

4.1. Beer Samples

One hundred samples of a commercial lager beer with an alcohol content of 5.4% *v*/*v*, 50 packaged in glass bottles and 50 packaged in aluminium cans, were delivered directly from a local brewery, in the freshest possible conditions, and used for the controlled natural ageing experiment. The samples were aged in the absence of light at 14 °C +/- 0.5 °C for 11 months. A total of 41 samples were used for the sensory analysis and 18 samples were used for the GC-MS analysis. Thirty samples of the same beer brand were purchased in the freshest possible conditions (less than 1 month of packaging) in a local supermarket in both packages: glass bottles and aluminium cans. The samples were used in the sensory analysis, to verify if the storage conditions imposed in the supermarket affected the sensory attributes of fresh beer. All beer samples were degassed by ultrasonication for 15 min prior to GC-MS analysis. Samples were analyzed in triplicate.

4.2. Headspace Solid-Phase Microextraction (HS-SPME)

The SPME holder, for manual sampling, and the divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm fiber used in this investigation were purchased from Supelco (Bellefonte, PA, USA). All fibers were conditioned prior to use and thermally cleaned between analyses by inserting them into the GC injection port at the temperature recommended by the manufacturer. All beer samples were degassed by ultrasonication for 15 min before the analysis. The optimal conditions that allowed the extraction of the largest number of odorants and with the highest intensity were achieved by placing 10 mL of sample into a 20 mL glass vial with 3.2 g of NaCl (saturation) and a small magnetic stir bar (the extraction was carried out under constant magnetic stirring). The vials were hermetically capped with a silicon septum under N_2 atmosphere and were pre-equilibrated for 10 min at 40 °C in a thermostatic bath. Then, a solid phase micro extraction (SPME) device was manually pushed through the vial septum and the fiber was exposed to the headspace vial for 1 h at 40 °C. Afterwards, the

fiber was pulled into the needle assembly and the SPME device was removed from the vial. Finally, the fiber was inserted into the injection port for thermal desorption of the analytes at 270 °C for 1 min in the splitless mode of the gas chromatograph.

4.3. Gas Chromatographic Analysis

Samples were analyzed with a GC-MS equipment from Agilent Technologies (Palo Alto, CA, USA). This was composed of a 7890 gas chromatograph and a 5977B HES mass spectrometric detector equipped with a high-efficiency ion source. To carry out the chromatographic separations a Chrompark (Varian, Middelburg, The Netherlands) CP-WAX 57 CB (50 m x 0,25 mm i.d., 0.2 μm film thickness) fused silica capillary column was employed. The oven temperature was programmed as follows: the initial temperature was 40 °C; after 5 min it was raised a rate of 3.5 °C/min to 120 °C, and finally a rate of 5 °C/min to 215 °C and held for 10 min. The split-splitless injection port was operated in splitless mode at 270 °C for 1 min. The mass spectra were recorded by electronic impact (EI) ionization at 70 eV with a temperature of 230 °C in the ion source and 150 °C in the mass quadrupole. The mass range analyzed was from 35 to 300 amu (atomic mass units).

4.4. Compound Identification

The odorants detected were identified using an Automatic Mass Spectral Deconvolution and Identification System (AMDIS) using a library of mass spectral databases (NIST MS Search version 2.3) by comparison with reference substances based on the retention index (RI) in the website of Flavornet [10], on two stationary phases of different polarity (CP-WAX 57CB and HP-5 MS). To calculate these RI values, a series of *n*-alkanes (from 8 to 20 carbon atoms) were injected under the same chromatographic conditions.

4.5. Sensory Analysis

The beer samples were sensory evaluated by a panel of untrained assessors, aged between 18 and 50 years, and the tests were performed following the methodology and statistics of the European Brewery Convention [12]. The assessors performed two

triangle olfactometric tests of difference to determine whether the samples were significantly different: (i) between aluminium cans and glass bottles, at different periods of shelf life (fresh, 6-months, and 11-months), and (ii) between the same beer samples without differentiating the type of container, comparing fresh samples and samples aged for 6 and 11 months. For the evaluation, samples were served (50 mL) in black glasses (300 mL; coded with 3-digit numbers) that were odorless and covered with watch glasses. The samples were served in a sensory laboratory in random order and under normal light and temperature conditions. The assessors were presented with a set of three coded samples, two of which were identical. Assessors were asked to identify the olfactometrically different sample. As this is a forced-choice method, if assessors could not identify a difference, they had to make a guess. The results were interpreted and analyzed according to the European Brewery Convention Analytica of Sensory Analysis (13.7) and the significance level used was ≤0.05 [12].

4.6. Chemometric Methods

The chromatographic profiles collected on the samples were processed using chemometric classification and prediction methods. The data obtained were exported to an Excel table and structured in a matrix of dimensions 18 samples × 34 columns (34 identified compounds). The chromatographic values of the matrix were the integrated peak areas of each identified compound. Before the chemometric analysis, the matrix values were preprocessed, since the difference between the high and low peaks was significant, affecting the analysis. The pre-processing of the values was based on the selection of a representative peak of the high peaks and one of the low peaks, with the smallest relative deviation in all samples. The high peak chosen was 2-phenylethyl alcohol (31), and the low peak selected was p-vinylguaiacol (33), with a median relative deviation of 12% for both compounds. Then, the values of the peak area of the high peaks in the matrix were divided by p-vinylguaiacol (33) and of the low peaks by 2-phenylethyl alcohol (31), creating a new matrix based on the original matrix. As no significant deviations were found in the data, only natural differences in the peak areas,

the only pre-processing applied to the data was autoscaling (i.e., mean centering and standardization to unit variance).

PCA was used for a preliminary visualization of the GC-MS data. PCA reduces the dimensionality of a data set by finding an alternative set of variables, called principal components (PCs), which retain most of the information contained in the original data. Each PC is a linear combination of the original variables and is orthogonal to each other. The relationship between samples, variables, and sample/variables was revealed when scores and loadings were plotted [27]. PCA can reveal groups and trends in the data and point out outlier samples.

PLS-DA was used to discriminate beer samples depending on the type of container (aluminium can or glass bottle) and to discriminate fresh beers from aged beers. PLS-DA is a discriminant method that is based on the partial least squares regression (PLSR) algorithm, as described below. To classify the samples, a PLS-DA model was built by correlating the matrix **X** of predictor variables (peak areas in this case) with a vector **y** of dummy variables, zeros and ones in a two-class problem, as in this work. In the first case, the value 0 was assigned to the aluminium cans and the value 1 to the glass bottles. In the second case, the value 0 was assigned to fresh beers and the value 1 to aged beers. Then, a PLS model was built between the experimental matrix **X** and the binary-coded vector **y**. For new samples, the predicted values were distributed around zero and one, and a threshold is a set to assign the samples to a given class. Finally, partial least squares regression (PLSR) was used to model and predict the ageing time of the samples. PLSR is a multivariate calibration method that correlates a matrix **X** of predictor variables (peak areas in our case) with a vector **y** containing the property of interest (in this study the ageing time) [14]. Four levels of ageing were considered: 0 months (fresh from the brewery), 1 month (fresh from the supermarket), 6 months, and 11 months. All calculations were performed using PLS Toolbox v8.7 (Eigenvector Research Inc., Eaglerock, LA, USA) running with MATLAB R2021a (The MathWorks, Natick, MA, USA).

5. Conclusions

In this paper, the effect of container and time of ageing of a beer stored under optimal conditions was sensorially and instrumentally monitored and evaluated. In general, the type of packaging influenced the olfactometric perception by the panellists, who were able to differentiate between canned and bottled samples at all ageing times analyzed, except the fresh samples from the brewery. However, instrumentally, the samples could not be differentiated by the type of packaging, but only by the ageing time. The olfactometric difference could be explained by the presence of varied esters in the sample, which could have interfered with the aroma profile of the beer, due to the synergistic effect that these esters have on individual flavors, which means that a slight variation in their concentration may have had a critical effect on the organoleptic perception of the product. On the other hand, the instrumental variation of the peak areas of the compounds in both types of container was not as marked as the variation presented for the ageing time. Despite the number of samples not being high (18 samples), it can be said that at an exploratory level, it was possible to detect some trends in the samples. In a general way, we can say that multivariate analysis proved to be a useful tool for discriminating beer samples based on the time of storage (fresh from the brewery, 1 month from the supermarket, 6 month-aged, and 11-month-aged) but not for discriminating by packaging type (aluminium cans or glass bottles). PLS-DA showed that the samples could be classified into two groups: fresh and aged. Finally, PLSR was able to relate the chromatographic peak areas with the ageing time (fresh from the brewery, 1 month from the supermarket, 6-month aged, and 11-month-aged) and predict the ageing time with an error of 1.1 months. To strengthen the models presented by the chemometric analysis and obtain conclusive results, it would be necessary to analyze more samples.

Further studies, including a descriptive sensory analysis by a trained panel, could lead to a better understanding of the most important differences that occurred during the natural ageing process and that led to a sensory difference between the canned and

bottled beers. Additionally, a better understanding of the ageing process could be achieved by applying other chromatographic techniques, such as gas chromatography-olfactometry (GC-O) or improving the HS-SPME/GC-MS technique. This would make it possible to quantify the aromatic compounds related to the ageing process, thus being able to determine with greater precision the ageing marker compounds for each type of container.

In conclusion, the combination of sensory, GC-MS, and multivariate analyses seems to be a valuable tool for discriminating beer samples at different periods of shelf life and could be used for monitoring and identifying possible changes in the volatile fraction during ageing.

Supplementary Material

1. Figure S1

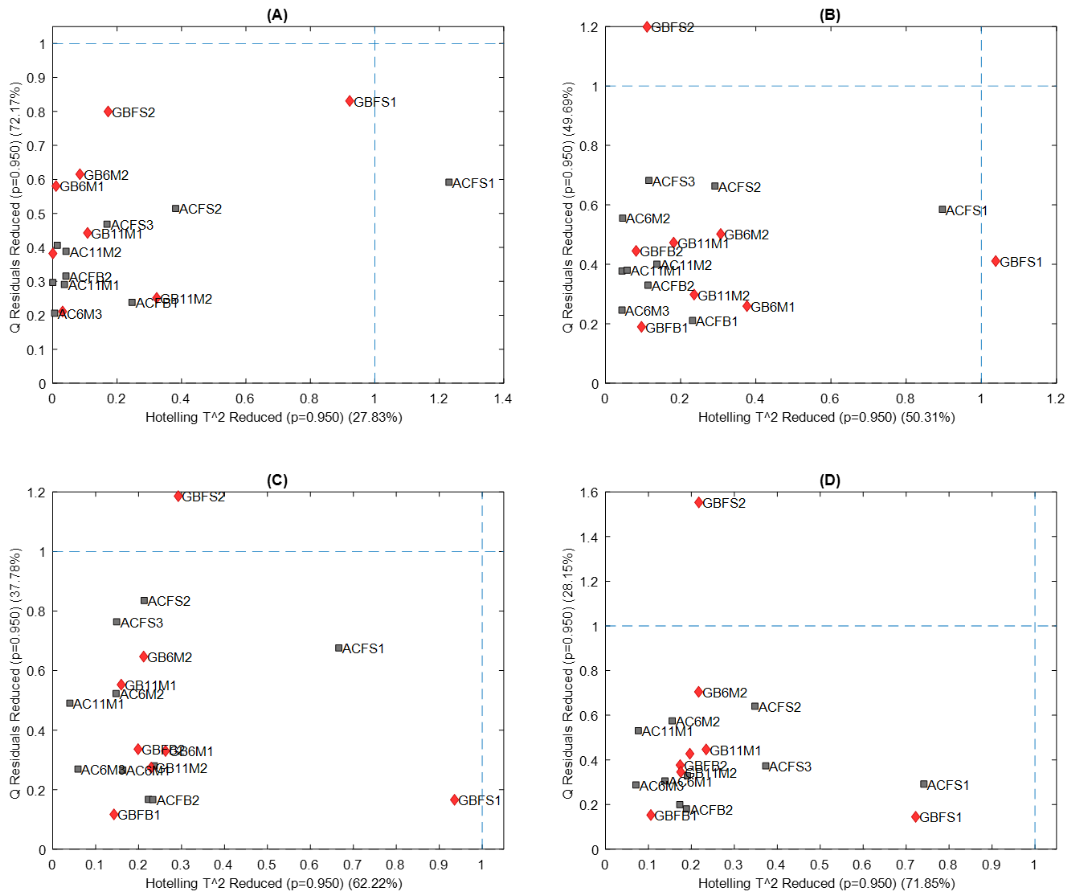

Figure S6: Hotelling T2 vs Q residual plots.

2. Figure S2

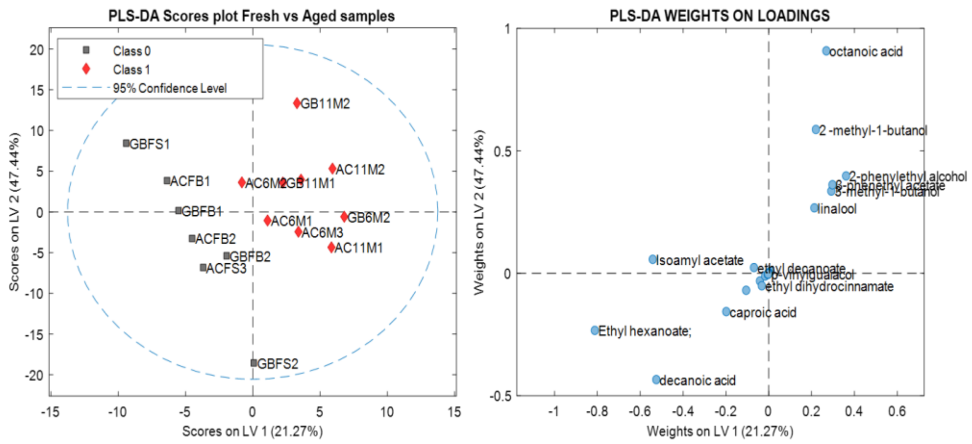

Figure S7: Score plot for the first two factors of the PLS-DA model Fresh vs Aged

3. Figure S3

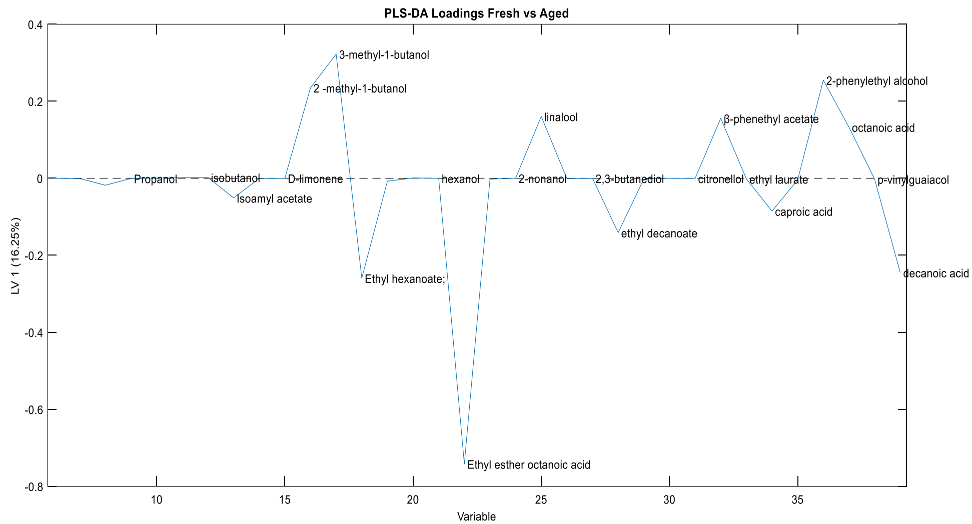

Figure S8: PLSDA Loadings plot of Fresh vs Aged samples.

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ARTICLE 3: A RAPID METHOD TO PREDICT BEER SHELF LIFE USING AN MS-DATA E-NOSE

Article 3: A rapid method to predict beer shelf life using an MS-data e-Nose

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Abstract

A rapid and efficient technique was applied, which used an electronic nose based on a mass detector (MS-based e-nose) combined with headspace solid-phase microextraction sampling and chemometric tools to classify beer samples between fresh and aged and between samples contained in aluminium cans or glass bottles, and to predict the shelf life of beer. The mass spectra obtained from the MS-based e-nose contained details about volatile compounds and were recorded as the abundance of each ion at different mass-to-charge (m/z) ratios. The analysis was performed on 53 naturally aged samples for eleven months without light and with a controlled temperature of around 14 °C ± 0.5 °C. Principal component analysis (PCA) was performed on the data and showed a grouping of samples between fresh and aged. Partial least square discriminant analysis (PLS-DA) allowed the discrimination of fresh from aged beers but could not discriminate between the samples according to the type of packaging. Finally, partial least squares regression (PLSR) proved to be an effective method for predicting beer shelf life.

Keywords: HS-SPME: MS-based e-NOSE; packaging; prediction; shelf life

1. Introduction

Beer aroma is defined by an intricate blend of volatile compounds that exhibit a diverse range in nature and concentration levels. Chemical compounds derived from raw ingredients such as malt, hops, and yeast are extracted during the brewing process [1]. The influence of each compound on the sensory experience of the final product depends on the balance between its concentration level and sensory threshold. This directly affects their odour activity [2].

Beer aroma is one of the most determinant factors of its quality. During its shelf life, the beer undergoes chemical reactions that can affect the aroma, leading to a decrease in sensory quality [3]. Due to its complexity, beer ageing is considered an important quality issue for the brewing industry since the rate of chemical reactions that occur during beer storage is determined by internal (e.g., raw material, brewing techniques, oxygen content, pH, and key odorants), and external factors (e.g., packaging, vibration, temperature, and light) [3–6].

To evaluate the aroma of a beer during its shelf life, different methods based on specialised equipment, such as gas chromatography (GC), gas chromatography–mass spectrometry (GC-MS), gas chromatography with olfactometric detection (GC-O), and high-performance liquid chromatography (HPLC), among others, can be used [7, 8]. Ncube *et al*. [9] studied the deterioration of an opaque traditional African beer using stir bar sorptive extraction and gas chromatography–high-resolution mass spectrometry. The study aimed to detect and monitor the changes in 84 volatile compounds throughout the day/shelf-life period. An additional example of the use of the gas chromatography technique is demonstrated in the research conducted by Ferreira *et al*. [10], where the authors monitored the impact of storage conditions on the chemical profile in beer samples using headspace solid-phase microextraction gas chromatography coupled to mass spectrometry (HS-SPME-GC/MS) and HS-SPME-GC-O. Samples were subjected to a controlled temperature of 37 °C ± 1 °C for 7 and 14 days

to replicate warm storage conditions. Additionally, samples were stored at 20 °C ± 1 °C and 4 °C ± 1 °C.

In some cases, a sensory panel is used in combination with instrumental and physicochemical methods to identify and quantify the aroma descriptors in beer. In the study of Schubert *et al.* [11], the authors analysed the evolution of some volatiles present in lager and India pale ale (IPA) in different storage conditions (room temperature, cold storage, and forced ageing) to understand how the ageing process influences the chemistry and flavour of hoppy ale-style beers. The authors used gas chromatography and physicochemical and sensory analyses to evaluate the samples. The authors concluded that noticeable increases in certain staling aldehydes were observed during storage. Moreover, the concentration of some volatiles in the hop aroma, such as terpenoids (e.g., linalool and geraniol), remained relatively constant throughout storage, mitigating the perception of "oxidised" qualities in ales with high concentrations of these compounds. By contrast, the hop aroma in certain ales was influenced by more volatile compounds, such as esters, known for their lower stability. Wauters *et al*. [12] tracked the evolution of 41 key aroma compounds during the ageing of a re-fermented beer using gas chromatographic techniques and sensory analysis. The authors used headspace gas chromatography with flame ionisation detection (HS-GC-FID) and HS-SPME-GC-MS to quantify esters and alcohol, as well as aldehydes and terpenes, respectively. The sensory analysis performed was a similarity test via a set of forced-choice triangle tests in a randomised block design. Barnet and Shellhammer [13] evaluated the impact of dissolved oxygen and ageing on dry-hopped aroma stability in beer using gas chromatography and sensory analysis to understand the degree to which this dissolved oxygen affects the chemistry of dry-hopped beer. In the study, commercially brewed dry-hopped beers were dosed with oxygen to create a range of dissolved oxygen concentrations from approximately 40 to 250 μg/L and then stored at 3 °C and 30 °C. In addition to physicochemical analysis, GC–MS was used to measure the hop aromas, and a trained panel was used to describe the samples. Another example

of using these techniques to evaluate the aroma profile during ageing can be found in Saison *et al.* [14], where the effect of 26 staling compounds on the flavour of aged beer was studied by determining their threshold values to assess the impact of these chemical compounds on beer flavour. The authors used an untrained and a trained panel to determine the threshold of added substances and headspace solid-phase microextraction coupled to gas chromatography–mass spectrometry (HS-SPME/GC–MS) to determine the concentration of flavour compounds. Additionally, a trained panel of eight members was used to perform a sensory analysis of fresh and forced-aged beers. In a previous study [15], we applied gas chromatography with mass spectrometric detection (GC–MS) in combination with a sensory evaluation test to monitor and compare the evolution of the aroma profile in beer stored in aluminium cans and glass bottles during the natural ageing of beer under controlled conditions of light and temperature. The results showed that through olfactometric analysis, panellists were able to distinguish each beer by the type of container. On the other hand, the results from the instrumental analysis indicated that the samples could not be distinguished based on the type of container but rather by the duration of the ageing process. A prediction model was built to determine the ageing time of the samples with an error of 1.1 months.

Due to the costly and time-consuming nature of the chromatographic and sensory analysis approaches, particularly the latter, which is susceptible to assessor fatigue and subjectivity, alternatives are sought [7, 16–18]. In this paper, we propose an alternative method centred on using an electronic nose (e-nose) based on the mass spectrometry (MS) detector. An e-nose is a device designed to mimic the human sense of odour by analysing the chemical signature of volatile compounds [19, 20]. The sensor array output is processed through pattern recognition algorithms that analyse the unique pattern of sensor responses, creating a distinctive fingerprint of the volatile fraction in the sample [21]. In the case of an MS-based e-nose, the responses are the abundances of the total fragmented ions at different mass-to-charge (m/z) ratios without prior chromatographic

separation [22]. The e-nose combined with headspace solid-phase microextraction (HS-SPME) as a sampling system provides a representative mass spectrometric fingerprint of the volatile fraction of the sample. As the MS-based e-nose has proven to be an efficient and rapid method to classify and characterise beers from different factories and brands using the aroma profile of the product [6, 23–26], in this study, we propose to investigate whether the technique is also capable of classifying beer according to the type of container, aluminium can or glass bottle, and the time of storage, fresh or aged. We also aimed to build a prediction model of the beer shelf life and compare the results with those obtained in our previous study to confirm the efficacy of this technique.

2. Materials and Methods

2.1. Samples

Since one of the aims of this study was to confirm the efficacy of the MS-based e-nose to predict each beer shelf life by comparing the results with those obtained in our previous study [15], all the samples used in both studies were of the same commercial brand and were received from the brewery company or bought in a supermarket and stored at the same time in the same storage conditions described below. The samples were analysed in three periods during their shelf life: fresh, after 6 months of ageing, and after 11 months of ageing.

For this study, a total of 108 samples of commercial lager-styled beers packaged in aluminium cans (54 samples) and glass bottles (54 samples) were sourced directly from a local brewery in optimal freshness, featured an alcohol content of 5.4% *v*/*v*, and were employed in controlled natural ageing experiments. The samples were aged for 11 months without light at 14 °C ± 0.5 °C. Eighteen samples were used in each ageing period analysis.

Additionally, forty lager beers of the same brand (20 in aluminium cans and 20 in glass bottles) were bought in optimal freshness (less than one month of packaging) from a local supermarket to have fresh samples when analysed after 6 and 11 months of storage.

Before analysis, ultrasonication at 0 °C (to avoid the loss of aroma compounds) was employed to degas all beer samples for 15 min. The preparation and analysis of each sample was performed in triplicate.

2.2. Sampling System: Headspace Solid-Phase Microextraction (HS-SPME)

The SPME holder, for manual sampling, and the StableFlex Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm fibre used in this investigation were purchased from Supelco (Bellefonte, PA, USA). Before use, all fibres underwent conditioning, and they were thermally cleaned between analyses by placing them into the GC injection port at the temperature specified by the producer.

The conditions for headspace extraction were 10 mL of sample placed into a 20 mL glass vial with 3.2 g of NaCl to achieve saturation. The vials were tightly sealed with PFTE/silicone septa in a nitrogen atmosphere. In order to establish the equilibrium of volatile compounds between the liquid and the headspace, the samples were maintained at 40 °C for 15 min. Then, the extraction and concentration of the volatile compounds using the HS-SPME were carried out under continuous magnetic stirring at 40 °C for 1 h.

2.3. MS-Based e-Nose Analysis

Beer samples were analysed using an MS-based e-nose from Agilent Technologies (Palo Alto, CA, USA), composed of a 7890B gas chromatograph system coupled to a 5777B mass spectrometer detector equipped with a high-efficiency source (HES) analyser. As the role of the gas chromatograph was to convey the volatiles from the injection port to the MS detector, the HP-5MS apolar analytical column (30 m × 0.25 mm × 0.25 μm) was kept at an appropriate temperature to ensure the rapid transfer of volatiles to the MS within less than 5 min, thereby preventing chromatographic separation. The oven temperature program, designed to transfer the volatiles to the MS within the shortest timeframe, was 70 °C (1 min), 70 °C·min^{-1} to 180 °C (2.5 min). The carrier gas was helium

with a flow rate of 1.6 mL·min^{-1}. The injection was made at 270 °C in the splitless mode for 1 min using an inlet of 1.5 mm i.d. The mass spectra were recorded by electronic impact (EI) ionisation at 70 eV with a temperature of 230 °C in the ion source and 150 °C in the mass quadrupole. The mass-to-charge ratio range (*m*/*z*) used was 50–150. Ratios below 50 *m*/*z* were excluded from the analysis to prevent interference from ethanol, the most abundant volatile compound (approx. 5%).

2.4. Chemometric Methods

The mass spectra fingerprints obtained from the analysis contain information about the whole volatile fraction of the sample and are presented on a plot illustrating the mass fragments (*m*/*z*) along the selected mass range on the *X*-axis, with the ion abundances for the mass fragments depicted on the *Y*-axis.

The data obtained were exported from the HS-SPME MS-based e-nose to an Excel table and were structured in a matrix of 53 rows (samples) × 100 columns (*m*/*z* ratios). The chromatographic values of the matrix were the values of the *m*/*z* ratios. Before the chemometric analysis, the spectra were pre-processed, as the difference between the highest and lowest peaks was significant, thus, affecting the analysis. The values of each *m*/*z* were divided by the total sum of the *m*/*z* values contained in the matrix. Then, the data were auto-scaled (i.e., mean-centred and standardised to unit variance).

First, principal component analysis (PCA) was applied to the matrix to visualise the data and to detect sample groups or trends. Then, partial least squares discriminant analysis (PLS-DA) was used to discriminate between fresh and aged beers and beer contained in aluminium cans or glass bottles. Finally, partial least squares regression (PLSR) was used to build a model relating the mass spectra to the ageing months, that is, to predict the freshness of the product during its shelf-life under optimal storage conditions and independently of the type of packaging.

All calculations were performed using PLS Toolbox v8.7 (Eigenvector Research Inc., Eaglerock, LA, USA) with MATLAB R2021a (The MathWorks, Natick, MA, USA).

3. Results

3.1. MS-Based e-Nose Analysis

The configuration of the HS-SPME sampling system is thought to maximise the sensitivity of the analysis, as SPME allows extracting and, unlike a static headspace, concentrating analytes in a single step. Although there is no strict chromatographic separation with the MS-based e-nose, there is a delay during the transfer of the volatile fraction from the injection port to the mass spectrometer. The mass spectrum is the sum of the abundances for every ion recorded in the time interval during the volatile transfer from the injector to the mass spectrometer plotted against their mass-to-charge ratios (*m*/*z*). As mentioned above, the range of *m*/*z* used was from 50 to 150 to avoid the effect of ethanol (*m*/*z* = 45 and 46) and because the main aromatic compounds in beer have fragment ions in that *m*/*z* range.

Figure 1 shows the mass spectrum of a given sample at different ageing times. In this figure, it can be observed that the mass spectra of the fresh samples from the brewery and the supermarket are different from each other. It can also be observed that the samples aged 6 and 11 months show just a slight variation in the peak area of some *m*/*z* ratios.

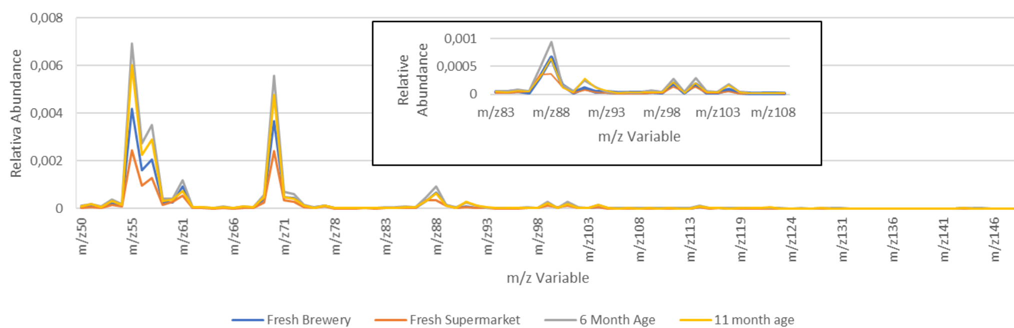

Figure 1. Comparison of the mass spectra of a given sample at different ageing times.

3.2. Chemometric Methods

3.2.1. Principal Component Analysis (PCA)

PCA was used for a preliminary observation of the MS data. This chemometric tool reduces the dimensionality of the original data matrix by compressing the information into a few new variables called principal components (PCs), which helps to reveal groups and trends in the data and highlight outlier samples. The two main plots obtained from PCA are the score and loading plots, which show the projection of the samples and variables onto the new PC space, respectively.

A first PCA was applied to the complete dataset and revealed the presence of outlier samples. After careful inspection of the data, we observed that the spectrum of some samples was different (lower abundance values) from other samples in the same group, so we decided to remove these samples and perform the analyses. Figure 2 shows the PCA score plot of the new dataset for the first two PCs.

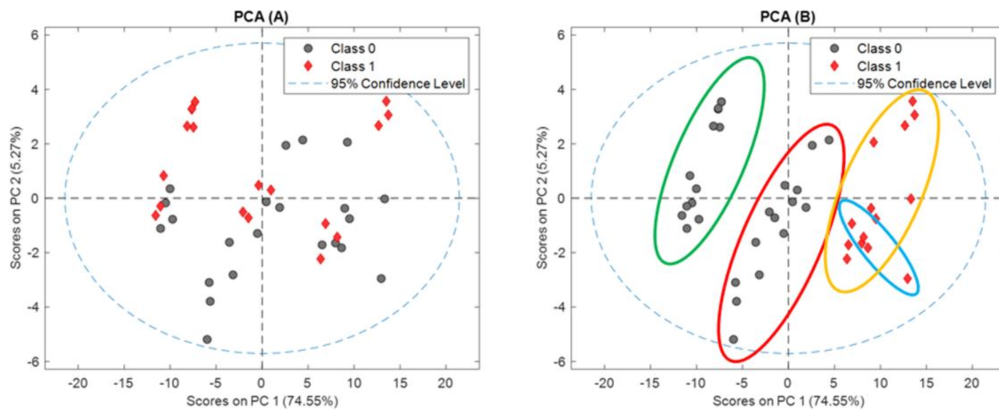

Figure 2. Score plot of the first two principal components in the PCA model (**A**): Aluminium can (Class 0) *vs* glass bottle (Class 1). (**B**): Fresh samples (Class 0) *vs* aged samples (Class 1). Green ellipse: fresh samples bought in a supermarket; red ellipse: fresh samples from a brewery; yellow ellipse: 6–month aged samples; blue ellipse: 11–month aged samples.

It can be observed that the two first PCs can explain 80% of the original information. In Figure 2A, we can observe no grouping between the aluminium cans or glass bottles.

On the other hand, in Figure 2B, the first principal component (PC1) shows a separation between the fresh and aged samples: The fresh samples from the brewery resided more in the centre, and the fresh samples bought in a supermarket were more to the left of the plot. The six and eleven-month-aged samples on the right side of the plot appear to cluster together.

3.2.2. Partial Least Squares Discriminant Analysis (PLS-DA)

Based on the promising PCA results, where some differentiation was observed between samples according to ageing time, we decided to apply PLS-DA to check if we could discriminate the data set between samples packaged in aluminium cans and glass bottles and between fresh and aged samples.

PLS-DA is a discriminant method that is based on the partial least squares regression (PLSR) algorithm described below. To classify the samples, the PLS-DA model is built by correlating the matrix **X** of predictor variables (abundances at the different *m*/*z* ratios in this case) with a vector **y** of dummy variables, zeros and ones, in a two-class problem, as in this work. In the first case, a value of 0 was assigned to aluminium cans and 1 to glass bottles. In the second case, the value 0 was assigned to the fresh samples and 1 to the aged samples. The PLS-DA models were built with auto-scaled spectra and validated using the random subsets cross-validation technique. The optimal number of latent variables (LVs) of the model was determined based on the minimum number of misclassified samples in the cross-validation set, resulting in models with two LVs (model fresh *vs*. aged samples) and two LVs (model aluminium cans *vs*. glass bottles). Figure 3 shows the results of the classification models. Figure 3A shows that the PLS-DA model could not discriminate between samples packaged in aluminium cans and glass bottles, with 14 misclassified samples in the validation set. However, the model in Figure 3B shows that PLS-DA allows discriminating fresh from aged beers, with a 100% correct classification for the validation set of samples.

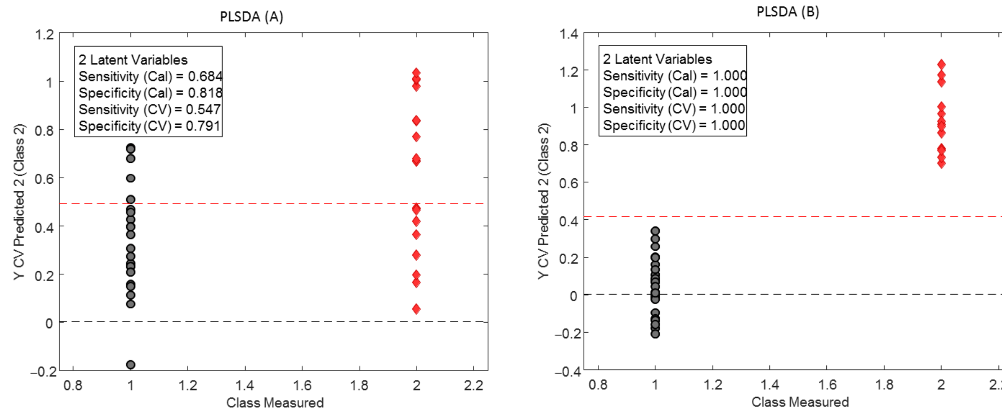

Figure 3. Results of the PLS-DA models. (**A**) Aluminium can (grey) *vs* glass bottle (red); (**B**) Fresh samples (from brewery and supermarket) (grey) *vs* aged samples (6 and 11 months aged) (red).

A classification model was attempted to classify the samples aged 6 and 11 months, motivated by the observation that these samples tended to cluster together (PCA shown in Figure 2B). However, the accuracy of this classification model was not satisfactory.

3.2.3. Partial Least Squares Regression (PLSR)

Finally, in order to predict beer shelf life, PLSR was used. The best model was found using three ageing classes: Class 1: 0 months (fresh samples from a brewery), Class 2: 1 month (fresh samples from a supermarket), and Class 3: 6 and 11 months aged samples. A regression model was built between the **X**-matrix (abundances of the different *m*/*z* ratios) and a **y**-vector containing the ageing classes: Class 1, Class 2, and Class 3. The model was cross-validated using random subsets. The optimal number of latent variables (LVs) of the model was determined based on the minimum value of the prediction error for the cross-validation set and expressed as the root mean square error of cross-validation (RMSECV):

$$\text{RMSECV} = \sqrt{\frac{\sum_i^{n_t}(y_{t,i} - \hat{y}_{t,i})^2}{n_t}}$$

$\hat{y}_{t,i}$ are the months predicted by the models, $y_{t,i}$ is the actual months, and n_t is the number of samples in the cross-validation set. RMSECV is an estimation of the average error to be expected in future predictions when the calibration model is applied to new samples.

Figure 4 shows the plot for the predicted *vs*. actual values for the validation set and some model parameters. The average prediction error to be expected for ageing time using this model is around 0.46 months for a model with three LVs. This means that a beer less than 11 months old could be safely predicted to be within its shelf life (1 year).

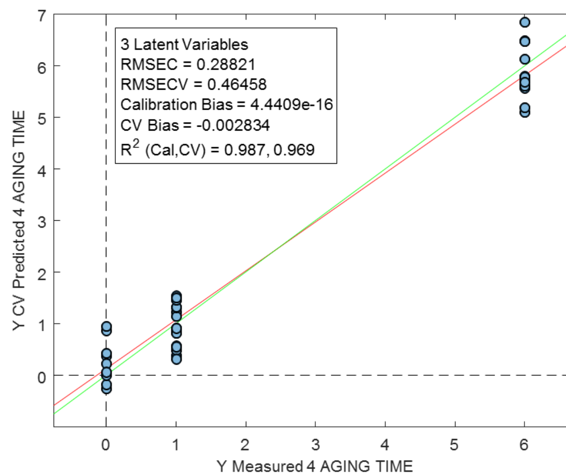

Figure 4. Plot of predicted *vs*. actual values for the validation set and some parameters of the PLSR model. Class 1: 0 months (fresh samples from a brewery); Class 2: 1 month (fresh samples from a supermarket); Class 3: 6- and 11 months-aged. Red line: regression line. Green line: target line.

4. Discussion

By observing the chemometric results of the PCA in Figure 2, it could be seen that the samples clustered according to their freshness but not according to the type of packaging used (aluminium can or glass bottle), as in our previous study mentioned above. The mass spectra of the samples in Figure 1 exhibit slight variations corresponding to the degree of product ageing. This trend was assessed and verified by the PLS-DA models. Two PLS-DA models were developed to classify samples based on their type of packaging (aluminium can and glass bottle) and their shelf-life ageing (fresh *vs* aged).

The results showed that the PLS-DA model can classify samples based on their ageing with a success rate of 100% but cannot classify the samples based on their type of packaging, misclassifying 35% of the samples.

During beer shelf life, the balance between the aromatic compounds of the volatile matrix changes; thus, the aromatic profile of a freshly packaged beer is not the same as that of a beer that has been stored [27]. Beer flavour begins to decline almost immediately after the production process concludes [28]. According to the literature [28–31], the changes during storage in the volatile matrix of a beer that leads to the degradation of its aroma are directly related to the storage conditions of light and temperature. Paternoster *et al.* [31] created a model to simulate the impact of temperature and time on the sensory quality of lager beer. The authors analysed data from lager beers stored between 20 and 30 °C for up to 365 days. The results indicate that at the lowest temperature, the degradation of the quality of a lager beer occurs to some extent. On the other hand, at 30 °C, the sensory quality of the product degrades faster, thus, reducing its shelf life. This could explain the difference between the fresh samples from the brewery and those bought in the supermarket. The samples delivered from the brewery were kept in darkness at a controlled temperature of 14 °C ± 0.5 °C. In contrast, samples bought in the supermarket were kept in the conditions of light and temperature imposed by the supermarket, that is, exposed to light and kept at room temperature (20 °C ± 0.5 °C). Therefore, varying storage conditions can result in different sensory attributes.

Beer flavour evolves over time due to chemical reactions that involve the consumption of reactants. The speed of this process diminishes as the available reactants become restricted following a period of ageing [31]. This observation could explain the pattern of grouping observed in the PCA plot—Figure 2B—for the six- and 11-month samples. The alterations in the volatile composition of the samples after six months of ageing could have been less pronounced compared to the changes observed in the samples up

to that point. Consequently, this led to the volatile composition of the 11-month samples resembling that of the samples aged 6 months.

The evolution and stability of beer flavour during its shelf life and the speed with which this evolution occurs depend on factors such as raw materials, content of dissolved oxygen, light, and temperature. Although aluminium cans and glass bottles share the common purpose of protecting and preserving the product's quality, they inherently differ in terms of material composition, UV light protection, oxygen pick-up, and permeability [27,32,33]. According to Fromuth *et al.* [27], the differences between aluminium cans and glass bottles affect the potential risk of numerous chemical reactions during ageing, considering the type of container as a variable that affects beer flavour stability. They studied the impact of the type of container on beer stability by employing a non-targeted metabolomic strategy in two relevant styles of craft beer—amber ale and India pale ale (IPA). Notable differences between cans and bottles were found in amber ale beer, whereas such differences were not observed in IPA beer, leading to the conclusion that the influence of packaging depends on beer style. The results from this study help clarify the reason for the non-distinction between cans and bottles in our chemometric study. This stands in contrast to other studies involving the lager-styled beers [32,33], where beers in aluminium cans and glass bottles exhibit significant differences in the volatile matrix during ageing. In other words, the manner in which beer undergoes changes throughout its shelf life in various types of containers is influenced by storage conditions, the brewing process, and the raw materials used in the style of beer.

The PLSR model was implemented to predict the beer shelf life. It showed a determination coefficient of $R^2 = 0.967$ and could predict the ageing time of a beer with an error of around 0.4 months. This prediction error is much lower than the 1.1 months obtained in our previous study. The results of the PLSR model showed the potential application of the MS-data e-nose in assessing the freshness of a beer during its shelf life.

UNIVERSITAT ROVIRA I VIRGILI
EVALUATION OF BEER SENSORY QUALITY AND SHELF LIFE STABILITY USING MULTIVARIATE ANALYSIS TECHNIQUES
Ana Carolina De Lima E Silva

5. Conclusions

Conventional methods for the comprehensive characterisation and prediction of beer shelf life are both time-consuming and expensive, needing the expertise of experienced professionals. In this study, we introduce a swifter and more efficient method, using an MS-based e-nose and multivariate analysis, to classify beers according to packaging type or freshness and to forecast the beer's shelf life by examining alterations in the volatile matrix of the samples. PLS-DA was used to classify the samples into fresh and aged with very good classification results, and PLSR was used to create a model to predict beer shelf life. The results showed that MS-based e-nose coupled with multivariate analysis seems to be a rapid, efficient, and effective tool for distinguishing between beer samples based on their storage duration and predicting the shelf life of samples.

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ARTICLE 4: MULTIVARIATE ANALYSIS OF THE INFLUENCE OF MICROFILTRATION AND PASTEURIZATION ON THE QUALITY OF BEER DURING SHELF LIFE

Article 4: Multivariate analysis of the influence of microfiltration and pasteurization on the quality of beer during its shelf life

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Abstract

Gas chromatography-mass spectrometry (GC-MS), physicochemical and microbiological analysis, and sensory descriptive evaluation, together with multivariate analysis were applied to evaluate the efficiency of the microfiltration and pasteurization processes during beer shelf life. Samples of microfiltered and pasteurized beer were divided into fresh and aged. A forced aging process was applied, which consisted of storing fresh samples at 55° C for 6 days in an incubator and then keeping them at ambient conditions prior to analysis. Physicochemical analysis showed that both microfiltered and pasteurized samples presented a slight variation in apparent extract, pH and bitterness. The samples that underwent heat treatment showed a lower color value than the microfiltered ones. Chromatographic peak areas of vicinal diketones increased in both fresh and aged samples. The results of the microbiological analysis revealed spoilage lactic acid bacteria (*Lactobacillus*) and yeasts (*Saccharomyces* and *non-Saccharomyces)* in fresh microfiltered samples. In the GC-MS analysis, furfural, considered by many authors as a heat indicator, could only be detected in the samples that underwent forced aging and could not be detected in the samples that passed through the thermal pasteurization process. Finally, in the sensory analysis, the panellists found differences in the organoleptic properties of the fresh microfiltered samples in relation to the rest of samples.

Keywords: microfiltration, pasteurization, forced aging, microbiological stability, colloidal stability, flavour stability

1. Introduction

Beer is an alcoholic beverage obtained from the fermentation of sugars (mainly maltose) extracted from the barley malt during the mashing. The quality and shelf life of beer are determined mainly by its appearance, aroma, flavour and texture, and are conditioned by its microbiological, colloidal and flavour stability [1, 2]. To achieve and maintain beer stability, it is necessary to eliminate any form of biological contamination in the brewing process [3]. The main sources of beer spoilage organisms in a brewery include air, soil, water, raw materials, malt, pitching yeast, brewery equipment and machinery. According to Spedding [4], the spoilage caused by microorganisms includes turbidity, haze formation, 'rope' formation, over-attenuation, gushing, souring of wort and beer and production of different off-flavours.

Due to globalisation and the growing export of beer, shelf-life problems have become an important issue for breweries. Despite that, some parameters in beer composition such as alcohol content, low pH, low nutrients and reduced oxygen content make it an inherently stable product. To achieve microbial stability and to guarantee product quality during its shelf life, breweries must adopt measures such as microfiltration and/or pasteurisation [5, 6]. Nowadays, in some craft breweries beer is microfiltered to achieve microbiological and colloidal stability. On the other hand, the brewing industries apply thermal treatments such as pasteurization at the end of the process to guarantee microbiological stability and extend the shelf life of the product.

The purpose of filtering beers is to clarify the product by removing yeast cells, some colloidal particles and hop residues without causing any significant change in the quality of the final product. On the contrary, the pasteurization of beers aims to reduce the microbial and enzymatic activity found in the final product, considerably extending its shelf life but at the expense of changes in the sensory profile.

Microfiltration is a membrane filtration process that consists of flowing a particle-rich liquid through a polymeric membrane designed to have pore diameters of 0.2-1.3 μm [7,

8]. The membranes can retain yeast cells and beer spoilage bacteria that have 5-10 μm and 0.45 μm, respectively, achieving microbiological stability [7]. Moreover, colloidal stability can be achieved by removing other large particles, such as polyphenols and protein flakes, which appear as a 'chill haze' at low temperatures in the clarified beer. According to dos Santos *et al.* [8], microfiltration is used for filtration and pasteurization of beers, properly retaining yeast cells, spoilage bacteria, polyphenols and protein flakes, avoiding significant changes in the sensory characteristics. It has been shown that microfiltration could provide flavour stability and at least six months of shelf life, avoiding the costs associated with conventional pasteurization [8].

Pasteurization is a thermal process that aims to inactivate yeast cells and spoilage microorganisms. Pasteurization allows the beer to remain stable for a longer period, thus increasing its shelf life. The intensity of this thermal process is measured in Pasteurization Units (PU), where 1 PU is defined as 1 minute treatment at 60° C [9]. Bottled/canned beers are generally pasteurized in pasteurization tunnels, which consist of progressively hotter zones, holding zones and progressively cooler zones to ensure microbiological control of the product and maintain most of its nutritional value and sensory properties [8, 10]. According to the literature, the application of a minimum of 15 PU is sufficient to achieve practical sterility regarding brewers yeast, *Pediococcus* sp., *Lactobacillus* sp., and wild yeast [6, 9].

In general, both microfiltration and pasteurization have advantages and disadvantages. The advantages of microfiltration include eliminating the use of filter aids, reducing beer losses, high solids handling capacity, and replacing thermal pasteurization for better product quality and cost saving [8, 11]. On the other hand, some authors argue that, on a large scale, this process still presents considerable technical and economic barriers, including severe membrane fouling, quality variation among different beer brands in the same membrane system and inconsistent quality of the product filtered in a system of membrane [7, 8, 11]. However, microfiltration is being used more and more by breweries with quite satisfactory results. Regarding pasteurization, despite favouring

microbiological stability and extending beer shelf life, this process presents some disadvantages. According to Stuart and Priest [12], pasteurization accelerates colloidal haze formation and breaks the stability between high-molecular-weight proteins and polyphenols. Additionally, pasteurization is one of the main causes of changes in the sensory properties affecting the qualitative characteristics of a beer [13].

Since the thermal process can have a negative impact on beer quality, some studies have reported the effect of this process on different beer quality characteristics. Cao *et al*. [1] analysed the impact of pasteurization intensity units (2 PU, 8 PU and 14 PU) on beer ageing and flavour stability during a 6-month storage period at room temperature. The key factors examined were colour, thiobarbituric acid (TBA) index, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, bitterness and total polyphenol composition, the concentration of beer volatile compounds, and 5-hydroxymethyl furfural (5-HMF). The authors concluded that, statistically speaking, the 14 PU samples exhibited the greatest flavour change and the highest degree of damage.

As the beer market is in steady expansion and consumers are more concerned about product quality, the brewing industry needs to offer a product that can maintain stability and the best organoleptic characteristics during its shelf life. In this study, the influence of microfiltration and pasteurization on the quality and stability of beer during its shelf life was compared in fresh and forced-aged beers. This research aimed to evaluate the efficiency of both processes during beer shelf life, correlating the main parameters that influence the sensorial quality and stability of the product, such as microbiological, colloidal and flavour stability, by using multivariate analysis.

2. Materials and Methods

2.1. Beer Samples

In total, 136 samples of a commercial Lager beer from the same batch, with an alcohol content of 4.8% v/v and packaged in glass bottles, were delivered directly from a local brewery of Belo Horizonte (Brazil), in the freshest possible conditions. Of them, 68 were

microfiltered and 68 pasteurized. The microfiltered samples were taken off the bottling line after the capping phase and pasteurized samples were kept on the line to pass through the pasteurization tunnel. For microfiltration, 0.22/0.45/1.0 μm membranes were used to ensure microbiological, colloidal and taste stability. The pasteurization process was carried out in a pasteurization tunnel for 40 min total, where samples were heated to 62 °C and then cooled back to the refrigeration temperature (4 °C). The 136 samples were divided into 68 fresh and 68 aged samples, and, in both cases, there were 34 microfiltered beers and 34 pasteurized beers. This information is summarized in Table 1. The forced aging process was performed by storing fresh samples at 55 °C for 6 days in an incubator (Incubator B.O.D NL-161.01 New Lab) and then keeping them at ambient conditions prior to analysis.

Table 1. Samples Applied Treatment*

Samples	Fresh	Forced Aging
Microfiltered	34	34
Pasteurized	34	34
Total	68	68

*: All beers were served at 10 °C during sensory analysis.

2.2. *Physicochemical Analysis*

To better understand the influence of the microfiltration and pasteurization processes on the quality of the product during its shelf life, physicochemical analyses were carried out using the official methodology indicated in section 9 of the Analytica Brewery Convention [14]. The parameters analysed were apparent extract (9.4); pH (9.35); alcohol (9.2.1); colour (9.6); IBU – International Bitterness Unit (9.8) and vicinal diketones (9.24.1) [14]. Before the analysis, all samples were degassed by ultrasonication for 15 minutes.

2.3. *Microbiological analysis*

Microbiological analysis was conducted according to the procedures described in Section 4.0 of The European Brewery Convention-Analytica Microbiologica for detecting contaminants in beer [15]. All samples were prepared and analysed in duplicate.

2.3.1 Culture media

For the detection of non-*saccharomyces* yeast, Lysine Agar (4.2.6) was used because the presence of lysine favours the development of wild yeast that does not belong to the *Saccharomyces* genus. Yeast Medium (4.2.5.1) was also used for the detection of *Saccharomyces* wild yeast, a medium with malt extract, glucose and peptone, with $CuSO_4$, because the presence of Cu inhibits the growth of culture yeasts and thus allows the growth of wild yeasts, including *Saccharomyces* [16]. For the isolation of aerobic and anaerobic bacteria, WLD (Wallerstein Laboratory Differential Medium) with cycloheximide was used to inhibit yeast growth. The medium was prepared according to the manufacturer's instruction (Difco™) by suspending 80 g of the powder in 1 L of purified water, mixing thoroughly, heating with frequent stirring, boiling for 1 minute to completely dissolve the powder and autoclaved at 121 °C for 15 minutes. To inhibit yeast growth cycloheximide was used. The colour appearance of the solution was blue to greenish-blue.

All culture media were prepared in the laboratory, sterilized in an autoclave, and distributed on a plate under sterile conditions in a laminar flow hood.

2.3.2 Sample preparation, inoculation, and plate reading

After going through the forced aging process, the beer was kept at a temperature of 20 ± 2 °C in the dark. Prior to analysis, beer containers were sprayed with alcohol before opening. All determinations were performed under sterile conditions in a vertical laminar flow hood. Direct sowing of beer was used on the culture medium, according

to the procedures described in the European Brewery Convention-Analytica Microbiologica, section 2.0 [15]. Seeding was performed using 0.1 mL of sample per plate, all in duplicate. All the inoculated culture media were incubated at a controlled temperature and atmosphere. Incubation is a necessary step, between the inoculation of the culture medium and the examination of the cells grown on the plates. The aerobic incubation, in atmospheric air, was used for the detection of non-*Saccharomyces* yeast, (Lysine Agar), *Saccharomyces* wild yeast (YM), and the aerobic count of bacteria (WL). The aerobic incubation conditions were 5 days for all samples, and the temperature used for the detection of non-*Saccharomyces* and *Saccharomyces* wild yeast was 27 ± 1 °C and for the aerobic bacteria was 37 ± 1 °C. For anaerobic bacteria count (WL), an air-tight jar with an anaerobic generator was used and the incubation process took 5 days at a temperature of 37 ± 1 °C.

At the end of the incubation period, the plates were inspected by direct visual examination. The colonies of yeast cells were identified and counted. To proceed to the differentiation of Gram-positive or Gram-negative bacteria the method applied was the Gram Staining (2.3.6.1) described in The European Brewery Convention Analytica Microbiologica [15]. Gram staining depends on differences in cell wall structure. The results may show a permanent blue or violet colour indicating gram-positive bacteria or a red or pink colour indicating a gram-negative bacteria. Once the Gram staining test was performed, the Catalase Test (2.3.7) for differentiating catalase-positive from catalase negative bacteria, was carried out with a 3% v/v hydrogen peroxide solution [15].

2.4 Gas Chromatographic analysis

2.4.1 Sample Preparation

The optimal conditions that allowed the extraction of the largest number and intensity of odorants were achieved by placing 50 mL of sample into a 250 mL round bottom flask, with 50 μL of a solution of 400 mg L^{-1} of 5-nonanol in distilled water as internal standard

(final concentration 0.4 mg L^{-1}). Samples were distilled until 10 mL of distilled solution were obtained and then injected into the GC-MS. All samples were degassed by ultrasonication prior to the analysis and were analysed in quintuplicate.

2.4.2 Gas Chromatographic Analysis

Samples were analysed with a GC-MS equipment, model Shimadzu® GCMS-QP2010 with an automatic injector. It was composed of an HP 7690 gas chromatograph and a 5977B HES mass spectrometric detector equipped with a turbo pump and a high-efficiency ion source. To carry out the chromatographic separations an Elite-WAX (30 m x 0,32 mm i.d., 0,25 μm film thickness) fused silica capillary column was employed. 1 μL of the distilled solution was injected in splitless mode at a temperature of 150 °C. The carrier gas used was Helium. The oven temperature was programmed as follows: the initial temperature was 30 °C; after 10 min it was raised from 15 °C min^{-1} to 150 °C and held for 2 min; finally, the temperature was raised at a rate of 15 °C min^{-1} to 220 °C and held for 5 min. The mass spectra were recorded by electronic impact (EI) ionization at 70 eV with a temperature of 230 °C in the ion source and 150 °C in the mass quadrupole. The mass range analysed in the scan mode was from 25 m/z to 200 m/z.

2.4.3 Compound Identification

The odorants detected were identified using the Automatic Mass Spectral Deconvolution and Identification System (AMDIS) using a library of mass spectral database (NIST MS Search Library version 2.3) by comparison with reference substances based on the retention index (RI) in the website of Flavornet [17].

2.5. Sensory descriptive analysis

Beer samples were sensory evaluated by a panel of 11 trained assessors, age between 30 and 50 years old, following the methodology and statistical analysis described in Sensory Analysis – Description Analysis (13.10) of the European Brewery Convention [14]. Four numbered samples, being 01 Fresh Microfiltered, 01 Fresh Pasteurized, 01

Aged Microfiltered and 01 Aged Pasteurized were presented to the panellists. Each assessment rated the intensity of descriptors on a ten-point scale (a score of 1 meant that the aspect analysed was slight, whereas a score of 10 meant that the aspect analysed was very intense). All samples were kept at 10 °C for 48 hours prior to the analysis. The attributes evaluated were described in Table 2.

Table 2. Attributes evaluated in the sensory analysis

Aspect Analysed	**Descriptor**
Visual	foam colour, beer colour, turbidity, foam size, Belgian lace and foam quality, scored from 1 to 10.
Aroma	alcoholic, sweetness, solvent, vegetables, species, nuts, toast, fruity, floral, biscuit, sulphurous, phenolic, aged, lactic acid, acetic acid, bread, butter. The panellists were asked to choose the aroma that best described the sample.
Flavour	alcoholic, sweetness, solvent, vegetables, species, nuts, toast, fruity, floral, biscuit, sulphurous, phenolic, aged, lactic acid, acetic acid, bread, butter. The panellists were asked to choose the flavour that best described the sample.
Taste	sweetness, bitterness, acidity, salty, umami, scored from 1 to 10.
Mouth sensation	body, carbonation, texture, alcohol, astringency, spicy, metallic, scored from 1 to 10.

2.6. Chemometric Analysis

The chromatographic, physicochemical and sensory information collected on the samples was used to discriminate beer samples by using chemometric classification and prediction methods. The data obtained were organized in a matrix **X** (20 x 70), where samples were placed in the rows and the columns represent the results of the integrated peak area of the 12 identified compounds, the physicochemical results for the six analyses, and the results of the 52 parameters evaluated in the sensory descriptive test. Since the differences in magnitude between the data was significant and could affect the analysis, before the chemometric analysis the values of the data matrix were pre-processed by dividing each value in the matrix by the total sum of the values contained

in the array. Then, data were autoscaled (i.e. mean centred and standardized to unit variance).

For a preliminary visualization of the data, Principal Component Analysis (PCA) was used, as this exploratory method may help to understand the correlation between variables, reveal the main differences between microfiltered and pasteurized samples and point out outlier samples.

Partial Least Squares (PLS2) regression was used to correlate data derived from instruments (GC-MS and physicochemical results) to the sensory descriptors. The data obtained from these analyses were organized in a matrix **X** (20 x 22), where samples were placed in the rows and the variables in the columns. Variables represent the integrated peak areas of the identified compounds with exception of ethanol, the physicochemical results (pH, VDK, colour, IBU and apparent extract), and the results of the sensory descriptive test (foam colour, turbidity, foam size, foam quality, lactic acid, biscuit, bread, and acidity). Partial Least Squares Discriminant Analysis (PLS-DA) was finally applied to classify the samples according to the conservation process applied, helping to understand which are the main parameters/compounds that make this differentiation possible. All the analyses were performed using the software MATLAB (MathWorks, Natick, MA, USA) supported by the PLS Toolbox 5.2.2 (Eigenvectors Research Inc., Manson, WA, USA).

3. Results

3.1. Physicochemical Analysis

Physicochemical analysis was performed to better understand the influence of the microfiltration and pasteurization processes on the quality of the final product during its shelf life. Figure 1 shows the physicochemical parameters studied.

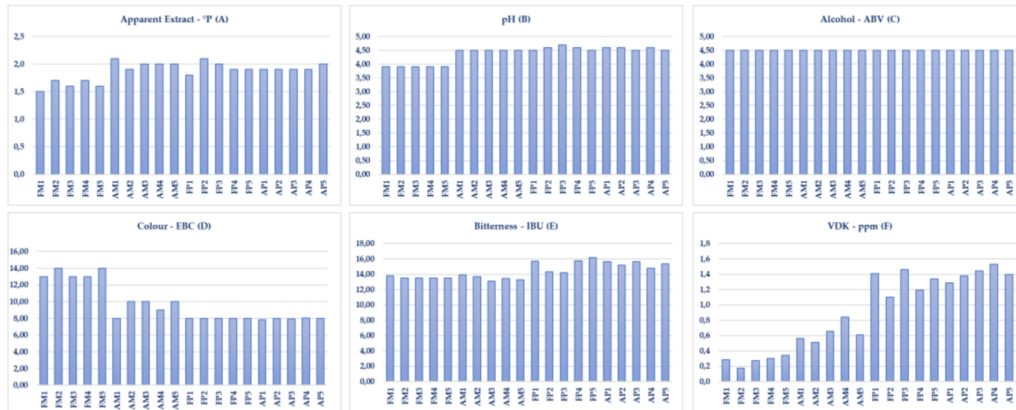

Figure 1. Physicochemical analyses of the beers studied: Apparent Extract °P (A), pH (B), Alcohol ABV (C), Colour EBC (D), IBU (E), VDK ppm (F). FM: Fresh Microfiltered; AM: Aged Microfiltered; FP: Fresh Pasteurized; AP: Aged Pasteurized.

From the analysis of the physicochemical results, we can observe that the values of apparent extract (A) and pH (B) of fresh microfiltered samples are slightly lower than those of the other samples. The alcohol (C) remains constant in all samples. Beer colour (D) decreased after heat treatment and this decrease was less affected by the forced aged process than by the pasteurization process. IBU (E) presented a slight variation between both microfiltered and pasteurized samples. Finally, VDK (F) showed a significant increase after heat treatment, especially between the fresh microfiltered and fresh pasteurized samples. Table S1 in Supplementary Material shows the ANOVA (Analysis of Variance) results for the physicochemical analysis of the different samples. The table shows that the samples of pasteurized beer, both fresh and aged, did not present significant differences between them for any parameter analyzed, with the exception of pH in which the AP samples showed similarities with the FP and AM samples. The microfiltered samples showed significant differences in apparent extract, colour, pH and VDK. Despite the slight variation in the apparent extract of AM samples shown in Figure 1, the ANOVA results indicate that AM samples and both pasteurized samples (FP and AP) are similar in terms of apparent extract.

3.2. Microbiological Analysis

The microbiological analysis was performed to assess the efficiency of both microfiltration and pasteurization processes regarding the microbiological stability of the product. For that, detection of *Saccharomyces* and non-*Saccharomyces* yeasts, isolation of aerobic and anaerobic bacteria, and the Gram staining and catalase tests were performed. The results of the microbiological analyses are shown in Table 3.

Table 3. Results of microbiological analyses. FM1 - Fresh Microfiltered sample 1; AM – Aged Microfiltered sample; FP – Fresh Pasteurized sample; AP – Aged Pasteurized sample. * Spoilage was not detected in any sample.

Samples	Analysis	YM (*Saccharomyces* yeast) CFU/ 10^6	LYSINE (non-*Saccharomyces* yeast)	WL (aerobic incubation) cfu per	WL (anaerobic incubation) CFU per	Gram Staining Test	Catalase Test
FM1	1	1	5	>300/>300	>300/>300	+	-
	2	2	3	>300/>300	>300/>300	+	-
FM2	1	2	2	>300/>300	>300/>300	+	-
	2	1	2	>300/>300	>300/>300	+	-
FM3	1	1	1	>300/>300	>300/>300	+	-
	2	5	0/0	>300/>300	>300/>300	+	-
FM4	1	3	0/0	>300/>300	>300/>300	+	-
	2	5	4	>300/>300	>300/>300	+	-
FM5	1	2	2	>300/>300	>300/>300	+	-
	2	8	2	>300/>300	>300/>300	+	-
AM* FP* AP*	1	0/0	0/0	0/0	0/0	0/0	0/0
	2	0/0	0/0	0/0	0/0	0/0	0/0

According to the results of the microbiological analysis (Table 3), beer spoilage bacteria and yeasts were only detected in the microfiltered fresh beer samples. The plates of YM and Lysine were analysed visually to count the colonies and then evaluated

microscopically. Some non-*saccharomyces* colonies were detected on some YM plates. All fresh microfiltered samples showed colony-forming units superior to 300 colonies/0.1 mL for both aerobic and anaerobic bacteria. All bacteria detected are Gram-positive and catalase negative being the most predominant the latic acid bacteria, *Lactobacillus* and *Pediococcus* [18]. Microscopic analysis of the samples suggests that the bacteria detected could be from the genera *Lactobacillus*. The other samples that went through thermal treatment, both aged and pasteurized samples, did not show any spoilage of bacteria or yeasts.

3.3. GC-MS analysis

The results of the GC-MS analysis show the evolution of the volatile compounds present in both fresh and aged samples of microfiltered and pasteurized beers. Table 4 shows the 12 volatile compounds identified in microfiltered and pasteurised beers.

Table 4. GC-MS compound identification of both microfiltered and pasteurized beer samples**.** FM - Fresh Microfiltered; AM - Aged Microfiltered; FP - Fresh Pasteurized; AP - Aged Pasteurized.

N.	Name	CAS	ODOR[17]	FM	AM	FP	AP
01	ethanol	64-17-5	sweet	X	X	X	X
02	2-methyl,1-butanol	137-32-6	malt	X	X	X	X
03	3-methyl,1-butanol	123-51-3	whiskey, malt	X	X	X	X
04	furfural	98-01-1	bread, almond, sweet	-	X	-	-
05	acetic acid	64-19-7	sour	X	X	X	X
06	2,3-butanediol	513-85-9	creamy, buttery	-	X	-	-
07	methyl benzoate	93-58-3	prune, lettuce, herb, sweet	X	-	X	X
08	β-phenethyl acetate	103-45-7	rose, honey, tobacco	X	X	X	X
09	caproic acid	142-62-1	sweet	X	X	X	X
10	2-phenylethyl alcohol	60-12-8	honey, spice, rosa, lilac	X	X	X	X

11	octanoic acid	124-07-2	sweet, cheese, rancid	X	X	X	X
12	benzoic acid	65-85-0	urine	X	-	X	X

The results of the GC-MS analysis showed the presence of furfural and 2,3-butanediol only in the AM samples. In the same samples, methyl benzoate and benzoic acid are absent.

3.4. Sensory descriptive analysis

Data from the descriptive analysis were evaluated according to the conservation process imposed, microfiltration or pasteurization, and its efficiency regarding organoleptic quality and stability of the product during its shelf life (fresh and aged). Figure 2 shows the overall impression of the sensory analysis.

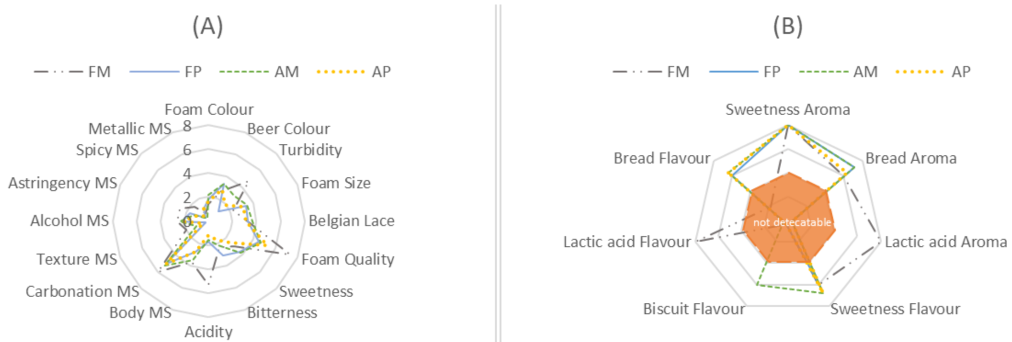

Figure 2. Overall Impression of Sensory Analysis. Visual Description, Mouth Sensation and Taste Description (A); Aroma and Flavour Description (B). FM - Fresh microfiltered; FP - Fresh Pasteurized; AM - Aged Microfiltered; AP - Aged Pasteurized; MS - mouthfeel sensations.

As can be seen in Figure 2, Fresh Microfiltered (FM) samples showed some differences in all descriptive analyses performed by the panellists. For visual description - Figure 2 (A), FM was qualified as the sample with the best foam quality and more turbid. For aroma description - Figure 2 (B), FM showed three compounds, including lactic acid, sulphurous and phenolic, which were not perceived with the same intensity in the other samples. For flavour description – Figure 2 (B), lactic acid and phenolic flavours were

perceived more strongly in FM samples. Finally, for taste analysis and mouthfeel sensation – Figure 2 (A), panellists described the FM samples as more acidic, spicier and more astringent than the others.

3.5. Chemometric Analysis

Principal Component Analysis (PCA) was applied to the chromatographic data matrix and the physicochemical results. Figure 3 shows the loading plot (A) and the biplot (B) of the chromatographic data and physicochemical results (scores and loadings) for the first two principal components of the PCA, which explains around 60% of the total variance in the data. In the plot, the correlation between the physicochemical results and the fresh and aged samples of both microfiltered and pasteurized conservation processes can be observed.

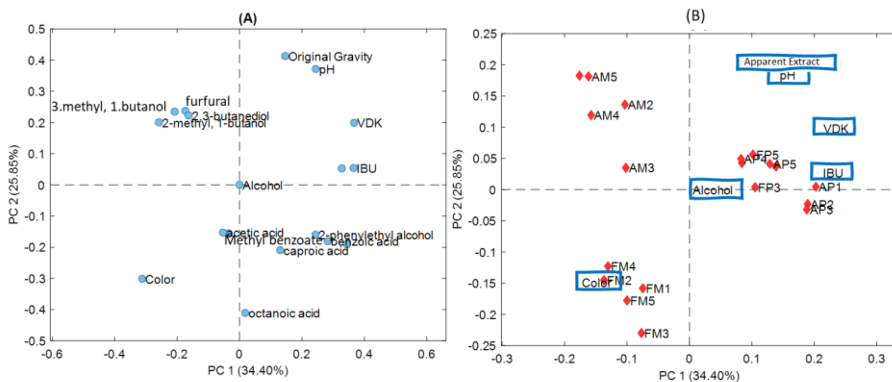

Figure 3. (A) Loading plot of PCA analysis of the chromatographic data and physicochemical results for both Microfiltered and Pasteurized samples; (B) PCA biplot of the chromatographic data and physicochemical results of samples and physicochemical results: Apparent Extract, pH, Alcohol, Colour, IBU, VDK. FM: Fresh Microfiltered; AM: Aged Microfiltered; FP: Fresh Pasteurized; AP: Aged Pasteurized.

Although the sample size (20 beers) was certainly not large, at least on an exploratory level some trends were detected. Figure 3 (A) shows the loading plot of the PCA. It can be observed that furfural (4) and 2,3-butanediol (6) are positively correlated to the AM samples. Methyl benzoate (7) and benzoic acid (12) had a positive loading on PC1, suggesting that it was positively correlated to the pasteurized samples and negatively

correlated to the microfiltered samples. Octanoic acid (11), which showed a decrease in the peak area in pasteurized and forced-aged samples, had a negative loading in PC2, suggesting that it was positively correlated to fresh microfiltered samples. Furfural (4), which is considered for some authors, such as Aron and Shellman [19] and Saison et. al. [20], as an indicator of thermal treatment, has a positive loading on PC2, suggesting that it was positively correlated to the samples that went through heat treatment.

The biplot in Figure 3 (B) shows that the group of microfiltered and pasteurized samples, both fresh and forced aged, have an opposite correlation on PC1. In the same figure we can observe that pasteurized samples (FP and AP) are more grouped than the microfiltered ones (FM and AM), which show more dispersion.

Table S2 in the Supplementary Material shows the ANOVA results for the GC-MS analysis of the different samples. It can be seen that, with the exception of the AM samples where these compounds could not be identified, all the samples show some similarities regarding the compounds 2-methyl 1-butanol (2), 3-methyl, 1-butanol (3) methyl benzoate (7), and 2-phenylethyl alcohol (10). For some compounds such as acetic acid (5) and benzoic acid (12) samples did not present any significant difference regardless of whether they were microfiltered or pasteurized. On the other hand, for octanoic acid (11) the microfiltered and pasteurized samples show differences between them.

By analyzing the correlation between physicochemical results and samples, we can observe that colour has a negative loading in PC1, suggesting that it is positively correlated to FM samples and negatively correlated to AM samples. IBU, VDK, Original gravity and pH have a positive loading in PC1, indicating that they are positively correlated to both fresh and aged pasteurized samples. Alcohol appears in the center of the plot, indicating that it has a low influence in the PCA model. We can also observe in the same figure that PC1 explains the difference between microfiltered and pasteurized samples and PC2 seems to explain the difference between the samples that underwent

heat treatment AM (forced ageing with temperature), FP (pasteurization process) and AP (pasteurization and forced ageing processes) and those which not such as FM (microfiltration process).

After the preliminary PCA, we applied a regression model to observe the correlation between instrumental data and sensory descriptors. For this, PLS2 was applied by regressing the instrumental **X** data matrix (GC-MS and physicochemical results) to the **Y**-matrix of sensory descriptors. The model was leave-one-out cross-validated. Figure S2 in Supplementary Material shows the correlation loadings of the PLS2 model with all X and Y variables (GC-MS, physicochemical results, and sensory analysis). The aim was to reduce the number of variables in the data matrix, keeping only those that have more weight when analyzing the efficiency of the microfiltration and pasteurization process in relation to the sensory quality of the final product during its shelf life. Table 5 shows the variables with more influence in the efficiency of the microfiltration and pasteurization process in relation to the sensory quality.

Table 5. PLS2 - Main variables responsible for determining the efficiency of the microfiltration and pasteurization process concerning the sensory quality.

GC-MS ANALYSIS	**Physicochemical Analysis**	**Sensory Analysis**
2-methyl, 1-butanol (2)	IBU	VISUAL DESCRIPTION
3-methyl,1-butanol (3)	VDK	Foam Quality
furfural (4)	OG	Foam Size
3-methyl,1-butanol (6)	Colour	Turbidity
Methyl benzoate (7)	pH	AROMA DESCRIPTION
β-phenethyl acetate (8)		Lactic acid
2-phenylethyl alcohol (10)		FLAVOUR DESCRIPTION
Octanoic acid (11)		Bread
Benzoic acid (12)		Biscuit
		TASTE DESCRIPTION
		Acidity

Finally, to better understand the effect of the studied parameters on beer quality during ageing regarding the conservation process (microfiltration and pasteurization), after the

selection of the most influential variables, Partial Least Squares Discriminant Analysis (PLS-DA) was applied to classify samples according to the conservation process applied and to ageing (fresh or aged). The aim was to understand how the samples are classified and what are the main parameters/compounds that make this differentiation possible. First, PLS-DA was applied to classify the four classes: Class 01 (FM samples), Class 02 (AM samples), Class 03 (FP samples), and Class 04 (AP samples). In this classification model, the pasteurized samples were not distinguished from each other (results not shown), so we joined FP and AP samples and performed a classification model with three classes: Class 01 (FM samples), Class 02 (AM samples), and Class 03 (FP and AP samples). The model was leave-one-out cross-validated and the optimal number of LVs was determined based on the percentage of correctly classified samples for the cross-validation set. Figure 4 shows the Threshold/ROC plot for the classification model with three classes. It can be observed that all samples in the three classes are correctly classified.

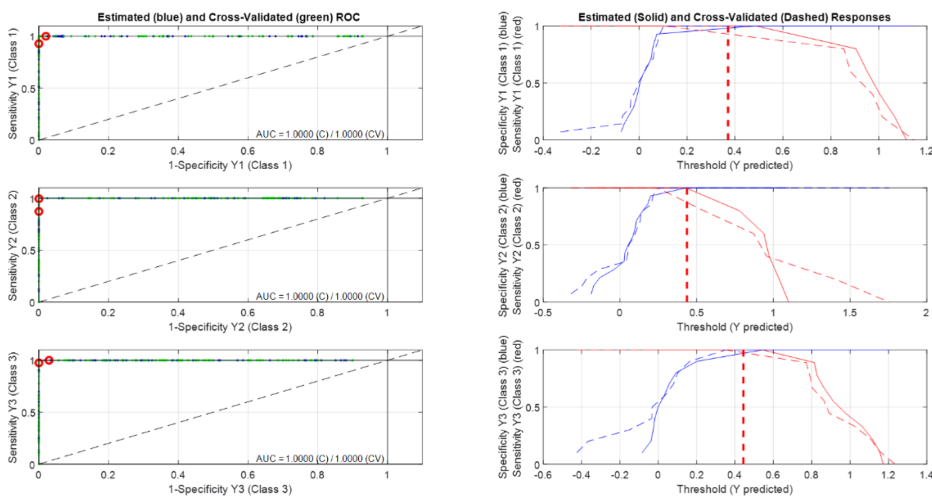

Figure 4: Threshold /ROC plot for the classification model with three classes: Class 1: FM; Class 2: (AM); and Class 3 (FP + AP).

Finally, Figure 5 shows the score and loading plots of the PLS-DA classification model.

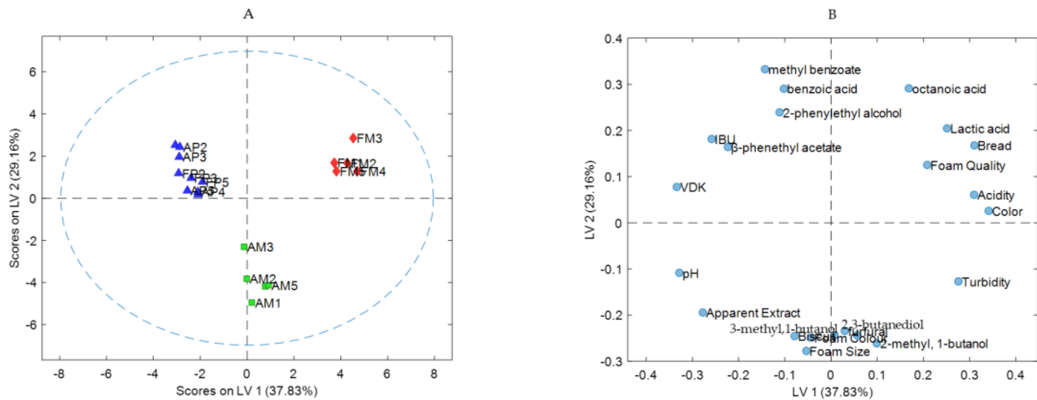

Figure 5: Score plot (A) and loading plot (B) of the PLS-DA classification model with three classes: Class 1: FM; Class 2: (AM); and Class 3 (FP + AP).

By observing the plots in Figure 5, it can be seen that 2,3-butanediol and furfural are positively related to the AM samples. Also, in the same loading plot the physicochemical parameters such as apparent extract and pH, and the sensory visual and aromatic descriptors like turbidity, foam size and colour, and biscuit respectively, are related to the forced aged microfiltered samples.

4. Discussion

Although the pasteurization process increases the colour of beer due to the Maillard reactions, in this study the pasteurized samples, both fresh and aged presented lower colour values than the microfiltered ones. This could be explained as a result of contamination due to spoilage in the microfiltered samples. Since the pasteurized samples went through thermal treatment just before the bottling process, the microorganisms that spoil the beer were inactivated before they could produce any substantial changes in the physicochemical characteristics of the product. The AM samples also went through a heat treatment to force ageing, but unlike the pasteurized ones this process was applied a few days after bottling which may have contributed to changes in some physicochemical parameters such as colour, IBU and VDK. On the contrary, the FM samples did not receive any heat treatment, which allowed the spoilage microorganisms present in those beers to act by altering the physicochemical and

sensory parameters to a greater degree than the AM samples. Analyzing the correlation loadings of the PLS2 model (Figure S1 in the Supplementary Material) it can be seen that colour is directly correlated to lactic acid (0.80), acidity (0.86), and turbidity (0.64) and inversely correlated to pH (-0.95), VDK (-0.86), IBU (-0.66) and apparent extract (-0.81). Looking at the physicochemical results, it is seen that the FM samples have lower values of VDK, IBU, and apparent extract than the other samples. This also happens in the AM samples but to a lesser degree. Additionally, FM samples are directly related to the sensory attributes lactic acid, acidity and turbidity. All these parameters are related in some way to spoilage contamination in beer. Regarding the physicochemical results of colour (Figure 1), although the FM samples had higher colour values than the other samples, in the descriptive sensory test the panellists could not detect this difference visually and scored almost all the samples equally.

Vicinal diketones (VDKs) are produced by yeasts during fermentation in presence of oxygen and enzymatically consumed in absence of oxygen. The most significant VDKs for the beer process are diacetyl (2,3-butanedione) and 2,3-pentanedione, due to their low flavour thresholds, 0.1 and 0.9 ppm, respectively, giving butter/butterscotch and toffee-like flavours to beer [21, 22]. Diacetyl, which has a significant effect on the flavour, aroma and drinkability, is re-assimilated and reduced via acetoin in 2,3-butanediol, which has a flavour threshold of around 4500 ppm [22–25]. According to the GC-MS results (Table 4), 2,3-butanediol could be detected only in the aged microfiltered (AM) samples. Because of its higher flavour threshold, the cream/buttery odour of this compound could not be detected by the panellists during the descriptive analysis, as shown in Figures 2(A). Despite the concentration of VDKs in the pasteurized samples being almost six times higher than in the microfiltered samples, neither diacetyl nor 2,3-pentanodione could be detected instrumentally or sensorially in any sample. The presence of VDKs could be explained as a result of yeast removal or inactivation before the maturation stage [26].

Despite being considered an unfavorable environment, a limited number of bacteria and yeasts are capable of growing and spoiling beer, especially if it is unpasteurized or microfiltered beer [27–29]. The microbiological analysis detected the presence of wild yeast (*Saccharomyces* and non-*Saccharomyces*) and Gram-positive bacteria (catalase negative) spoilages in the fresh microfiltered samples. The ideal microbiological situation for a beer is to have only one species of organism present in the product, namely the culture yeast pitched into the wort. Although these types of yeasts are desirable in some kinds of beer such as lambic and gueuze beer, in a lager beer wild yeasts are considered spoilage organisms and must be avoided. The *Saccharomyces diastaticus* is considered the main *Saccharomyces* spoiler in beer [30]. Contamination of beer by wild yeasts is manifested by the fermentation of residual carbohydrates, including dextrins and starch, by haze formation, by production of phenolic off-flavours and over-attenuation leading to gushing [5, 30]. This could explain the lower value of the apparent extract in the FM samples [30, 31]. The most detected non-*saccharomyces* wild yeast is *Brettanomyces*. Its presence changes the organoleptic properties of a beer due to the production of secondary metabolites when performing alcoholic fermentation [32]. Undesirable flavours such as horse sweat, barnyard, medicinal or leathery could appear when beer is spoiled by this yeast species.

According to the literature [9, 18, 28], Gram-positive bacteria are the most threatening contaminants in the brewery due to their rapid growth rate and tolerance to high temperatures and low pH. The predominant Gram-positive with catalase-negative beer spoilers are the lactic acid bacteria (LAB) *Lactobacillus* and *Pediococcus,* being responsible for almost 70% of all spoilage incidents in brewing industries [9, 18, 33]. Based on microscopic analysis, the lactic acid bacteria that spoiled the samples could be *Lactobacillus.* These bacteria produce lactic acid that lowers the pH value of beer (often below 4.3), which could explain the lower pH value of the fresh microfiltered samples (around 4.0). *Lactobacillus* could also produce turbidity, and off-flavours making beer sour via lactic and acetic acid production, which explains that in the sensory descriptive

test panellists described, in the FM samples, turbidity, lactic acid, and acidity/spicier in visual, aroma/flavour and taste description, respectively [5, 33]. In Figures 5A and B we could observe that, except for turbidity, which is more related to the AM samples, all other sensory descriptors above mentioned are positively related to the FM samples. Aged and pasteurized samples did not present any spoilage of bacteria or yeasts. Since the membranes used in the microfiltration process are capable of retaining bacteria and yeasts we could say that the source of contamination is located between the filter and the pasteurization tunnel. According to Suiker [30], most cases of spoilage involving *S. diastaticus* are related to contamination that occurs post-fermentation, most likely during filling, being biofilms the main source of this contamination. Moreover, in a review written by Suzuki [5] about the emergence of new spoilage microorganisms in the brewing industry, the author reported *Lactobacillus linderi* as one of the lactic acid bacteria that represents a potential threat to unpasteurized beers, since due to its small size it is able to more easily penetrate the sterile membrane filter.

The GC-MS results show that turbidity, mentioned as a sensory characteristic of the FM samples in sensory analysis, here is positively related to the AM samples. According to Jaluska et. al. (2018) [34], in their study about the influence of transport and storage conditions on beer quality and flavour, haze formation (turbidity) could be linked to an increase in temperature. Moreover, we could observe that furfural (4) and 2,3-butanediol (6) were detected only in the AM samples. Furfural (4) presence in aged beer seems to be directly related to sensory changes, namely flavour staling, and its increased levels seem to occur independently from oxygen concentration [26]. The chemometric analysis confirmed that both compounds are positively correlated to the AM samples. Furfural (4) is considered a heat indicator in beer and its presence almost invariably results from exposure of the beer to higher temperatures than the AM samples. According to Madigan *et al*. [35] the higher the temperature the greater the formation of furfural, which explains the presence of this compound in the force-aged microfiltered samples. On the contrary, in pasteurized samples, both FP and AP, despite having been subjected

to high temperatures, furfural (4) could not be detected in their matrix. The presence and absence of furfural (4) in the microfiltered and pasteurized samples, respectively, could be explained by the difference in the way that temperature was applied in both samples. While in force-aged microfiltered samples (AM) the temperature increase occurred abruptly (from 10 °C to 55 °C in less than 1 hour) and the samples were maintained at 55 °C for 6 days, in the pasteurized samples (FP and AP), the temperatures increased and decrease gradually in the pasteurized tunnel.

Regarding the presence of benzoic acid (12) and methyl benzoate (07) in all samples except for AM samples, it could be observed in Figures 5 A and B that both compounds have a positive loading in LV2, being positively related to the FM, FP and AP samples. The correlation matrix in Figure S1 (Supplementary Material) shows that these compounds are not related to each other.

5. Conclusions

In this paper, the influence of microfiltration and pasteurization on the quality of beer during its shelf life was analysed using Multivariate Analysis. Regarding the efficiency of the microfiltration and pasteurization process to achieve colloidal, microbiological and flavour stability to guarantee beer quality during shelf life, the results of the analyses performed showed that applying only microfiltration as a conservation method in a non-sterile-bottling process, may not be enough to guarantee the sterilization of the product and consequently its stability during shelf life. This is because contamination may happen after this step due to the presence of contaminating microorganisms in the equipment or the air. Since some microorganisms that spoil beer are resistant to its intrinsic hurdles in a non-sterile-bottling process, applying pasteurization as a preservation process is more efficient than just applying microfiltration.

It could be observed that AM samples did not present any spoilage of bacteria or yeast after the forced-ageing process. Based on this result, it can be said that the force-age process used to accelerate the ageing of a beer is capable of inactivating some

microorganisms that could influence the beer's sensory quality profile. So, we recommend further studies using natural ageing to evaluate the effects of spoilage contamination on the quality parameters of microfiltered samples during shelf life and compare them with pasteurized samples.

In conclusion, despite some disadvantages presented above in the use of heat treatments to improve the stability of the product during its shelf life, pasteurization is a conservation process recommended for its ability to inactivate the spoilage contaminants that are responsible of the rapid decline in beer quality during its shelf life.

UNIVERSITAT ROVIRA I VIRGILI
EVALUATION OF BEER SENSORY QUALITY AND SHELF LIFE STABILITY USING MULTIVARIATE ANALYSIS TECHNIQUES
Ana Carolina De Lima E Silva

Supplementary Material

Table S1. ANOVA results of the comparison of the physicochemical analysis for the different samples: FM – Fresh Microfiltered: AM – Aged Microfiltered; FP – Fresh Pasteurized; AP – Pasteurized.

Samples	Repetitions	Apparent Extract		Bitteness - IBU		Colour	
		Mean*	standard deviation	Mean*	standard deviation	Mean*	standard deviation
AM	5	2,0^{a}	0,07	13,49^{b}	0,32	9,40^{b}	0,89
FM	5	1,62^{b}	0,081	13,55^{b}	0,13	13,40^{a}	0,55
AP	5	1,92^{a}	0,04	15,33^{a}	0,37	7,95^{c}	0,08
FP	5	1,94^{a}	0,11	15,24^{a}	0,90	8,00^{c}	0

Samples	Repetitions	pH		VDK	
		Mean*	standard deviation	Mean*	standard deviation
AM	5	4,30^{b}	0	0,64^{b}	0,11
FM	5	3,90^{c}	0	0,28^{c}	0,04
AP	5	4,56^{ab}	0,05	1,40^{a}	0,07
FP	5	4,58^{a}	0,08	1,30^{a}	0,16

*By column, means with same letter are not signifficantly different according to Fisher test ($p < 0,05$).

Table S2. ANOVA results of the comparison of GC-MS data for the different simples: FM – Fresh Microfiltered: AM – Aged Microfiltered; FP – Fresh Pasteurized; AP –Aged Pasteurized.

Samples	Repetitions	2-methyl, 1-butanol		3-methyl, 1-butanol		Acetic acid	
		Mean*	standard deviation	Mean*	standard deviation	Mean*	standard deviation
AM	5	10645723[a]	425690	69872988 [a]	6845601	4361536 [a]	3040205
FM	5	8378586[ab]	951401	57915673 [ab]	8620330	4017503 [a]	991809
AP	5	6753296[b]	3137184	53411466 [b]	10064468	3431667 [a]	1633763
FP	4	7931926[ab]	2759565	59762112 [ab]	11268427	1826468 [a]	128794
Samples	Repetitio	Methyl benzoate		β-phenethyl acetate		Caproic acid	
		Mean*	standard deviation	Mean*	standard deviation	Mean*	standard deviation
AM	5	n.d.	-	363159 [b]	117157	304043[c]	42330
FM	5	15338787 [b]	7288922	322563 [b]	52044	747808 [a]	77081
AP	5	27232921 [a]	4267484	631270 [a]	101789	585589 [b]	64394
FP	4	21387463 [ab]	2492559	509323 [a]	66059	696037 [a]	53493
Samples	Repetitions	2-phenylethyl alcohol		Octanoic acid		Benzoic acid	
		Mean*	standard deviation	Mean*	standard deviation	Mean*	standard deviation
AM	5	4729680[b]	1217907	431522 [c]	93969	n.d.	-
FM	5	5684452 [ab]	1006138	2060345 [a]	698630	3274700 [a]	1837820
AP	5	6348493 [a]	485698	1057990 [b]	109607	6246513 [a]	3643163
FP	4	6083649 [a]	729478	509323 [b]	108382	3008385 [a]	287580

*By column, means with same letter are not signifficantly different according to Fisher test (p < 0,05).

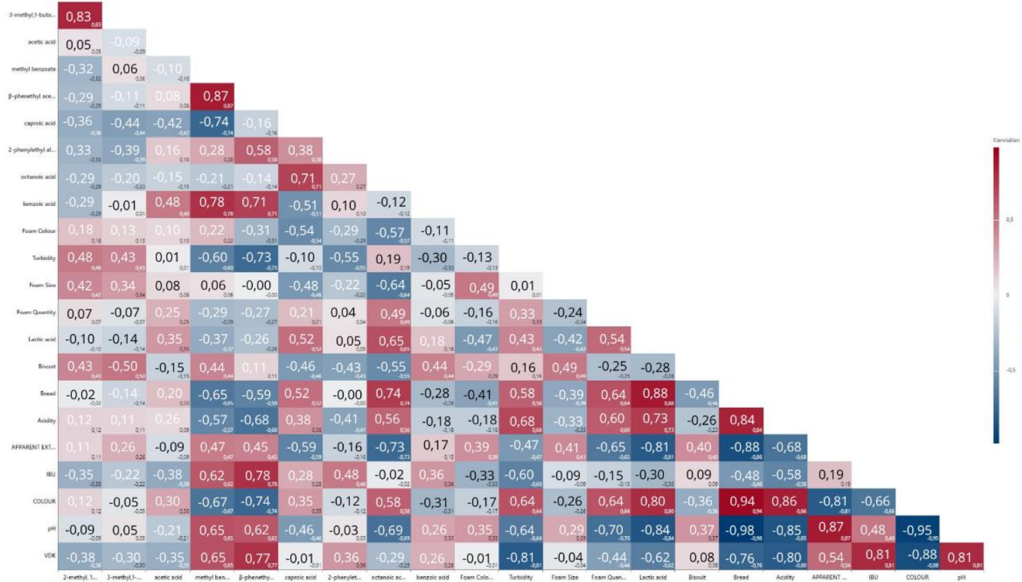

Figure S1. Correlation matrix of the physicochemical parameters, GC-MS peaks and sensory attributes.

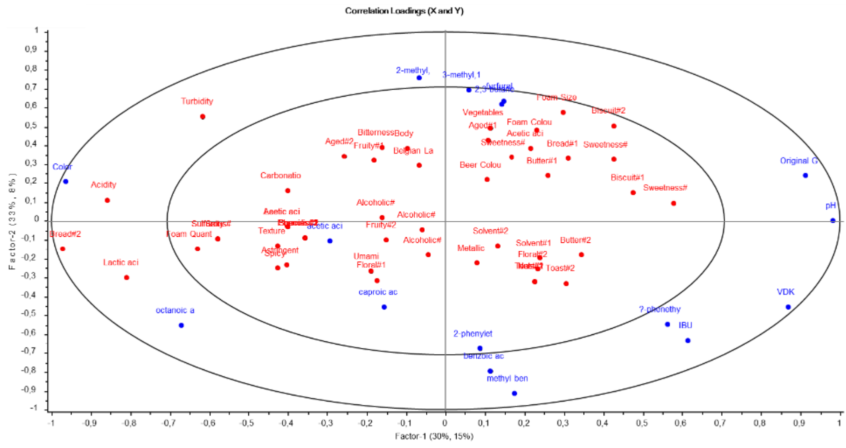

Figure S2. Correlation loadings of the PLS2 model (X variables in blue and Y variables in red).

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Chapter 6
GENERAL DISCUSSION

6.1. Introduction

Beer sensory quality is defined by its appearance, aroma, taste and texture, and it is influenced by several parameters such as raw materials, brewing process techniques and shelf life conditions [1, 2]. Freshness is a key factor in determining beer quality and is directly related to the aroma compounds present in the final product. To guarantee beer quality during its shelf life it is necessary to achieve microbiological, colloidal and sensory stability during the brewing process [3, 4].

As stated in the previous chapters, the expansion of the beer market and the rise of independent craft breweries worldwide in the last decades has led to changes not only in the beer industry but also in the perception of quality by consumers [5]. Craft breweries focus on product innovation by creating a variety of unpasteurized flavoured beers, introducing the idea of a fresh and live product. Due to the growing export of beer and especially, unpasteurized beers, guaranteeing quality during beer shelf life has become an important issue for breweries.

Since the ageing of beer is a complex phenomenon influenced by the raw materials, the brewing process and the conditions of storage and transport, the development and improvement of analytical methods to better understand and control the brewing process and shelf life of beer has become a critical factor for breweries to achieve and maintain quality and stability. The application of multivariate data analysis techniques makes it easier to extract and interpret the information from brewing databases and to manage the connections between sensory and instrument data, thus allowing the creation of predictive models that helps understand the production process and the quality of the product during its shelf life [6, 7].

6.2. Chemometric Methodologies Applied to the Evaluation of the Sensory Quality and Stability of Beer

Achieving beer quality and stability during its shelf life is a challenge for the brewing industry. The quality of the product is defined by several parameters such as appearance, aroma, taste, and texture and is influenced by the raw material, microbial activity, light, oxygen and temperature. This review focused on the sensory quality of beer linked to different brewing stages such as malting, boiling and fermentation. Additionally, it reviews and describes the use of chemometrics for a better understanding of the process and to monitor different quality parameters during beer shelf life.

From the literature review, the main chemometric techniques applied for the study of beer quality and stability are:

- Principal Component Analysis (PCA): characterization of raw materials and aroma compounds, exploratory purposes, classification of samples when combined with other techniques, and extraction of chemical features from Nuclear Magnetic Resonance (NMR) data [8–10].
- Partial Least Squares Discriminant Analysis (PLS-DA): verification of product authenticity, classification according to the raw materials and/or production methods, characterization markers of the production phase, and evaluation of flavour profiles [11–13].
- Linear Discriminant Analysis (LDA): characterization of beer aroma profile during the ageing process, classification of beers by style, production process (commercial *vs* craft), and fermentation process (bottom or top-fermented beers) [14, 15].

- Partial Least Squares Regression (PLSR): correlation of parameters related to raw materials, brewing process, storage conditions and transportation, prediction of beer shelf life, and prediction of palate fullness intensity in beers [16, 17].
- Multiple Linear Regression (MLR): data processing to determine parameters related to the brewing process, storage conditions and transportation [18].

Despite being extensively studied, beer quality remains a challenge for the brewing industry due to the complexity of the brewing process, and especially of the ageing process. The large volume of data obtained by studying beer quality and stability demands a powerful tool such as chemometrics to help extract, process and understand all the information acquired.

6.3. Chromatographic Analysis

Freshness is one of the key factors that determines beer quality and it is directly related to the content of aromatic compounds in the final product. The decrease in the freshness of a beer during shelf life depends on the type of packaging, the time of storage and the storage conditions such as light and temperature. The evolution of the aroma profile of beer packaged in aluminium cans and glass bottles was monitored during the natural aging process, and the sensory results were correlated to the instrumental analyses obtained by HS-SMPE/GC-MS.

Regarding chromatographic analysis, despite a slight variation in the peak area of some esters between aluminium cans and glass bottles at 11 months of storage, no other differences were observed between both types of packaging during the natural ageing process. The results showed that the main variations in the volatile matrix of samples were observed in the peak area of some compounds already present in fresh samples instead of the formation of new molecules, as confirmed by the literature [6, 19–21]. The difference between the furfural peak area in fresh beers from the brewery and fresh beers from the supermarket may be an indicator that the supermarket samples were exposed to a sudden temperature change, as this compound has been considered as a heat indicator for some authors [22, 23]. Furthermore, furfural was correlated to sensory scores of flavour staling compounds which could explain the difference perceived by panellists when comparing brewery and supermarket samples [24].

The sensory tests carried out showed that temperature and light storage conditions do have a negative impact on the sensory quality of beer even in a short period of time (one month), as the panellists were able to perceive differences between both types of containers, aluminium cans and glass bottles, only in fresh samples bought at the supermarket. In a general way, under optimal storage conditions, panellists did not find differences related to the types of packaging for the fresh beer. However, in samples aged 6 and 11 months, the differences between the types of packaging could be

perceived by the panellists. According to the literature [25], due to their lower oxygen content, aluminium cans retain all organoleptic characteristics and show a weaker fading and a slight increase in bitterness after 10 months of storage. The results of the tests carried out with samples in the same type of container showed that, for naturally aged beers in optimal light and temperature conditions, glass bottles seem to be a better container than aluminium cans, since they better preserve olfactory the product up to 6 months of ageing, which did not occur with samples packed in aluminium cans.

Concerning the chemometric results, PLS-DA showed that samples could be classified based on the time of storage but not on the type of container. Finally, the PLSR model built was capable of predicting the ageing period of lager beers up to 11 months with an error of 1.1 months.

6.4. e-Nose Analysis

A rapid and efficient technique using an electronic nose based on a mass detector, combined with headspace solid-phase microextraction sampling and chemometric tools, was applied to classify beer samples according to the ageing time and the type of container (aluminium cans or glass bottles). Additionally, a prediction model of beer shelf life was created and the results were compared with the prediction model obtained in our previous study using a more time-consuming HS-SPME-GC-MS technique [26]. To compare the chemometric results of both studies, beer samples in aluminium cans and glass bottles were acquired simultaneously and subjected to the same light and temperature conditions as in our previous study. The samples were analysed fresh and aged 6 and 11 months using the HS-SPME MS-based e-nose technique.

A PLS-DA classification model was not able to differentiate the samples by the type of container (aluminium cans or glass bottles), as only 65% of them were correctly classified. However, the model classified the samples according to their freshness (fresh *vs* aged) with 100% success. Since light affects the sensory characteristics of beer and degrades the product, we could say that the absence of light during the ageing time was the key factor for the canned and bottled beer samples to not present differences.

Although the PLS-DA model classified the samples bought in the supermarket as fresh, in the PLSR model they were predicted as to be older than the brewery fresh samples. This could be explained by the temperature conditions in which the supermarket samples were stored compared to the samples from the brewery. Samples purchased from the supermarket had been stored at supermarket temperature conditions for one month before being subjected to optimal storage conditions. On the contrary, the samples from the brewery were delivered directly after packaging and were kept at a controlled temperature of 14 °C +/- 0.5 °C. Many studies have proven that an increase in storage temperature can deteriorate the aroma profile of a beer affecting the quality and stability of the product [1, 2, 27, 28].

The PLSR model was able to predict the ageing time with an error of about 0.4 month, which is better than the 1.1 months predicted in our previous study using a GC-MS technique, showing the potential of this technique to predict beer ageing.

6.5. Conservation Processes

Microfiltration and pasteurization are both conservation processes applied to beers to guarantee the quality of the product during its shelf life. Generally, microfiltration is applied in craft breweries, while brewing industries usually apply thermal treatments such pasteurization to stabilize and extend the shelf life of the product. To study the efficiency of both processes, several physicochemical, instrumental, microbiological and sensory parameters were analysed in lager beers produced in Brazil.

Regarding the physicochemical results, we could observe that despite beer colour increases during the pasteurization process, in this study the pasteurized beers had lower colour values than the microfiltered ones. This could be explained by the spoilage contamination in the fresh microfiltered samples. From the ANOVA results, we could observe that colour is directly correlated to lactic acid (0.80), acidity (0.86), and turbidity (0.64) and inversely correlated to pH (-0.95), VDK (-0.86), IBU (-0.66) and apparent extract (-0.81).

VDKs are formed during beer fermentation and could be reabsorbed during the maturation step. If yeasts are removed from beer before the maturation process VDKs may appear later in the finished product. Also, an excessive or unexpected VDK level can be a sign of infection by bacteria or wild yeast [29]. Improper fermentation and/or bacterial/wild yeast infection could explain the presence of VDKs in the samples and their increase during ageing.

Although beer is considered a microbiologically stable beverage due to the presence of ethanol and bitter hop compounds, low pH and reduced concentration of oxygen, a limited number of yeast and gram-positive species are able to grow and spoil beer especially if the product is not pasteurised [30, 31]. The microbiological analysis showed that only the microfiltered samples were spoiled by yeast (*Saccharomyces* and non-*Saccharomyces*) and by Gram-positive bacteria. Since the microfiltration process occurs

before bottling in a non-aseptic area, there is a possibility that the product is contaminated by yeasts and/or bacteria present in the machinery or in the air until the moment of sealing. Aged and pasteurized samples did not present any spoilage contamination by bacteria or yeasts, suggesting that these microorganisms did not resist the temperature/time applied in the forced aged process (55 °C for 6 days) and in the pasteurization process (from 10 to 60 °C in less than 1 h).

The lowest pH value found in the fresh microfiltered samples (around 4.0) could be explained by the contamination of lactic acid bacteria (LAB), which is the predominant Gram-positive beer spoilers [32, 33]. LAB can also produce turbidity and make beer sour by producing lactic and acetic acids, which explains why in the sensory descriptive test panellists described, in the fresh microfiltered (FM) samples, turbidity, lactic acid and acidity in the visual, aroma/flavour and taste description, respectively.

However, from the chemometric analysis turbidity was found to be positively correlated to the aged microfiltered (AM) samples. According to Jaluska *et al.* (2018) [2], in their study about the influence of transport and storage conditions on beer quality and flavour, haze formation (turbidity) could be linked to an increase in temperature. This could also help explain the presence of furfural in the AM samples that were subjected to a higher temperature to force the ageing process. According to Madigan *et al*. [34] the higher the temperature the greater the formation of furfural. On the other hand, in pasteurized samples, both fresh pasteurized (FP) and aged pasteurized (AP), despite having been subjected to high temperatures, furfural could not be detected in their matrix. The presence and absence of furfural in the microfiltered and pasteurized samples, respectively, could be explained by the difference in the way the temperature was applied to both samples. While in force-aged microfiltered samples (FM) the temperature rise was abrupt (from 10 °C to 55 °C in less than 1 h) and the samples were kept at 55 °C for 6 days, in the pasteurized samples (FP and AP), the temperatures increased and decreased gradually in the pasteurization tunnel. These results indicate

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that furfural could be considered as a heat indicator for samples that passed through a sudden temperature change.

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Chapter 7

CONCLUSIONS

7. Conclusions

The main conclusions based on the objectives defined initially and derived from the results obtained throughout the development of this Doctoral Thesis are:

1. The use of chemometric techniques in combination with sensory analysis and GC-MS analysis appears to be a valuable tool to monitor and determine the main alterations in the aroma profile of a beer during its shelf life.
2. Even under optimal storage conditions, beer quality deteriorates significantly as the product approaches its expiration date, regardless of the type of container in which it is packaged.
3. The HS-SPME MS based e-nose technique together with multivariate analysis has proved to be an efficient method to classify beer samples and to create a beer shelf life prediction model.
4. In a non-sterile bottling process, applying pasteurization as a conservation process to guarantee the sterilization of the product seems to be more efficient than just applying microfiltration.
5. A forced process applied to accelerate the ageing of a beer (55 °C for 6 days) was able to inactivate spoilage contamination in fresh microfiltered samples that could influence the beer sensory quality profile.
6. The application of high temperature to force the ageing of a beer could change the sensory profile of the product.

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