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DEVELOPMENT OF NEW ANALYTICAL PROTOCOLS FOR CHEMICAL-COMPOSITIONAL AND TOXICOLOGICAL FINGERPRINTING OF FERMENTED BEVERAGES AND RELATED RAW MATERIALS IN A CONTEXT OF SUSTAINABILITY IN THE BREWING WORLD OF CRAFT BEERS

PH.D. THESIS

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Abstract

Beer stands as the world's most widely consumed alcoholic beverage, with a notable surge in the proliferation of craft breweries in recent years. In today's market consumers are on a constant quest for health-conscious, high-quality products, amid an ever-expanding array of choices. This surge in demand has prompted craft breweries to innovate by experimenting with diverse grains, both raw and malted, and elevating beer flavors and aromas through the addition of fruits, spices, and more.

The primary objective of this research is to bolster the regional beer production chain. This research centers on the study of indigenous hops and the development of novel analytical methodologies for profiling, which encompass bioactive constituents that define the health aspects, sensory characteristics, and toxicological considerations related to potential contaminants in craft beers. In the exploration of indigenous hops, a preliminary study was undertaken at the UNIRC Soil Laboratory, involving soil analyses, chemical-physical assessments, enzymatic parameters, and the establishment of a dedicated cultivation area. Subsequently, at the Research Institute of Brewing and Malting (RIBM) in Prague (Czech Republic), native hops from Calabria and commercially grown hops in the region were characterized from a phytochemical perspective.

Concurrently, innovative analytical protocols were forged at the FoCuSs UNIRC Laboratory to monitor the chemical, toxicological, and aromatic profiles of specially crafted beers in collaboration with the Technology Hall of Wilde Orange srl, a project partner in support of this doctoral research (ARS01_582 - E-Brewery). As a final model of our work, we conducted comparative analyses of various lager-style beer samples using bio-mimetic systems, differentiating the aromatic profiles achieved by employing both native and non-native hops.

In particular, nutraceutical studies on autochthonous hops revealed significant potential in terms of antioxidant and antimicrobial properties, suggesting that

these underrated cultivars could innovate the brewing industry with new functional ingredients, thereby enhancing the regional "terroir."

In the production of craft beers, the importance of considering the product type, addition phase, and diversification of raw materials emerged, with significant influences of hops, malt, and flavoring ingredients on the analyzed indicators.

The application of biomimetic and optical systems demonstrated non-destructive methods with potential applications in brewing production, yielding distinctive results in the evaluation of beer characteristics, including the discrimination between styles and types.

A novel analytical protocol for the simultaneous identification of over thirty polyphenols indicated the presence of nutraceutical compounds, revealing potential applications for improving taste, flavor, and preventing food fraud.

The developed method for aromatic fingerprinting, using HS-SPME extraction and chromatographic techniques coupled with GC/MS and GC-QTOF/MS, confirmed that the presence of flavoring ingredients significantly influences the aromatic discrimination of the final product.

The LC-MS/MS multi-mycotoxins method was identified as a useful tool for a comprehensive screening of mycotoxins in craft beer.

The final working model involved creating a lager-style beer enriched with various hops, including native Calabrian varieties. The objective was to assess, through biomimetic systems, the aromatic differences between the various samples. This multidisciplinary approach contributes to understanding and innovating in craft beer production.

Riassunto

La birra è la bevanda alcolica più consumata al mondo, con un notevole aumento nella proliferazione di birrifici artigianali negli ultimi anni. Nel mercato odierno, i consumatori sono alla costante ricerca di prodotti salutari e di alta qualità, in una gamma di scelte in continua espansione. Questo aumento della domanda ha spinto i birrifici artigianali a innovarsi sperimentando diversi cereali, sia crudi che maltati, ed esaltando i sapori e gli aromi della birra attraverso l'aggiunta di frutta, spezie e altro ancora. L'obiettivo primario di questa ricerca è rafforzare la filiera regionale della produzione della birra. Questa ricerca è incentrata sullo studio del luppolo indigeno e sullo sviluppo di nuove metodologie analitiche per la profilazione, che comprendono componenti bioattivi che definiscono gli aspetti sanitari, le caratteristiche sensoriali e le considerazioni tossicologiche relative ai potenziali contaminanti nelle birre artigianali. Nell'esplorazione del luppolo autoctono è stato effettuato uno studio preliminare presso il laboratorio suoli dell'UNIRC, prevedendo analisi del suolo, valutazioni chimico-fisiche, parametri enzimatici e la costituzione di un'area di coltivazione dedicata. Successivamente, presso l'Istituto di ricerca sulla produzione della birra e sul malto (RIBM) di Praga (Repubblica Ceca), il luppolo autoctono calabrese e quello coltivato a fini commerciali nella regione sono stati caratterizzati da un punto di vista fitochimico. Allo stesso tempo, presso il Laboratorio FoCuSs UNIRC sono stati messi a punto protocolli analitici innovativi per monitorare i profili chimici, tossicologici e aromatici di birre appositamente realizzate in collaborazione con la Technology Hall di Wilde Orange srl, partner del progetto a sostegno di questa ricerca di dottorato (ARS01_582 - E -Birrificio). Come modello finale del nostro lavoro, abbiamo condotto analisi comparative di vari campioni di birra in stile lager utilizzando sistemi biomimetici, differenziando i profili aromatici ottenuti utilizzando luppoli sia nativi che non nativi.

In particolare, gli studi nutraceutici sui luppoli calabresi hanno rivelato un significativo potenziale in termini di proprietà antiossidanti e antimicrobiche, suggerendo che queste cultivar sottovalutate potrebbero innovare l'industria birraria con nuovi ingredienti funzionali, potenziando così il "terroir" regionale.

Nella produzione di birre artigianali, è emersa l'importanza di considerare il tipo di prodotto, la fase di aggiunta e la diversificazione delle materie prime, con influenze significative di luppolo, malto e ingredienti aromatici sugli indicatori analizzati. L'applicazione di sistemi biomimetici e ottici ha dimostrato metodi non distruttivi con potenziali applicazioni nella produzione birraria, fornendo risultati distintivi nella valutazione delle caratteristiche della birra, inclusa la discriminazione tra stili e tipi. Un nuovo protocollo analitico per l'identificazione simultanea di oltre trenta polifenoli ha indicato la presenza di composti nutraceutici, rivelando potenziali applicazioni per migliorare gusto, sapore e prevenire frodi alimentari. Il metodo sviluppato per l'impronta aromatica, mediante estrazione HS-SPME e tecniche cromatografiche abbinate a GC/MS e GC-QTOF/MS, ha confermato che la presenza di ingredienti aromatici influenza significativamente la discriminazione aromatica del prodotto finale. Il metodo LC-MS/MS multi-micotossine è stato identificato come uno strumento utile per uno screening completo delle micotossine nella birra artigianale. Il modello di lavoro finale ha coinvolto la creazione di una birra in stile lager arricchita con vari luppoli, compresi quelli autoctoni della Calabria. L'obiettivo era valutare, attraverso sistemi biomimetici, le differenze aromatiche tra i diversi campioni. Questo approccio multidisciplinare contribuisce a comprendere e innovare nella produzione di birra artigianale.

Keywords: Autochthonous Hops; Craft Beer; polyphenols, sensory attributes, aromatic compounds; mycotoxins.

CHAPTER 1

1. General Introduction

Beer stands out as one of the most widely consumed and esteemed beverages globally. Alcohol-free, low-alcohol, gluten-free, and craft beers are among the non-alcoholic segments that are driving the ongoing expansion of the global beer market. [1]. Craft beers, specifically crafted by small and independent microbreweries, have experienced rapid growth on a global scale due to heightened interest in artisanal products [2]. However, various factors, including market competition, energy expenses, scale-related challenges, and taxation, pose threats to the economic viability of microbreweries. Consequently, there is a growing emphasis on diversification and broadening the product range to discover new beer styles and enhance the market value of the product.

Considering these challenges, there is an increasing focus on the ingredients and raw materials involved in the beer production process, which can be simplified into four main steps: malting, mashing, fermentation, and maturation. The primary ingredients typically include water, barley malt, hops, and yeast. Additionally, depending on the beer style and brewery practices, the list of traditional ingredients may be extended, as specific raw materials contribute distinct flavors, aromas, and sensory attributes [3]. Exploring the use of indigenous biological inputs holds potential advantages, such as cost reduction in production and supply, and fostering a strong connection with local territories that enhance the final product's value. Water, including mineral water, plays a crucial role in the mashing stage and is frequently utilized in the process.

The brewing qualities of indigenous biological resources, encompassing cereals, malts, hops, microbes, and adjuncts of biological origin, are influenced in diverse ways by specific soil characteristics and environmental factors [9,10]. This variability demonstrates that regional attributes can be uniquely emphasized in the beer production chain, fostering a connection with the concept known as "terroir."

This association, previously identified in other products tied to specific regions, notably in the wine industry, can be advantageous in the production of regional craft beers, establishing a vital link with a particular geographical site.

In accordance with the law of August 16, 1962, no. 1354, and subsequent amendments, beer is the result of alcoholic fermentation involving strains of Saccharomyces Carlsbergensis and Saccharomyces Cerevisiae, acting on a wort prepared with barley or wheat malt or their combinations and water, bittered with hops or their derivatives, or a combination of both. The law permits the use of roasted malt extracts and food additives allowed by the Ministry of Health's Decree no. 209/96 in beer production. Some countries and certain beers allow the use of sugar. Additionally, the law allows for the complementation of alcoholic fermentation with lactic fermentation [11].

Throughout centuries, two enduring characteristics have defined the history of beer: its nearly universal presence and its popularity across all social classes. The primary composition of beer is water, and its quality significantly contributes to the beverage's goodness. A beer is considered excellent when the water contains a rich array of minerals, organic substances, and microorganisms such as bacteria and yeast. While minerals may not directly impact the taste of beer, they play a role in diastatic and colloidal reactions during production, specifically the processes of substance disintegration and reaggregation.

1.1. The composition of beer

Beer, primarily constituted of water, derives its quality from the mineral and organic attributes it contains. The chemical parameters of water play a pivotal role in influencing various aspects, including wort pH, yeast flocculation, α-amylase activity, hop utilization, color, mouthfeel, and the overall palatability of the final product [3,4,5,6]. Consequently, renowned brewing cities like Dublin, Dortmund, Vienna, Munich, London, and Edinburgh often serve as benchmarks for their respective beer styles, owing to the distinctive characteristics of the water used

[7]. However, adjustments are frequently made to the mineral composition and the concentration of specific ions, such as carbonate, sulfate, iron, and manganese, to prevent undesirable sensory attributes, colors, and tastes [8]. These adjustments may also align with regional guidelines, as seen in Germany, or conform to the parameters specified by regions/cities to achieve consistent beer styles [4].

While barley remains the most commonly used cereal in brewing, other grains like wheat, rye, rice, corn, and more are also utilized to produce beer. Among the various barley varieties, two-row, six-row, and multi-row are the ones predominantly employed in the brewing world. Each barley spike consists of a series of nodes or rachis, with each supporting six potential flowers [12]. The classification into two-row, six-row, or multi-row barley depends on whether two, four, or six flowers are made fertile, resulting in an equal number of grains on the rachis [13]. Essential characteristics of barley include its moisture content (which must be less than 15%), germination capacity (not less than 95%), enzymatic richness, and protein content [14].

The beer production process Is divided into five key phases:

1. Malting: Malt is essential to enrich the wort with enzymes. Barley germination is followed by a crucial toasting process to determine the type and character of the malt.

2. Boiling: This phase sterilizes the wort, eliminates unwanted microorganisms and allows hops to release bittering substances. It is vital to ensure the success of fermentation.

3. Hopping: Adding female inflorescences of Humulus lupulus imparts distinct aromas and the typical bitter taste to beer. 14op salso act as a preservative, ensuring beer stability.

4. Fermentation: Yeast takes center stage, converting sugars and amino acids into alcohol, carbon dioxide, and aromatic substances. Both tumultuous fermentation and secondary fermentation or maturation contribute to shaping the beer’s profile.

5. Filtration and Packaging: Beer is clarified to remove residues through filtration or centrifugation. Pasteurization at 70°C for 30-60 seconds contributes to microbiological stability before bottling or canning.

Beer production is an art that combines tradition and innovation. From malting to bottling, each phase is crucial in creating a unique and globally appreciated beverage. The balance between high-quality ingredients and mastery in applying brewing techniques gives each beer its distinctive character. In a world where beer is cherished by every social class, its production remains a fascinating blend of science and passion.

From 1980 to the 2000s, beer production continued to grow, surpassing the previous per capita consumption record, which had reached 23.8 gallons (about 90 liters) in 1981. In the subsequent years, there was an increase in breweries across the American territory: by the late 1990s, hundreds of new breweries were recorded in the United States. However, the majority of these new breweries were classified as small businesses, as their annual production level was well below the average. Consequently, these new enterprises came to be known as “microbreweries” [15].

These microbreweries, in fact, were born as tools for the enhancement of local identity. When we talk about “microbreweries”, we must distinguish two different categories of the same activity: the microbrewery (microbrewery) and the craft brewery (brewpub). By microbrewery, we mean a brewery that produces up to fifteen thousand barrels a year and sells no more than twenty-five percent of the product in its restaurant. By craft brewery, on the other hand, we mean a brewery that sells more than 25 percent of the beer in premises that are not necessarily located near the production site [16].

With market report conducted by “AssoBirra” and “Unionbirrai” for the year 2018, in recent years the beer market in Italy is continuing to grow, bringing more and more the use of beer in the Italian consumer habits. The flavor of the beer, being a combination of smell and taste impression, is a crucial factor in the acceptance of this product by the consumer. A list of beer constituents includes> 800 compounds, many of which contribute to its aromatic characteristic.

According to a recent consultation on microbreweries.org, in Calabria there are currently 50 microbreweries (divided between Microbreweries, brewpub and Beer Firm), representing a strong point for the regional economy, and a future perspective that aims to promote this industry [3].

The enhancement of the craft beer supply chain represents a crucial support for various aspects, significantly contributing to the economy, culture, and the environment. Firstly, craft beer embodies the authenticity and diversity of local flavors, promoting gastronomic culture and territorial traditions. By supporting craft brewers, the economic growth of local communities is fostered, creating employment opportunities and stimulating enogastronomic tourism.

Moreover, craft beer stands out for its attention to the quality of ingredients and sustainable production. Valuing this supply chain means promoting responsible agricultural practices, encouraging the cultivation of local barley and hop varieties. This contributes to the preservation of biodiversity and reduces the environmental impact associated with the transportation of raw materials over long distances.

Thus, enhancing the craft beer supply chain not only enriches the offer of high-quality products on the market, but also contributes to environmental sustainability, local economic growth and the maintenance of unique traditions.

1.2. The aim

The primary aim of this PhD thesis was to enhance the regional beer production chain. The research focused on the investigation of indigenous hops and the development of innovative analytical methodologies for profiling. This profiling encompassed bioactive constituents crucial for defining health aspects, sensory characteristics, and toxicological considerations related to potential contaminants in craft beers.

In the culminating phase of our study, we conducted comparative analyses of various lager-style beer samples using bio-mimetic systems. This allowed us to

differentiate the aromatic profiles achieved by employing both native and non-native hops.

The doctoral work is structured into two key sections. The first section concentrates on the valorization of native hops, comparing them with commercial hops. The second section is dedicated to the development of new analytical protocols for analyzing craft beers produced with both native and non-native hops, with a specific focus on the chemical profile and sensory aspects.

1.3.Experimental design

Experimental design played a pivotal role in this study, serving as the blueprint for conducting scientific investigations.

It encompassed the careful planning and organization of experiments to ensure meaningful results, minimize errors, and draw reliable conclusions. In the realm of study, where precision and reproducibility are paramount, a well-structured experimental design was of utmost importance.

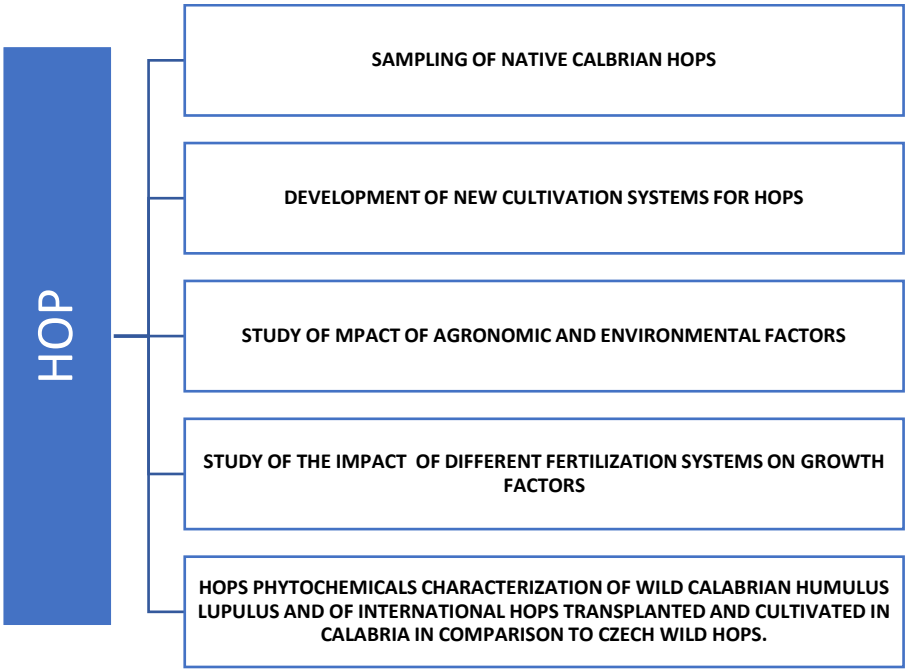
The first step in experimental design was formulating a clear hypothesis about the expected outcome of the experiment. It was set the direction and purpose of the investigation.

Identifying and controlling variables was crucial. Also, it was defined the equipment, instruments, and materials required for the experiment. Conducting multiple repetitions of the experiment was essential to evaluate the consistency and reliability of results.

Replication minimizes the impact of random errors and validates the findings. Statistical methods were applied to data to determine the significance of observed differences.

A well-designed experiment was critical for achieving meaningful and reliable results, advancing understanding of processes, and driving innovation in the field

of brewing. Considering that the main goal of the thesis was to enhance the sustainability and competitiveness of craft breweries by raising the competitive and qualitative standards of raw materials, primarily hops, to be used in the production of 100% Made in Italy craft beers, the experimental design was divided into different macro steps further divided into different subgroups (Figure 1).



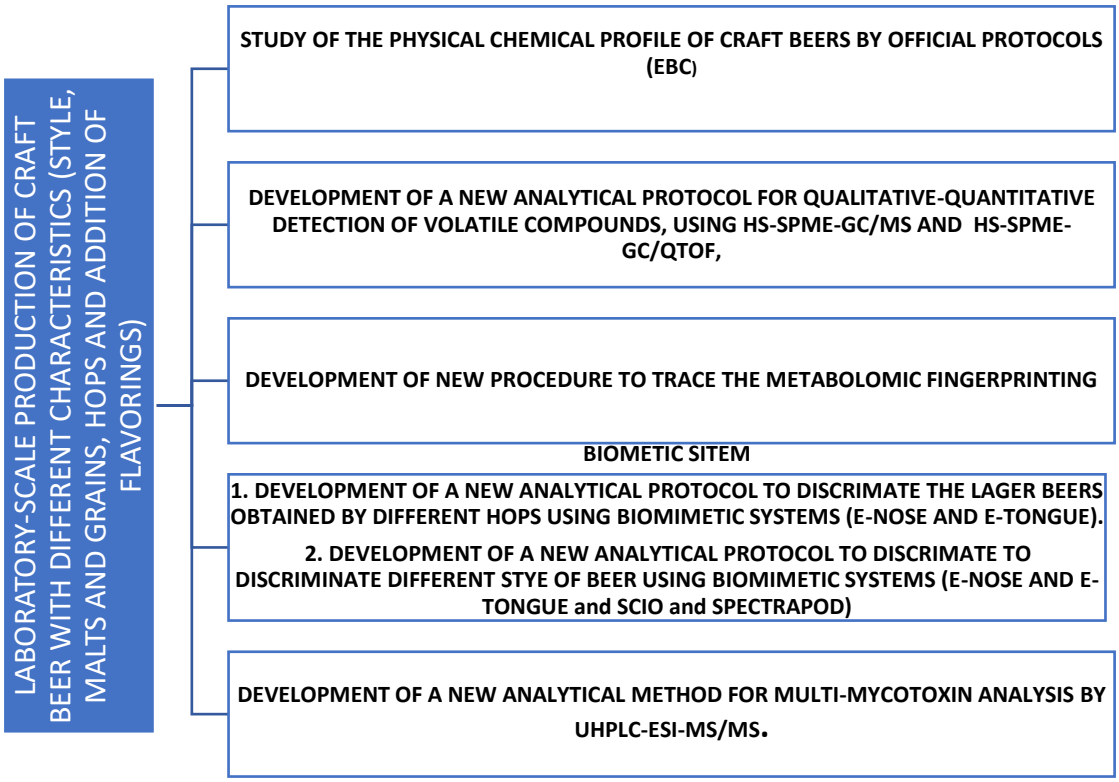


Figure 1: *Experimental Design.*

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CHAPTER 2

2. Assessment and Agronomic Characterization of Indigenous Hops Rediscovered in Calabria

Abstract

In the year 2022, a native hop variety discovered in Calabria underwent a careful process of extraction and subsequent cultivation in a controlled environment. This initiative took place at the Laboratory of Pedology, University of Reggio Calabria Mediterranea (under the scientific supervision of Adele Maria Muscolo).

The extracted rhizomes were then transplanted into pots filled with soils enriched with NPK and Horse Manure fertilizers. Additionally, two control groups of rhizomes were maintained under standard conditions for comparison as control.

The research also encompassed a comprehensive analysis of the soil samples from which the rhizomes originated. This analysis included assessing soil texture, dehydrogenase activity, hydrolytic determination of Fluorescin diacetate, catalase activity, and the determination of various physical-chemical soil parameters such as pH, electrical conductivity, water-soluble phenols, carbon percentage, nitrogen percentage, and the C/N ratio.

Throughout the cultivation process, meticulous attention was given to monitoring the growth of the rhizomes. This involved weekly measurements of stem growth, monitoring SPAD levels, and keeping track of the number of leaves on the plants, providing valuable insights into the plant's development and health.

Keywords: *Humulus lupulus L.,* Autochthonous hop, SPAD, Growth index, Soil Texture, soil enzymatic activity.

2.1. Introduction.

Humulus lupulus L. (common hop) is a perennial liana that is herbaceous and belongs to the Cannabeaceae family. All three species, the other two being H. Yunnanensis Hu. and H. Japonicus Siebold & Zucc., are present in China, which has generally been agreed to be the region of origin for the genus Humulus [1,2].

Hop production is generally restricted to regions between 35° and 55° latitude in both hemispheres for commercial production, the plant is sensitive to chilling and day-length for optimal growth and flowering [3-5].

The plant ascends in a clockwise manner, using its sturdy, downward-facing trichomes (hairs) for support, and it has the potential to attain a height ranging from 10 to 18 meters. The leaves are arranged oppositely or alternately, featuring 3 to 5 lobes with serrated edges. The plant's extensive root system thrives in deep, well-drained, fertile soil, and its horizontal roots tend to produce numerous delicate rootlets. [6-8].

Hops in combination with beer brewing were frst mentioned in 736 CE in a monastery document from the Hallertau region in Bavaria, Germany [9].

As much as 98% of the H. lupulus crops produced worldwide are utilized by the brewing industry today. [1-10]. In regions with a moderate and humid climate, lupulus grows at its best [11]. When dormant, this plant is quite sturdy, but it requires a lot of work for growers, and severe spring frosts can cause young shoots to perish. [12].

The impact of climate change and heightened occurrences of droughts has led to a halt in crop yields in certain regions. Globally, the annual production of hops stands at approximately 180,000 tons. The leading contributors to this production are the United States with 51,000 tons, Germany with 49,000 tons, and Ethiopia with 44,000 tons, according to FAOSTAT 2019. [13].

The aim of modern cultivar development is to keep hop characteristics relatively stable, as industrial-scale brewing demands consistent aroma qualities. [14].

Nevertheless, it has been demonstrated that the aroma of hops can be significantly influenced by the growing conditions, including factors such as soil composition, moisture levels, microclimate, and more. Notably, the aroma of the American cultivar Amarillo (Virgil Gamache Farms Inc., WA) has exhibited noticeable variations based on its cultivation location. Specifically, studies have indicated that the polyphenol and bitter acid contents in hops can change depending on the geographical location of growth and the year of harvest. [14,15].

Potopová et al. (2021) [16] highlighted the heightened susceptibility of hop yields to drought and heat waves, emphasizing the slower adaptive rate of hops compared to field crops. Climatic models predict an increased frequency of droughts in the future. The hop plant, being highly sensitive to drought, swiftly responds to even short-term water scarcity through anatomical alterations in its conductive elements, specifically tracheids. These conductive elements play a vital role in growth and storage, and any dysfunction rapidly impacts productivity and overall yield.

The assessment of hop quality primarily revolves around the alpha acid content found in the strobili, a parameter that significantly influences the crop's value and market price. In certain cultivation regions, such as the Czech Republic, the alpha acid content has experienced a decline, attributed to shifting climatic conditions, notably insufficient precipitation or unfavorable temperatures [17].

2.2. Materials and Methods

2.2.1. Plant Material

Autochthonous hops were collected during the flowering stage in Petrizzi (CZ, Calabria region, Italy). A voucher specimen was deposited in the herbarium at the Department of Agraria, Pedology laboratory, of University of Reggio Calabria (Italia Meridionale, 38°07'16.3"N 15°39'42.1"E, 31m s.l.m.).

2.2.2. Hop transplant and subsequent transplantation of the cuttings

Pots 50 cm diameters were filled with soil chemically analysed for their reproduction.

To verify if the addition of different fertilizers can increase the growth and quality of hops, the pots have been fertilized with NPK and horse manure. All the fertilizers were purchased from the agrarian consortium of Reggio Calabria.

2.2.3. Soil analysis

The soils were collected, after the hop rhizome collection, in Petrizzi site (SR1) and analyzed. Soil samples, before the analyses, have been air dried and sieved at 2 mm. Soil water content, soil physicochemical parameters (pH, electrical conductivity, organic matter, soil texture) and soil biochemical properties were detected using the methods listed below:

- Determination of soil texture: Bouyoucos method, G. J. (1927) [18].
- Determination of soil pH (Muscolo et al 2019) [19].
- Determination of water content (Muscolo et al., 2019) [19].
- Determination of organic carbon (Muscolo et al., 2019) [19].
- Determination of total nitrogen (Muscolo et al., 2019) [19].
- Determination of water-soluble phenols (Muscolo et al., 2019) [19].
- Determination of dehydrogenase activity: Von Mersi, W., & Schinner, F. (1991) [20] method
- Determination of Fluoresceine diacetate activity: Dick R.P., Brekwell D.P., Turco R.F. (1996); & Perucci P., (1992) method [21,22].
- Determination of catalase activity: Back T. (1971) [23] method.

2.2.4. Hop Growth Parameters

All hops transplanted were monitored detecting growth index in terms of number of leaves and stem length (cm), content of chlorophyll (SPAD) and soluble phenols [24,25].

2.2.5. Statistical analysis

Analysis of variance was carried out for all the data sets. One-way ANOVA with Tukey's Honestly. Powerful Statistical Analysis and Graphics Software for Windows 7 was used for all the statistical analyses. Efects were signifcant at $p \leq 0.05$

2.3. Results and discussions

2.3.1. Hop cultivation

The native soil where the hops grew belong to sandy textural class (Table 1). Data in Table 2 show that soil pH was neutral, soils were not saline, and kept water. SR1 had also a good content of organic carbon and consequently of organic matter and contained 0.26% of total nitrogen. SR1 had a C/N ratio, indicating that humification and mineralization processes were balanced and a high CEC (20 meq/100 g ds) generally a CEC above 10 meq/100g is preferred for plant production Total phenols were elevated. Catalase, considered a marker of soil stress condition, that increase in a stress dependent manner, was in the range of not stressed soils. In relation to the data obtained, SR1 had also a high FDA and DHA activities, indicating a good hydrolytic and oxidoreductases activity. The tables 3 and 4 shows the soil used to transplant the hops (SRT) was sandy with alkaline pH an elevated content of organic matter, a low content of water-soluble phenols not saline but with high content of carbonate (23%), a high cation exchange capacity and high hydrolytic activity. Catalase data indicated a not stressed soil.

Table 1: *Particle size analyses of soil where the hops naturally grow Petrizzi location (SR1).*

Sample	Clay (%)	Silt (%)	Sand (%)	Texture
SR1	2	8	90	Sandy

.

Table 2: *Physico-chemical-parameters of Petrizzi soil*

Sample	WC	pH	EC	WSP	C	N	C/N	CaCO3	OM	CEC	DHA	FDA	CAT
SR1	17	6.96	153	314.40	2.84	0.26	11.0	2	4.89	18	1.36	25.7	1.29

Texture (USDA classification), water content (WC, %), eletctic conductivity (EC, µS/cm), soluble phenols (WSP., µg TAE g-1 s.s.), Total Carbon (C,%); total nitrogen (N%); Carbon/nitrogen ratio (C/N); Carbonate (CaCO3,%), Organic Matter (OM.%), Cation exchange capacity (CEC, meq/100g ds) fluoresceine diacetate hydrolase activity (FDA, µg fluorescein * g-1 s.s) , Dehydrogenase activity (DHA, µg INTF * g-1 s.s. h-1), Catalase activity(CAT, %O/3min/g- s.s.).

Table 3. *Particle size analyses of soil where the hops have been transplanted.*

Sample	Clay (%)	Silt (%)	Sand (%)	Texture
SRT	3	9	88	Sandy

Table 4: Chemical physical analysis of soil used to transplant the rhizomes collected in Petrizzi.

Sample	WC%	pH	EC	WSP	C	N	C/N	CaCO3	OM	CEC	DHA	FDA	CAT
STR	18%	8.55	107.3	56.095	3.01	0.28	10.75	23	5.17	21.57	1.31	44.47	1.54

Texture (USDA classification), water content (WC, %), eletctic conductivity (EC, µS/cm), soluble phenols (WSP., µg TAE g-1 s.s.), Total Carbon (C,%); total nitrogen (N%); Carbonate (CaCO3,%) Carbon/nitrogen ratio (C/N); Organic Matter (OM.%), Cation exchange capacity (CEC, meq/100g ds) fluoresceine diacetate hydrolase activity (FDA, µg fluorescein * g-1 s.s) , Dehydrogenase activity (DHA, µg INTF * g-1 s.s. h-1), Catalase activity(CAT, %O/3min/g- s.s.).

Table 5: *Stem length (cm) and leaf number of rhizome hops grown with different fertilizers (NPK and HM) and on soil without fertilization (CTR). Data monitoring started 1 month after hop transplant.*

Date	16/03/2022		23/03/2022		28/03/2022		06/04/2022		14/04/2022		21/04/2022	
Value	Stem (cm)	Leaf number	stem (cm)	Leaf number	stem (cm)	Leaf number	stem (cm)	Leaf number	stem (cm))	Leaf number	stem (cm)	Leaf number
CONTR	59.67	8.33	100.83	12.67	111.67	15.33	129.5	15.67	143.5	17.33	161.33	19.33
NPK	72.33	9.67	112.83	16.67	127,33	16.33	134.83	21	147.5	22.67	169.5	23.66
HM	53	13	62	17	69.5	19	77.5	22	89.66	22	101.33	24

Data evidenced that even if the stem is smaller in HM treated rhizomes, the number of leaves was greater than the other conditions. These data were also supported and confirmed by SPAD values, indicating that over time the leaves of HM treated rhizomes had the greatest SPAD value. Since the SPAD index, is intended as an indicator of the nutritional status of the plant (depending on the content of chlorophyll and foliar nitrogen), it possible to conclude that the hops grown on substrates fertilized with horse manure, and at minor extent with NPK showed a better vegetative state than control. In short, sandy soil with high content of organic matter and CEC and a good hydrolytic enzyme activity can be an optimal incubator to reproduce the hop rhizomes.

Table 6: *SPAD values (medium) of hop rhizomes differently grown.*

Date	**16/03 2022**	**23/03 2022**	**28/03 2022**	**06/03 2022**	**13/04 2022**	**20/04 2022**	
Value	average Stems	average Stems	average Stems	average Stems	average Stems	average Stems	std. Dev.
CONTR	25	23	25	25	26	26	±144
NPK	28	28	30	31	31	31	±1.12
HM	41	42	40	40	41	42.4	±1.13

2.4. Conclusions

There are many more research findings that focus on characterizing the quality of hops grown in Mediterranean climatic conditions than there are agronomic studies.

A majority of the research efforts have been directed towards characterizing the quality and properties of hops in these regions, while comparatively fewer studies have delved into the agronomic aspects of hop cultivation. This skewed emphasis in research is primarily due to the craft beer industry's burgeoning interest in unraveling the unique "terroir" that each distinct growing location can bestow upon hops and, consequently, the resulting beer products.

A significant development highlighted by Rossini et al. in 2021 [26] is the feasibility of growing hops under organic farming conditions in Italy. However, this approach requires a comprehensive understanding of various agronomic strategies that can effectively alleviate pathogen pressure, manage pest infestations and weed control, and enhance soil fertility, all without resorting to chemical interventions. This is particularly crucial because, as of the present, Italy has not registered any chemicals for hop protection. In light of this, the sole distinguishing factor between organic and conventional hop cultivation lies in the use of chemical fertilizers.

In a forthcoming study, there are plans to carry out a nutraceutical evaluation of these native Calabrian hops. This evaluation will include a comparative analysis with commercial hops cultivated in the same rhizome discovery regions. It's important to emphasize that, to the best of our current knowledge, this study stands as the inaugural exploration into the agronomic factors specific to native Calabrian hops, marking a pioneering endeavor in this domain.

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CHAPTER 3

3. Phytochemical Characterization of Native and Commercial Hops Grown in Calabria

Abstract

The traditional application of female hop inflorescence within the brewing industry has long revolved around the practice of adding it to beer, a process that serves to enhance the beer's bitterness, aroma, flavor, and overall shelf life. The chief compounds responsible for elevating the quality of beer are the bitter acids. However, in recent times, there has been a discernible shift towards an increased focus on identifying a diverse array of bioactive compounds that can be found in female hop flowers. This shift has been predominantly driven by the growing interest of consumers in natural ingredients, leading to extensive research endeavors aimed at uncovering new sources of functional biomolecules.

Consequently, it becomes evident that hops boast substantial potential as bioactive components that extend beyond their conventional brewing applications. They can play a pivotal role as natural preservatives in fresh food products, effectively prolonging their shelf life while maintaining their quality. Furthermore, hops hold significant promise in cosmetic formulations for skincare products, adding to their versatility and application in the cosmetic industry. Beyond these practical applications, the nutraceutical and health-related potential of hops is also a subject of growing interest.

Within the scope of this study, four distinct hop samples were thoughtfully selected, two of which were commercially cultivated hops originating from Calabria (cv. Cascade), while the other two were native hops cultivated in the same region. The primary aim of this research endeavor was to conduct a comprehensive nutraceutical analysis of these four samples. This analysis was conducted with the overarching objective of assessing and characterizing their

respective nutraceutical profiles, thus shedding light on their potential applications in various domains.

Keyword: Hop, Nativ hop, bitter acids, essential oils, DPPH, Total Polyphenols.

3.2. Introduction

Hop cones, which are essentially the inflorescences of the female plant scientifically known as *Humulus lupulus L.*, play a pivotal role within the brewing industry, primarily functioning as both a flavoring and bittering agent. The significance of these hop cones in brewing is underscored by the fact that approximately 98% of the global hop production is dedicated to supporting the brewing sector [1].

Common hop is commonly referred to as *Humulus lupulus L.*, which is a perennial liana that belongs to one of three distinct Humulus species found in the family Cannabiaceae. These three species, including *H. Yunnanensis Hu*. and *H. Japonicus Siebold & Zucc.*, originate from China, recognized as the genus Humulus' region of origin [2,3]. The growth pattern of this remarkable plant involves clockwise climbing around any available support, aided by its distinctive downward-facing stout trichomes, or hairs. Under favorable conditions, it can achieve impressive heights ranging from 10 to 18 meters. The leaves of the plant appear either in opposite or alternate arrangements and are characterized by their 3 to 5 lobes with serrate margins. The extensive root system requires deep, well-drained, and fertile soil, with horizontal roots producing numerous fine rootlets [4,5,6].

The remarkable combination of hops and beer brewing has a rich historical backdrop, with the first documented mention dating back to the year 736 CE in a monastery document originating from the Hallertau region in Bavaria, Germany [7]. As of today, it's estimated that the brewing industry consumes a staggering 98% of the global H. lupulus crop production [2,8]. The growth of H. lupulus thrives most optimally in temperate regions featuring a moderate and humid

climate [9]. The plant showcases its resilience when dormant, yet the process of cultivating it is characterized by labor-intensity, and the vulnerability to severe frosts in the spring poses a genuine threat to young shoots [10].

In the context of our research and to the best of our knowledge, this document stands as the pioneering effort to comprehensively report on the nutraceutical composition of both native and commercial hops, specifically the Cascade variety, cultivated in the region of Calabria.

3.3. Materials and methods

3.3.1. Hop samples

Four samples of hops grown in Calabria (Tab.1) were used for analysis. Two samples were native cultivars and were recovered during the 2021 harvest year in different areas of Calabria. The other two samples were commercial hops (cv. Cascade) grown in Calabria, which also came from different areas.

The hop cones were harvested at the state of technological maturity and were dried at a temperature of 55-60°C. The hop cones were stored in vacuum packs without access to air at +4 °C and +20 °C.

Once opened from the vacuum, all hop samples, were stored at temperatures of -18°.

Shortly before analysis, the dried hop cones were ground.

Table 1- Sample Hops.

Sample's Hop			
	Hop's	**Origin**	**ABB.**
1	Autochthonous Hop-1	Italia-Calabria-(CZ)	AHP
2	Autochthonous Hop 2	Italia-Calabria-(VV)	AHM
3	Cascade-1	Italian-Calabria-Zona 1	C1
4	Cascade-2	Italian-Calabria-Zona 2	C2

3.3.2. Bitter acids

All samples were analyzed by liquid chromatography according to the EBC 7.7 method (Analytica EBC, Milan, Italy) [11]. (Analytica EBC, 1998), using the external calibration standard ICE 3. In the following text, the following analytical method is referred to as HPLC (EBC 7.7).

Weigh 10 g of sample and add with 20 ml methanol, 20 ml 0.1 M HCL and 100 ml diethyl ether.

Shake the solution for 40 minutes at a speed of 250 rpm.

Weigh about 0.5-0.6 g of Standard and make up to volume with HPLC-grade Meoh in a 100-mL conical flask (50 ppm).

Once stirring of the sample is complete, phase separation will occur. Take 5 ml of supernatant and make up to volume with MEOH. Filter and put into 1.5 ml vials.

HPLC conditions

Table 2: *HPLC conditions*

System	**HPLC-DAD**
Mobile phases	**100% B** 0% A
Mobile phase	**B=** 900 ml MeOH, 140 ml H_2O, 5 ml H_3PO_4 **A=** MeOH
Column	ARION® Plus C_{18} 250×5.1mm, 4 µm
Signal Polarity	+
Flow rate	0.4 mL/min
PDA detector range	190-400nm
Cell temperature	40°C
Volum injected	5 µL

The equipment consisted of a photo diode detector (RP-UHPLC-Dray) (RP-UHPLC-DAD, Shimadzu, Milan, Italy), equipped with a column oven (CTO-20AC), an autosampler (SIL-30AC), an in-line degasser (DGU-20A5R), a communication module (CBM-20A), two parallel flow double piston pumps (LC-30AD) and a photosensitization system (LC-30AD), and a photodiode array detector (SPD-M30A).

Chromatographic separation was performed with an ARION® Plus C18 250×5.1mm, 4 µm column. Analyses were conducted using the following optimized chromatographic conditions: MeOH (mobile phase A), : 900mL MeOH,140 mL H2O, 5 mL H3PO4 (mobile phase B), flow rate 0.4 mL/min, and oven temperature 40 °C (table 2).

3.3.3. Essential Oils

The official EBC method 7.12 [12] was used for the quantification and identification of essential oils.

Sample preparations

Sample homogenized and finely grounded hops. Hop oils are extracted from hop samples by solid-liquid extraction. A portion of 50 mg of a grounded sample with 5 µl of internal standard 1-hepten-3-ol (7.8 g/L) mix with 400 µl of dichloromethane:acetonitrile (2:1, v/v) solution. The mixture has to be heated at 50 °C for 60 min, then cooled down and finally transferred into a vial containing 400 µL of water to wash the extract from highly polar interferents by liquid-liquid extraction for 1 min (three times). The phases of water and organic extract separate by centrifugation (3 min. 1200 rpm). Separated org. extract is ready analyze.

GC-MS method

Determination of analytes in the hop sample is performed on a Trace GC Ultra instrument with a DSQ II mass spectrometer. Conditions:

Carrier gas He with a purity of 5.0 with a flow rate of 1.2 ml/min; Splitless dosing, splitter opening time 1 minute with a total flow of 50 ml/min, temperature of injection and transfer lines 250°C, injection volume 1 µl; Trace GC Ultra DSQ II instrument; TG WAX MS capillary column, polar; temperature set to go from 45°C after 2 minutes to 100°C at a rate of 10°C/min and further increased to 230°C at a rate of 15°C/min and a final duration of 6 minutes; MS detector: source heated to 200°C.

3.3.4. Total polyphenols

The official EBC method 7.14 [13] was used for the quantification of total polyphenols.

Reagents: Acetone (70%), Carboxylmethyl Cellulose, Ammonia (1:2 with H2O), Ammonium Iron (III) Citrate (3,1 g in 100 ml of H2O).

0.8 g of hops are ground and then weighed. The powder is poured into 500-mL flasks. 250 ml of 70% acetone is added. Hydrogen (gas) is added inside and sealed with glass stoppers.

It is then filtered with filter paper.

White preparation: 10 ml of sample, 8 ml of carboxymethyl cellulose is added to a 25 ml Erlenmeyer flask, then 0.5 ml of ammonia is added. Make up to volume with distilled water and allow to react for 10 minutes.

Sample Preparation: 10 mL of sample, 8 mL of carboxymethyl cellulose are placed in 25 mL flasks, then 0.5 mL of ammonia, 0.5 mL of CITRAK (iron ammonium (III) citrate 3.1 g in 100 mL of water) are added. Make up to volume with distilled water and allow to react for 10 minutes.

Read absorbance at 600 nm.

The following application of the readings was used for quantification:

$P= (A_H-A_{SL}) * 820$

Where:

A_H: mean absorbance of the sample;

A_{SL}: mean absorbance of blank.

3.3.5. DPPH

The assessment of antioxidant (antiradical) activity involved employing the DPPH free radical, following a methodology established by Krofta et al. (2008) [14]. This procedure is specifically designed to quantify substances with gradual reduction, with a particular emphasis on polyphenols. The calculation of ARP (antioxidant potential), representing the overall reduction in DPPH value during the 0–10 minute reaction period, was conducted:

Reagents: Acetone (70%), BUFFER, DPPH solution.

BUFFER: 6.01 g acetic acid in 1 L of water. Adjust the pH up to 4.3 with NaOH. Then dilute in EtOH 1:2.

DPPH solution: 0,0163 g of 2,2-difenil-1-picrylhydrazyl in 200 mL of H2O.

Preparation:

0.8 g of hops are ground and subsequently weighed. The powder is poured into 500 ml flasks and we add 250 ml of 70% acetone. We add hydrogen (gaseous) inside and seal with glass stoppers.

Then it is filtered through filter paper. WHITE: 5.6 mL of buffer + 0.4 mL of sample. React for 10 minutes and read at 525 nm.

Sample: 2.8 mL of DPPH solution + 0.2 mL of sample. React for 10 minutes and read at 525 nm.

The reading lasts 10 min with readings every 10:10 seconds at the end of the 10 min there will be many results deriving from the readings every 10 sec due to the degradation reaction of the antioxidants caused by the 2,2-diphenyl-1-picrylhydrazyl radical.

The final result will be represented by a line indicating, at a specific time, the percentage concentration of degraded DPPH.

3.4. Results and discussion

3.3.1. α and β-acids

Table 3 highlights the data obtained for alpha and beta acids. The samples that showed the best results were the Cascade hops, in particular for the α-acids content. Zone 2 (C2) Cascade hops had higher contents, while Zone 1(C1) Cascade hops performed better in β-acids content.

Native hops had low levels of α and β acid content. Of the two, Maierato (AHM) presented the best results.

Table 3: *α and β-acids results*

SAMPLE		AHM	AHP	C1	C2
α	**alfa-co**	0.54345 ± 0.002	0.42655 ±0.0006	12.2811 ± 0.049	20.32935 ± 0.025
	alfa n+ad	1.34395 ± 0.005	0.99225 ± 0.002	34.0751 ± 0.016	44.50905 ± 0.041
β	**beta-co**	0.36175 ± 0.008	0.25085 ± 0.0089	23.46355 ± 0.009	22.6594 ± 0.039
	beta n+ad	0.56325 ± 0.001	0.3519 ± 0.007	30.34195 ± 0.0019	22.4219 ± 0.051

Table 4 shows the results in % alpha and beta acids content. From a comparison with an article by Vladimír Nesvadba et al., 2023 [15], which deals with Evaluation of the variability of α and β acids content in Czech bittering hop varieties (Humulus lupulus L.), it is possible to note that commercial hops (CV Cascade) grown in Calabria, has a good content of beta acids, in particular C1 hops.

Table 4*: Average α and β-acids results.*

		SAMPLE			
		AHM	**AHP**	**C1**	**C2**
α	Average (% w/w)	0.198	0.149	4.863	6.802
β	Average (% w/w)	0.097	0.063	5.644	4.729

3.3.2. Total polyphenols

Table 5 shows results of total polyphenols present in hops. The best results, as they presented higher values, were found in native hops. In particular AHM presented the best result.

***Table 5**: total polyphenols in hops.*

TOTAL POLYPHENOLS	
SAMPLE	**mg of total polyphenols/g**
C1	67.36 ± 0.058
C2	76.54 ± 9.74
AHP	99.96 ± 1.85
AHM	125.091 ± 2.49

3.3.3. DPPH

Table 6 shows the results of DPPH understood as loss due to oxidation after 1 and 10 minutes and expressed as ARP (Antioxidant Potential). The best results were found in native hops, in particular, the AHP sample showed the lowest oxidation loss after 1 and 10 minutes, followed by AHM.

***Table 6:** ARP (Antioxidant Potential) hops*

DPPH			
DPPH concentration degraded over time in % (ARP)			
SAMPLE	**loss after 1 minute**	**loss after 10 minute**	**built-in leak after 1 minute**
C1	28.56	41.88	34.79
C2	31.51	43.25	36.42
AHP	19.19	30.55	25.09
AHM	23.67	39.14	31.86

From a comparison with the data obtained by Alexandr Mikyška, Marie Jurková, 2019 [16] in their work «Varietal specificity of polyphenols, free phenolics and antioxidant potential in hops», dealing with the antioxidant activity of commercial cultured hops grown in CZ, the data of the hops grown in Calabria (IT) presents the higher value as regards the Total Polyphenols, especially the autochthonous hops presenting the greatest quantities.

While instead as regards the DPPH, expressed as ARP (Antioxidant Pontential), the method measures in particular slowly depleting substances, especially polyphenols; i.e. an integrated decrease in DPPH value within 0-10 minutes of reaction. In this case, hops grown in CZ exhibit the best results.

From a general perspective, however, samples of native and commercial hops grown in Calabria appear to have a high antioxidant potential.

3.3.4. Essential oils

Table 7 shows the results obtained using GC techniques for the quantification of essential oils in hops.

Table 7. *Essential oils compounds*

Essential oils in hops [mg/kg]				
ID	**Sample (mean results [mg/kg])**			
	C1	**C2**	**M1**	**P1**
	mean	mean	mean	mean
isobutyl isobutyrate	0.04 ± 0.02	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.015
methyl hexanoate	0.00	0.00	0.00	0.00
alpha-pinene	1.90 ± 0.14	7.18± 0.002	0.00	0.00
isoamyl isobutyrate	1.61 ± 0.11	2.75 ± 0.002	0.27 ± 0.002	0.00
methyl heptanoate	15.21 ± 2.58	21.29 ± 3.46	1.23 ± 0.09	0.58 ± 0.02
2-nonanone	1.12 ± 0.23	1.39 ± 0.2	9.24 ± 0.87	5.06 ± 0.45
linalool	39.51 ± 5.23	56.32 ± 6.34	1.53 ± 0.3	0.96 ± 0.02
methyl octanoate	8.85 ± 1.02	17.18 ± 3.41	1.29 ± 0.23	0.60 ± 0.1
methyl nonanoate	5.70 ± 0.68	8.84 ± 1.23	0.58 ± 0.12	0.41 ± 0.09
cis-geraniol (nerol)	16.20 ± 1.94	11.42 ± 1.83	4.87 ± 0.67	1.00 ± 0.12
methyl geranate (Z-)	0.00	0.00	0.00	0.00

2-undecanone	18.61 ± 2.36.	24.97 ± 1.98	57.23 ± 6.87	19.04 ± 2.12
geranyl acetate	34.63 ± 4.15	117.46 ± 9.47	0.00	0.00
2-dodecanone	3.36 ± 0.58	4.74 ± 0.77	3.71 ± 0.42	2.90 ± 0.25
alpha-ionone	0.41 ± 0.16	1.84 ± 0.61	6.53 ± 1.15	2.78 ± 0.34
beta-caryophyllene	794.02 ± 15.88	890.10 ± 33.21	35.77 ± 3.26	10.59 ± 1.28
beta-farnesene	462.08 ± 41.52	531.79 ± 27.46	4.21 ± 0.78	0.96 ± 0.24
alpha-irone	0.38 ± 0.21	2.58 ± 0.96	5.29 ± 1.12	1.17 ± 0.45
beta-caryophyllene oxide	0.00	0.00	0.00	0.00
farnesol	108.79 ± 19.04	162.09 ± 14.48	41.45 ± 6.87	0.00
Beta-pinene	21.94 ± 3.94	25.32 ± 4.74	0.95 ± 0.23	0.34 ± 0.12
Myrcene	9643.29 ± 85.42	12090.07 ± 125.42	9.11 ± 2.03	4.32 ± 1.12
Limonen	0.18 ± 0.08	0.28 ± 0.04	0.06 ± 0.04	1.09 ± 0.24
Ocimene	5.70 ± 0.67	1.13 ± 0.19	0.85 ± 0.25	0.10 ± 0.06
3-caren	4.84 ± 0.84	3.46 ± 0.52	0.18 ± 0.08	0.10 ± 0.07
2-decanone	2.45 ± 0.29	2.05 ± 0.35	5.33 ± 0.59	2.39 ± 0.47
Terpine-4-ol	24.23 ± 2.95	22.05 ± 3.42	2.49 ± 0.72	0.85 ± 0.41
Alpha-terpineol	95.39 ± 11.09	130.65 ± 9.37	0.00	0.00
methyl geranate (E-)	95.39 ± 8.23	130.65 ± 9.74	0.00	0.00
Alpha-humulene	1615.02 ± 119.02	1783.72 ± 73.24	84.23 ± 6.28	30.06 ± 4.07
2-tridecanone	17.25 ± 4.28	25.76 ± 3.79	30.68 ± 5.62	11.47 ± 2.00
Beta-ionone	0.29 ± 0.18	0.00	0.65 ± 0.27	0.30 ± 0.12

Table 8: *Primary Compounds Found in Hop Essential Oils*

analytes [g/Kg]	C1	C2	AHM	AHP
beta-caryophyllene	0.79	0.89	0.04	0.01
beta-farnesene	0.46	0.53	0.00	0.00
Myrcene	9.64	12.09	0.01	0.00
Alpha-humulene	1.62	1.78	0.08	0.03
Selinenes	n.d.	n.d.	n.d.	n.d.

Table 8 highlights the quantities of the main compounds present in different commercial hop cultivars.

In comparison, with the work of Vladimír Nesvadba et al., (2021) [17] commercial hops grown in Calabria show good concentrations of these compounds, falling within the range values of the data reported in the literature. The compound Selinenes is not present.

Table 9: *Percentage Quantities of Selected Analytes*

compound in %	C1	C2	AHM	AHP
β-Pinene	0.17	0.16	0.31	0.35
β-Myrcene	73.96	75.20	2.96	4.45
Linanlol	0.30	0.35	0.50	0.99
β-Caryophyllene	6.09	5.54	11.62	10.91
α-Humulene	12.39	11.09	27.37	30.95

Table 9 describes the quantity in % of the analyses.

The commercial cultivars C1 and C2 appear to be the closest compared to the data present in the work of Vladimír Nesvadba et al., (2021) [16]. In particular, β-Myrcene is present in high quantities, resulting in the predominant compound (73.96 and 75.20, respectively).

In native hop samples, however, the predominant compound is represented by α-Humulene, with values which, by comparison, seem close to those found in the literature. The results for essential oils, especially for commercial hops grown in Calabria, appear to present an interesting terpenoic profile. They are in fact characterized by compounds that could greatly influence the aromatic and nutraceutical profile of the sample.

However, they remain the object of study and future study.

3.4. Conclusions

In the context of the samples under examination within this study, it is noteworthy to emphasize that the assessed cultivars exhibit significant potential in the realm of food preservation. Their documented antioxidant and antimicrobial properties make them particularly promising in this regard. Moreover, it's imperative to highlight that these cultivars hold immense untapped potential in terms of their volatile compounds and other chemical constituents, which have yet to be fully explored. This untapped resource could potentially revolutionize the beer industry, offering a valuable and natural means of preservation and a novel source of raw materials, thereby expanding the horizons of this thriving sector.

3.5. Acknowledgements

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CHAPTER 4

4. Physical-chemical characterization of craft beers

Abstract

Currently, the production and consumption of craft beer is increasing as a result of its valuable sensory characteristics.

Unlike traditional beer, craft beers offer better advantages not only from an aromatic point of view, but also from a visually and nutraceutical point of view.

Despite the growth in craft beer production, there are few studies on the subject; in fact, there is still much to be explored.

The countries with the most research is the United States, followed by Italy, Brazil, the United Kingdom and Spain [1].

Despite continued growth on the knowledge of characteristics on the difference between conventional and craft beers. There is still little knowledge on some important factors, which have been considered in this paragraph related to secondary metabolites due to craft beer production.

A study was conducted on the general chemical and physical characteristics of several styles of craft beers including: Alcoholic degree, Total Carbohydrates, Total Protein, Unit of Birtness, Color, Total Phenolic Content, DPPH (Radical-scavenging activity) Vicinal Diketons (VDK) and Free Ammino Nitrogen (FAN).

The objective of this part of the work was to compare different styles of handcrafted beer from a chemical-compositional point of view, going by the expected limits for FANs and VDKs.

However, other studies with more robust designs are required to produce more generalizable and conclusive results. In the end, the challenges of craft brewing were discussed, and alternative solutions were discussed.

Keywords: Craft Beer's, VDK, FAN, Total Polyphenols, Bitterness, Alcoholic Degree, Total Protein, Color, Total Carbohydrate.

4.2. Introduction

Evidence suggests that beer was produced thousands of years before Christ [2]. Beer production around the world has led to its current status as the most widely consumed alcoholic beverage due to discoveries and changes [3]. The brewing process is standard [4] with limited beer styles that are not responsive to consumer needs and preferences. Since the emergence of microbreweries in the United States, brewing has undergone significant changes in the early 1990s [5]. This has led to the production of artisan beers with more flavor like India Pale Ale (IPA) beers and more bitterness like Hazy IPA beers [2]. New Zealand consumers (22-60 years old, 39 percent female) had a preference for new, intriguing, and complex beers over ordinary and simple beers [6]. Italian consumers (aged 18-72, 52% women) indicated a preference for craft beers because of their outstanding authenticity (41.8% frequency), Their natural (22.9%) and tastier (22.3%) qualities make them better than commercial beers [7]. Italian consumers (aged 25-40 years, 50% women) have indicated a preference for craft beer due to its distinctive sensory traits [8]. Authenticity, creativity and innovation are characteristic of artisanal beers [2].

The production equipment contributes to the success of the brew by playing a key role in the pH of beer. Measuring pH from the very beginning of production, in fact, provides a clear warning about the environment in which the enzymes responsible for transforming sugars in the must are stored. The proper environment is necessary for them to perform their job effectively, which will result in a qualitatively superior end result.

During mashing, starches are broken down into sugars that are more or less complex. The enzymes -amylase and -amylase are responsible for this work,

which require a pH between 5.2 and 5.5 (measured at room temperature) to work optimally.

Two reasons make the pH value of Sparge water, especially if it is too high, equally important: the final beer may have sourness and astringency due to the extraction of tannins that it promotes; and, together with the pH of the puree, it would inevitably affect the acidity of the must.

In this regard, in order not to create large pH variations in the mash, acidifying the Sparge water is a good idea to reach a pH value of less than 6 points (maybe even around 5.5), The measurement is always taken at a temperature of 25°C in a room. By using this expedient, the overall pH won't be too high after the boil.

The EBC and ASBC units are used throughout Europe and the United States to describe the color (more precisely, the color intensity) of beer and wort. The value established by the EBC (European Brewery Convention) or the ASBC shows the quantity of light absorbed by the beer containing a given original must. The actual color of each beer brewed corresponds to a gradation of brown, which, at lower concentrations, gradually takes on a red, coppery and amber hue, until golden yellow and light yellow. In addition to the color of the malt and the original wort, the color intensity of the fermented beer is influenced by several other factors, such as the wort preparation, pH value, and fermentation process used. Although it may seem like a trivial task, measuring color is the element that determines the customer's initial impression before tasting the beer.

Various stages of the fermentation process can be monitored to monitor beer color conformity [9].

The most important bitter substances in wort and beer are iso-α-acids. Other α-acids and δ-acids may also be present, especially in wort. In addition, wort and beer contain other hop bitter acid derivatives, particularly oxidation products, which also contribute to the bitter taste [10].

The sum of bioavailable nitrogen components in wort is the free amino nitrogen (FAN) value. Excessive FAN content can create problems both in terms of taste and in terms of microbiological stability of the beer. Industrial and natural yeasts

ferment by converting amino acids into long-chain alcohols (propanol, isobutanol). FAN levels are also a good indicator of the completion of the fermentation process.

Typical FAN content is 200 to 1250 mg/L in wort and 10 to 120 mg/L in beer. [11-13].

Protein together surfactants are among the factors that influence the structure of the beer foam.

Electrostatic or hydrophobic forces, hydrogen bonds, or covalent bonds are used by the proteins to bind and interact with the interface.

Proteins interact with one another so that no free molecules remain [14].

Monitoring the carbohydrate content in wort and beer is a requirement of modern brewing, particularly for the development of new flavors and the selection of raw materials [15].

About 3.3-4.4 percent of beer is composed of carbohydrates, which are 75-80 percent dextrins, 20-30 percent monosaccharides and oligosaccharides, and 5-8 percent pentosans, making carbohydrates the primary nonvolatile component of beer [16,17].

Valine and isoleucine are the by-products of amino acid synthesis in Saccharomyces yeast during beer fermentation, resulting in the production of vicinal diketones (VDK) such as Diacetyl (2,3-butandione) and 2,3-pentanedione, and it can significantly affect the taste and aroma of beer. Diacetyl is renowned for its buttery or butterscotch-like flavor [18]

It is generally considered a defect to have VDK above the flavor threshold in beer, its flavour is undesirable in many styles of beer and may also indicate microbial contamination [19,20].

Beer is a beverage that contains precious nutrients like carbohydrates, amino acids, minerals, vitamins and polyphenols. About 30% of beer polyphenols come from hops, and 70% come from malts [21].

Antioxidants and polyphenols combined with a low alcohol content determine the functional quality of beers [22].

The primary phenolic compounds are hydroxybenzoic acids, cinnamic acids, like ferulic acid, and flavonols [23]. The quantity of hops added during production determines the hop polyphenol content of the beer. Decarboxylation and isomerization are chemical modifications that occur during the brewing process and fermentation of certain polyphenols. In the European Prospective Investigation into Cancer and Nutrition cohort study, Beer is a good source of polyphenols and was found to be the main food source for hydroxybenzoic acid intake [24].

Basic analyzes were conducted regarding the content of Total Polyphenols and DPPH, to generally evaluate the antioxidant activity of craft beers and how it could vary according to the different types of beers.

4.3. Materials and methods

4.3.1. Sample Preparation

The present work analyzed and compared the chemical and physical properties of three Craft Beers produced in Calabria produced in a pilot-scale plant at the Wild Orange Fermented Beverage Technology Room (Vibo Valentia, Calabria, Italy) through the collaboration between FoCuSs Laboratory and the Pedology Laboratory of the Mediterranean University of Reggio Calabria.

This category represents the most widely consumed beer styles in Europe.

The Craft Beer Sample was:

(CBS1), ingredients: water, barley malt hops, unmalted wheat, bitter orange peel and coriander seeds (Blanche Style).

(CBS2), ingredients: water, hops, barley malt, wheat, oat flakes, bergamot peels (Ipa Style).

(CBS3), ingredients: water, hops, barley malt (Lager Style).

The selection of samples was made, in addition to being the most consumed styles in Europe, based on enrichment with different types of raw materials, such as cereals (malted and non-malted), hops (for bitterness and aroma), and flavoring ingredients, which imparted differences that could be characteristic from a physicochemical, nutraceutical, and aromatic perspective.

The analysis was carried out in triplicate for each batch and for each type of Craft Beer. Samples were degassed at 20°C before testing, using a magnetic stirrer until all the gas was released.

Both the barley malt and the yeasts and hops used were the same for all three beer styles, while the grains and flavor bases were modified.

4.2.2 Beer analytical methods

The methods for the analysis of the beers, and precisely bitterness, color, pH, foam, haze, Total Protein, total Carbohydrates, FAN, VDK are described in Analytica EBC by European Brewery Convention [25, 26].

Per quanto riguarda la determinazione del contenuto antiossidante, è stato utilizzato il metodo Muscolo et al., 2018 [27] per il DPPH ed il metodo Singleton method V.L., & Rossi, J.A., 1965 [28] per il contenuto di polifenoli totali.

Beer pH was measured with pH-meter.

The method used to quantify the alcohol content is that of Mansur et al. 2022 [29], was a modification of the technique developed by Park et al. 2016 [30].

4.2.3. Alcoholic Degree.

Preparation of ethanol stock and working solutions

Ethanol stock solution (0.79 mg/mL) was prepared by mixing 1 mL of certified ethanol standard in 9 mL of water. Ethanol working solutions were prepared by dilution of ethanol stock solution with water in appropriate quantities.

Preparation of samples

The sample preparation technique used in study of Mansur et al. (2022), was a modification of the technique developed by Park et al. (2016). Briefly, a 0.5 ± 0.01 g of sample, 1 mL of internal standard (0.1% v/v 1-propanol), and 8.5 mL of water were consecutively added into a 20 mL clear vial.

GC-FID analysis

Quantitative analysis of ethanol in sample was performed using a GC-FID (GC-2010 Plus; Shimadzu Corporation, Kyoto, Japan) with an OMEGAWAX column [30 m (L) × 0.25 mm (ID) × 0.25 μm film thickness; Supelco.] as a stationary phase. Each sample (1 μL) was injected in split mode with a split ratio of 13:1. The gas flow rates were maintained as follows: carrier gas (helium, 10 mL/min), hydrogen (30 mL/min), and air (300 mL/min). The injector and detector temperatures were maintained at 180 and 250 ◦C, respectively. The oven temperature was initially held at 50 ◦C for 1 min, increased from 50 to 160 ◦C at a rate of 5 ◦C/min, and held for 2 min. Total run time was 25 mins [29].

4.2.4. Color determination.

Color of the degassed beer was measured at 430 nm by a UV–VIS spectrophotometer according to the EBC method [25]. Color was expressed in EBC units and calculated according to the formula: C=A430·f·25 where C gives the color (EBC), f is the dilution factor, and A430 is the absorbance at 430 nm.

4.2.5. Turbidity and bitterness assay.

Beer turbidity was carried on by nephelometric method. The amount of turbidity is expressed in EBC units. The amount of turbidity was measured in NTU units and was expressed in EBC unit. Bitterness was measured using a spectrophotometer (UV–Vis) at 275 nm [25].

4.2.6. Total carbohydrates and ascorbic acid detection.

Total carbohydrates were detected using the anthrone method with minor modifcations [31]. Sugars react with the anthrone reagent under acidic conditions to yield a blue–green color. The samples were mixed with sulfuric acid and the anthrone reagent and then boiled until the reaction was completed. The solution was then cooled the absorbance measured at 620 nm. There was a linear relationship between the absorbance and the amount of sugar present in the sample.

This method determines both reducing and non-reducing sugars because of the presence of the strongly oxidizing sulfuric acid. Like the other methods, it is non-stoichiometric, and therefore, it is necessary to prepare a calibration curve using known glucose concentrations. For ascorbic acid determinations, the method reported in Muscolo et al. [27] was used.

4.2.7. Total Protein.

Kjedahl method was used for the determination of total protein. This method involves three steps: a mineralization of the sample, a distillation and finally a titration to calculate the final result and quantification of total Nitrogen [32].

4.2.8. Vicinal diketones (diacetyl, 2,3-pentanedione).

The ECB method was used [26].

The basis of the method is the reaction of diacetyl or 2,3-pentanedione with 1,2-phenylenediamine at form 2,3-dimethylquinoxaline, which is measured by spectrophotometry.

First of all, a distillation of the beer is carried out, and the reference white is prepared from the product obtained.

For the blank, pipette 10 ml of the distillate and add 2.5 ml of 4 mol/l hydrochloric acid (4 N).

For the test sample, pipette 10 mL of the distillate and add 0.5 mL of phenylenediamine solution and mix. The mixture is left to rest in the dark for 30 min (reaction time)

Add 2 ml of hydrochloric acid 4 mol/l (4 N), mix and proceed with the determination within 20 min. Absorbance reading at a wavelength of 335 nm. The results are expressed in mg of vicinal diketones/kg.

4.2.9. Free Amino Nitrogen

The ECB method was used [25].

Low molecular weight nitrogenous compounds affect the fermentation process and the formation of related by-products. It follows that the concentration e the composition of the amino acids play an important role in defining the aromatic profile of a beer; also the reactivity with reducing sugars (Maillard reaction), exerts a certain influence. These reaction products determine the redox potential, color and aroma of the beer.

In methods based on color reactions, the various amino acids show different color intensities. The results are related to the so-called "standard amino acid", usually glycine.

In the ninhydrin method, the color production by individual amino acids varies between 70% and 105% compared to glycine.

Solution preparation

• *Color development reagent*

Dissolve 10.0 g of sodium hydrogen phosphate dodecahydrate, 6.0 g of potassium dihydrogen phosphate, 0.5 g of ninhydrin and 0.3 g of fructose in about 80 ml of H2O; check the pH (it must be between 6.6 and 6.8; correct with 6 mol/l hydrochloric acid or 4 mol/l sodium hydroxide solution if necessary) and make up to 100 ml with H2O in a graduated flask (the solution remains stable for 2 weeks if stored at +4 °C in dark bottles).

• *Dilution solution*

In a glass container, dissolve 2 g of potassium iodate in 600 mL of H2O, add 400 mL of 96% ethanol and mix (the solution remains stable for 1 week if stored at +4 °C in dark bottles).

• *Stock solution of glycine, 200 mg of amino nitrogen/ l*

Dissolve 107.2 mg of glycine and make up to volume with H2O in a 100 ml graduated flask (the solution is stable for 1 week if stored at 0 C in dark bottles)

• *Standard solution of glycine, 2 mg of amino nitrogen/ l*

In a 100 ml graduated flask, make up to 1.0 volume of glycine stock solution (200 mg amine nitrogen/l) with H2O and mix (prepare fresh every day).

Sample Preparation.

Dilute the beer 50 times with H2O.

Dilute the beer 100 times with H2O.

Reagent blank

• Pipette 2.0 mL of H2O into a test tube;

• Add 1.0 mL of Color Development Reagent and mix;

• Close the tube with the glass stopper loosely, to prevent losses by evaporation;

• Heat in a boiling water bath for exactly 16 minutes, then leave to cool in a water bath at 20 °C for 20 min;

• Add 5.0 mL of dilution solution and mix;

• Leave to rest for 3 minutes; proceed with the determination within 30 min.

Sample

• Pipette 2.0 mL of the diluted sample into a test tube;

• Add 1.0 mL of Color Development Reagent and mix;

• Close the tube with the glass stopper loosely, to prevent losses by evaporation;

• Heat in a boiling water bath for exactly 16 minutes, then leave to cool in a water bath at 20 °C for 20 min;

• Add 5.0 mL of dilution solution and mix;

• Leave to rest for 3 minutes; proceed with the determination within 30 min.

The results are expressed in mg FAN/l, the result already takes into account the dilution.

Typical FAN content is 200 – 1250 mg/L in wort and 10 – 120 mg/L in beer.

The absorbance is measured at a wavelength of 570 nm.

4.2.10. Total phenolic content (TP).

The method described by Singleton and Rossi [33] performed the TP analysis. The absorbance was measured at 725 nm. Quantifcation was carried out based on the standard curve of tannic acid, and the concentration of TP was expressed as tannic acid (TA) milligram per L of extract.

4.2.11. Anti-oxidant activity assays.

The method reported in Muscolo et al. [27] was used to determine the 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH·) scavenging assay. DPPH· concentration in the cuvette has been chosen to give absorbance values of~1.0.

The reaction mixtures were composed of: 10 μL of each extract, 700 μL DPPH· and 95% ethanol brought to 1.0 mL.

The change in absorbance of the violet solution was measured at 517 nm after 30 min of incubation at 37 °C. DPPH activity was expressed as μM of Trolox (T) using a calibration curve (1.0–50 μM T).

4.2.12. Statistical analysis

Analysis of variance was carried out for all the data sets. One-way ANOVA with Tukey's Honestly. Powerful Statistical Analysis and Graphics Software for Windows 7 was used for all the statistical analyses. Efects were signifcant at $p \leq 0.05$.

4.4. Results and discussions

Table I: Basic physical-chemical characterization of craft beers.

Analysis	Unit	SAMPLE		
		CBS 1	CBS 2	CBS 3
pH		4.18 ±0.014a***	4.4 ±0.014b***	4.535 ±0.035c***
Protein	g/100ml	0.29 ±0.02a***	0.37 ±0.04b***	0.53 ±0.05c***
Bitter	BITTER mg iso-alfa acids/beer	11.305 ±1.14a***	28.98 ±0.44b***	22.725 ±0.43c***
Colour	EBC average*	8.14 ±0.59a***	45.075 ±0.55b***	7.395 ±0.17a***
Colour	ASBC average**	4.13 ±0.30a***	22.89 ±0.28b***	3.76 ±0.09a***
Total Polyphenols	mg/100 ml gallic acid	124.94 ±6.82a***	261.11 ±6.96b***	222.27 ±6.62c***
Total Carbohydrates	g/100ml	2.65 ±0.10a***	2.81 ±0.032ab***	2.88 ±0.038b***
DPPH	IC 50 mg/KG Trolox	44.29b***	14.25a***	17.41a***
EtOH	ml/100ml	6.71 ±0.37a***	8.15 ±0.26b***	9.32 ±0.16c***
VDK	mg Vicinal Diketons/ kg	0,046 ±0.002a***	0,15 ± 0.002b***	0,12 ±0.002c***
FAN	mg FAN/L	103.5 ± 0.18a***	110.79 ± 1.61b***	108.57 ± 0.87b***

*Beer absorbance at 430 nm × 25 = colour in EBC units; **Beer absorbance at 430 nm × 12.7 = colour in ASBC units Data are the mean of three independent experiments±standard errors ***Diferent letters, by Tukey test, in the same row, indicate signifcant diferences at p≤0.05.

The main factors that characterize beer are alcohol content, bitterness of color, and variety and intensity of flavors, as well as factors to be monitored such as FANs and VDKs.

These characteristics are standardized and allow uniform determination of the overall qualities of any beer. The data show that all the beers had different alcohol

content. In contrast, significant differences were observed in color intensity, expressed as mean EBC and ASBC, which is also a measure of beer turbidity, and in IBU this is not a sensory perceived parameter of beer bitterness, but rather is an expression of the amount of iso-alpha acid presents, which not only plays an essential role in improving foam stability, but, as reported by Ano et al. [34], has an antioxidant role. Among the beers analyzed, both the highest EBC and ASBC value was observed for sample CBS2, followed by CBS1 and CBS3. These data showed lower turbidity of CBS1 and CBS3 Blanche and Lager compared to CBS2 Ipa style.

Bitterness (expressed as mg iso-alpha acids/beer) was the highest in CBS2 and CBS3 beers, while it was far lower in CBS1 beer, probably due to the fact that unmalted grains were used in CBS1 beer.

Bitterness (expressed as mg iso-alpha acids/beer) was the highest in CBS2 and CBS3 beers, while it was far lower in CBS1 beer, probably due to the fact that unmalted grains were used in CBS1 beer.

The average pH values of the analyzed beers were within the normal range for the category to which they belonged (between 4.18 and 4.55). pH is indeed an important parameter, not only because it conditions beer favor and taste, influencing its quality, but also because it functions it as a preservative by creating an adverse environment for many pathogenic and food-altering microorganisms [35]. The addition of bergamot peels in CBS2 and bitter orange peel and coriander in CBS1 definitely decreased pH values, compared to CBS3 (Classic Lager).

The protein values of beer should be close to zero, because proteins, binding to polysaccharides, form insoluble complexes that cause turbidity, compromising the age stability of the beverage [34]. In fact, the data obtained show for all three types of beer, very low values as reported in Table I.

Total carbohydrates present values ranging from 2.65 to 2.88 g/100 mL; values very similar to each other, but significantly different statistically for the three types of beers (Table 2), but still lower than the 3.3-4.4 g/100 mL interval recommended in the literature [36].

Regarding FANs, the values as reported in Table I ranged from a minimum of 103.5 mg FAN/L for CBS1 to a maximum value of 110.79 mg FAN/L for CBS2, values that fall within the recommended target range for beers (10-120 mg FAN/L for chare beers), indicated by the official ECB methods.

Concerning Vicinal Dyketones, the values of the three samples ranged from 0.046 mg VDK/Kg for CBS1 to a maximum value of 0.15 for CBS2, reflecting the recommended maximum contents indicated by the official ECB methods (<0.15 mg Vicinal Dyketones/ kg for ales).

Regarding nutraceutical activity, Total Polyphenols (mg/100 ml gallic acid) and DPPH (IC 50- mg/Kg Trolox) were considered. The beers that had the highest content of total polyphenols was CBS2 261.11 mg/100ml gallic acid followed by CBS3 222.27 and CBS1 124.94. This was definitely related to the malt and the different qualitative and quantitative factors used, as well as the fact that bergamot peels were used in CBS2 typology.

Accordingly, to the content of Total Polyphenols, the result of DPPH, obtained by oxidation of 2,2′-diphenyl-1-picrylhydrazyl radical, mirrored the data obtained in terms of IC50; in that the best results obtained were for CBS2 followed by CBS3 and CBS1 respectively of 14.25, 17.41 and 44.29 mg/Kg of Trolox.

4.5. Conclusions

In short, the production of these craft beers requires a combination of the type of product added, the stage of addition, and the diversification of the starting raw materials used.

The fact realative to the low content of total polyphenols and consequently a high DPPH value for sample CBS1 suggests that the use of flavoring ingredients is not solely responsible for the alterability of these indices; rather, these data could also be related to hops and malts. In that in the CBS1 sample, unmalted grains were

also used and therefore the development of melanoidins was not carried out thus affecting the antioxidant result of the CBS1 sample.

The beer, on the other hand, that showed the best results seemed to be CBS2, an IPA-style beer in which oats and wheat were used in addition to barley malt. This could be the reason for the excellent results obtained both in the antioxidant aspect and in the general chemical-physical characterization.

The CBS1 beer, on the other hand, was made with barley malt only, and still presented better results than the CBS1 beer.

Obviously, this is preliminary work, and needs further study and analysis.

4.6. Aknowlodgementes

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CHAPTER 5

A new analytical protocol through the application of biomimetic systems for discriminating craft beers.

Abstract

As is well known, the organoleptic characteristics of a beverage involve both quantitative and qualitative aspects. The evaluation of the latter is primarily based on the perception of our senses. In the case of beer, key parameters to assess include color, aroma, and flavor, which respectively engage our sense of sight, smell, and taste. Additionally, factors related to effervescence, which trigger somatosensory perceptions mediated by trigeminal receptors stimulated by carbonic acid on the surface of taste cells, are also crucial.

Over the past 10 years, Italians' relationship with alcoholic beverages, particularly beer, has gradually evolved. There has been a progressive increase in the age of consumers, coupled with a higher socio-cultural profile and greater awareness. Consumers, increasingly attentive, are oriented towards seeking quality, reading labels, and making informed choices.

Within this context, a study of the organoleptic characteristics was conducted using a biomimetic and sensoromic approach, employing tools such as the e-nose (electronic nose), E-Tongue (electronic tongue), Scio, and SpectraPod.

As a final working model various samples of lager-style beer were compared using the newly developed analytical protocol, employing both native and non-native hops, from a discriminative perspective of the aromatic profile.

Keyword: E-Nose, E-tongue, Aromatic compound, Sensor Response, craft beer, Principal Component Analysis.

5.1. Introduction

The brewing and maturation of beer involve intricate processes that necessitate the control of multiple parameters to ensure consistent taste and quality in the final product [1]. While sensory evaluation provides the most authentic representation of how humans perceive a product's taste, it is associated with practical challenges and disadvantages, including high costs, lack of reproducibility, and palate fatigue [2]. Consequently, there is a notable focus on developing efficient methods for routine beer analysis, encompassing both chemical composition and taste characteristics [3, 4].

Multisensory systems like the Electronic Tongue (ET) hold promise for evaluating the taste of food and beverages. The ET comprises an array of interconnected chemical sensors with cross-sensitive (partially selective) responses. These sensor responses are subject to analysis using pattern recognition techniques and multivariate calibration methods [5].

An electronic nose (E-nose) is currently an appealing technology. Developed by Persaud and Dodd in 1982 with the aim of simulating the human olfactory system [6], the concept involves using multiple sensors to generate diverse patterns of volatile compounds detected by these sensors. Additionally, it includes a pattern recognition system for identifying odors based on their patterns. Typically, an E-nose consists of three main components: (1) an array of gas sensors for detection, (2) a data processing unit to handle the sensor data, and (3) a pattern recognition unit [7]. Today, E-nose technology has applications in various odor-related fields, including the food industry, environmental monitoring, medical diagnostics, and explosive detection. In the food industry, E-nose technology has been applied to tasks like quality control, product characterization, freshness prediction, shelf-life estimation, and more [8–21].

Beer is undeniably a highly popular alcoholic beverage on a global scale, with an annual production and consumption of around 1.6×1011 liters per year [22]. Characterizing beer, particularly in terms of its aroma and taste, is of great

importance to the brewing industry [23]. However, few studies have concentrated on beer characteristics, especially its classification, using E-nose technology.

The electronic tongue (e-tongue) is an instrument designed to measure and compare tastes. It was created to emulate human olfactory and taste sensory functions and comprises an array of sensors. The e-tongue employs taste sensors to capture data from chemicals on the tongue, which is then relayed to a pattern recognition system. This process facilitates the identification of the tastes that constitute the human palate, typically categorized into five groups: sourness, saltiness, bitterness, sweetness, and umami (savoriness) [24].

For several years now, optical spectroscopy has firmly established itself as a reliable tool for comprehensive food analysis, particularly in rapidly and non-destructively evaluating nutraceutical and safety indicators. Notably, prominent and efficient spectroscopic techniques encompassing absorption, reflection, fluorescence, LIBS and Raman have been successfully tested and are progressively being integrated into numerous industrial process control systems. Furthermore, the extensive adoption of chemometrics and machine learning for processing spectroscopic data has significantly propelled the advancement of optical spectroscopy in food-related applications. [25].

The current state of the art highlights a widespread application of optical spectroscopy in beer analysis, particularly in the context of cereal breeding (assessing parameters such as protein, starch, polyphenols, lipids, minerals, and vitamins). Optical spectroscopy has also found utility during key stages of beer production, including the incorporation of hops for flavor stabilization, mashing, fermentation, and post-production processes. These aspects have been succinctly summarized in a recent review article cited as Reference [26]. Most of these successful examples have relied on the utilization of traditional spectrophotometers with wide spectral ranges and high-resolution capabilities.

With the aim of paving the way for future utilization of multi-spectral sensors in beer-related applications, we conducted a preliminary study using SCiO and SpectraPod devices on a small selection of beer.

The primary objective was to ascertain whether and to what degree fundamental distinctions between various beer types could be discerned through the employment of cost-effective devices. These devices hold the potential to be suitable for settings with limited resources, such as numerous artisanal producers.

For multispectral analysis and design purposes, two distinct commercially available spectral sensors were used: SCiO, developed by the Israeli company Consumer Physics Inc., and the more recent SpectraPod, produced by the Dutch company Mantispectra.

This exploratory pilot study presents results obtained through the use of two commercially available multispectral sensors for the evaluation of different beer categories.

Moreover, the study aims to assess the viability of an electronic tongue utilizing potentiometric chemical sensors as a swift analytical instrument for gauging the taste of beer and determining bitterness. Simultaneously, the electronic nose will be employed for the classification of aromatic fingerprints.

As a final working model, three types of craft Lager-style beers were analyzed using E-Nose and E-Tongue to assess aromatic and taste discrimination based on the raw materials used. These three craft beer models started from the same base (water, malt) but had different hop aromatization:

1- Commercial Cascade hops.

2- Cascade hops grown in Calabria.

3- Native hops found in Calabria.

The ultimate goal of this test is to incorporate these innovative devices into the brewing workflow in the near future.

5.2. Materials and Methods

5.2.1. E-noise

An electronic nose, or E-nose, serves the purpose of simulating the sense of smell found in humans. Instead of relying on the human olfactory system, E-nose technology employs multiple gas sensors to establish unique patterns for different scents and categorize specific odors.

Generally, an E-nose includes three primary components: a headspace sampler, a sensor array, and a pattern recognition system. These elements collaborate to create patterns of sensor signals, which are subsequently used to define different odors.

To provide more detailed information, the E-nose encompasses the following three major parts:

1. Odor Delivery System: This component is tasked with introducing the odors under examination into the system.
2. Detection System: It comprises an array of gas sensors that respond to the odors and produce sensor signals.
3. Processing System: The processing system plays a pivotal role in pattern recognition. It processes the sensor signals and generates distinct patterns that are associated with various odors. The development of an effective pattern recognition system within this component is crucial for the recognition and identification of more intricate odors.

In essence, an E-nose emulates the human sense of smell by utilizing sensors to detect and categorize odors, making it a valuable tool in various applications where the recognition and identification of scents are significant [27].

It consists of eighteen metal oxide semiconductor (MOS) gas sensors, which were readily obtained from Alpha MOS. In the case of MOS-type gas sensors, when they come into contact with an odor, typically a volatile chemical compound, the odor molecules adhere to the surface of the sensor's sensing material. As a result, this adhesion leads to a modification in resistance. This alteration is generally in proportion to the quantity of odor molecules attached to the sensor. The system's airflow direction was regulated using solenoid valves.

Three distinct beer varieties were considered: Blanche, IPA, and Lager, respectively.

To prepare the samples, the beers were diluted in a 1:3 ratio with distilled H2O and inserted into 10 mL vials with metal headspace caps (Headspace-Flasche, 46 x 22.5mm, purchased at Lab Logistics Group GmbH). Nine samples were used as technical replicates for the three different types of beer. Blank samples, composed of empty vials were inserted between the different beer styles for cleaning and reinitialization of the sensors in the final position. The acquisition time for both samples and blanks were 180 seconds.

5.2.2. E-Tongue

A device known as an Alpha Astree electronic tongue (e-tongue) manufactured by AlphaMOS in Toulouse, France, was employed to assess the flavor characteristics of the beer samples. The measurements were conducted within the FoCuSs Lab at the University "Mediterranea" in Reggio Calabria, specifically in the Department of Agraria.

Each sensor, of the chemFET type (chemically sensitive field-effect transistors), is composed of an organic coating that is sensitive to the substance responsible for the taste present in the sample and a transducer capable of converting the membrane's response into the signal to be analyzed. The electronic tongue operates by measuring the potential variation between the different sensors and the reference electrode (Ag/AgCl) [28]. The comprehensive signal of each sample, comprising a vector with the determinations of the seven sensors, is recorded as the sensor output and subsequently processed by the software (Astree II software, Alpha Mos, version 3.0.1, 2003).

This apparatus is equipped with a 48-position auto-sampler and incorporates seven food-grade sensors (AHS, SCS, ANS, CPS, NMS, CTS, PKS) for detecting variances in potential changes.

Three distinct beer varieties were considered: Blanche, IPA, and Lager, respectively.

For the preparation of the samples, three distinct beer varieties were considered: Blanche, IPA, and Lager, respectively. These beers were diluted at a 1:5 ratio with

distilled H2O. Eight samples were used as technical replicates for the three different types of beers. Blank samples containing only distilled H2O were inserted among the individual beer samples for sensor cleaning and re-initialization in an alternating sequence. The acquisition time for both the samples and blanks was 120 seconds.

5.2.3. SCiO

Multi-spectral sensors – SCiO and SpectraPod

A multi-spectral sensor is inherently designed as a device that observes a limited set of spectral bands with relatively broad resolution. This is achieved through the integration of a detector paired with an array of filters. Usually, these devices are characterized by a modest spectroscopic resolution and can function within either the visible or near-infrared regions. The specific spectral feature (bands, resolution signal-to-noise ratio, etc.) covered are contingent upon the filters and detector employed within the sensor.

The SCiO spectral sensor

SCiO was launched through a Kickstarter campaign in April 2014; however, it only became widely available in 2016. The underlying technology behind this spectral sensor remains shrouded in mystery due to numerous patents guarding the intellectual property rights. Unfortunately, the manufacturer has not released any detailed information about the technology's specifics.

Figure 1 presented herein, showcases an image of the device along with pertinent details that were contributed by tech geek enthusiasts who disassembled the device and shared their insights online. This device encompasses a notably luminous LED or potentially a downsized tungsten light source (exact nature unspecified). It integrates a silicon-based detector, along with an assembly of 12 filters equipped with a masked arrangement featuring differentially sized apertures to facilitate signal equalization. A microlens array is also incorporated to ensure optimal coupling of light with the detector. The operational range spans from 740 to 1070 nm. Operating this device requires a smartphone or tablet (compatible with either iOS or Android) through a dedicated cloud platform. The operational procedure involves activating the device and calibrating it using a provided white

reference sample. Reflectance data acquired by the spectral sensor from a given sample are sent to the cloud, which then transmits back a reconstructed spectrum. This reconstruction is formulated by considering the filters' transfer functions, which unfortunately remain undisclosed to the end user.

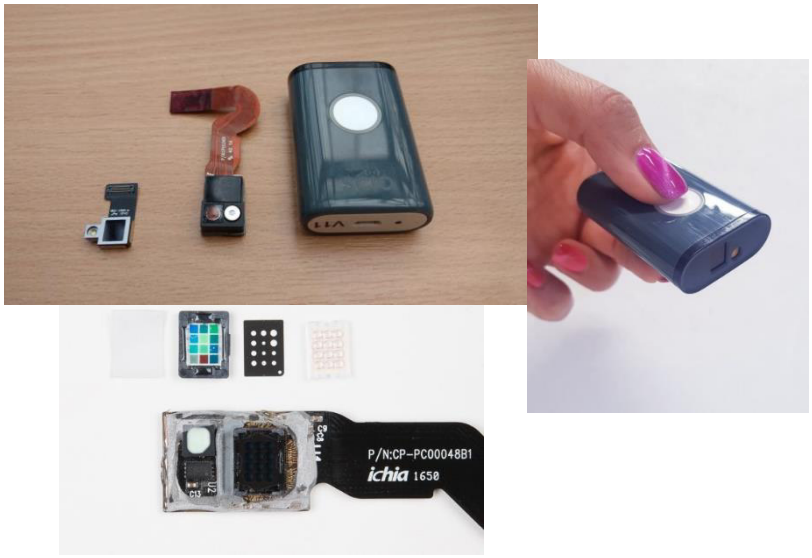

Figure 1. The SCiO spectral sensor and some details of the inside components including source, detector, filters with mask for signal equalization, and a microlens array for optimized light coupling to detector.

5.2.4. The SpectraPod spectral sensor

The SpectraPod stands as an independent handheld module, encompassing a 16-pixel integrated chip, an internal light source, and micro-optics elements for light collection. In Figure 2, the SpectraPod is depicted alongside some details of the 16-pixel integrated chip. The array of 16 pixels exhibits a custom-designed spectral response spanning the wavelength range from 850 to 1700 nm. Each pixel is fashioned within a solitary monolithic element that includes a thin absorbing layer and a tuning layer situated within an optical cavity. The individual filters within this configuration show a form of bimodal transmission.

This innovative technique involves the direct co-integration of detector and filter elements at the wafer level, culminating in a resilient system that can be efficiently manufactured at large scales using established semiconductor

processing techniques. In essence, the sensor produces a total of 16 signals, each corresponding to the integrated spectroscopic response of a distinct pixel.

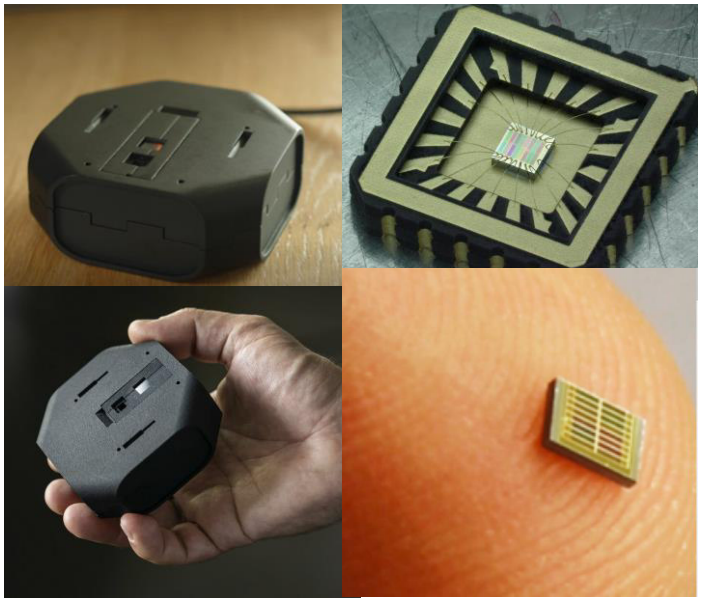

Figure 2. The SpectraPod spectral sensor, and details of the sensor chip and filters.

5.2.5. Main differences between SCiO and SpectraPod

Table I provides an overview highlighting the principal distinctions between the two spectral sensors. Beyond aspects like wavelength range and pixels, the most noteworthy difference lies in data accessibility: specifically, data captured by the SCiO are exclusively accessible via the proprietary cloud platform, whereas data acquired by the SpectraPod are immediately available in raw format, devoid of any reliance on internet connectivity. Additionally, considering prospective system integration, it's worth noting that the SpectraPod offers an OEM sensing board option, enabling seamless integration into tailored devices. Unfortunately, this capability is not provided by the SCiO, unless one engages with the producer for licensing arrangements.

Table I. Summary of the main differences between SCiO and SpectraPod.

Spectral sensor **Main features**	***SCiO***	***SpectraPod***
Wavelength range	740-1070 nm	850-1700 nm
Pixels	12	16
Connectivity and data access	Stand-alone, data achieved by internet-connection to cloud computing which provides the computed reconstructed spectrum	USB-connected to Laptop/PC – no internet connection needed – direct access to measured data
Data availability	Measured data sent to cloud computing – raw data not available, only reconstructed spectrum sent to smartphone	Raw data available as reflectance pattern – every pixel provides the integrated intensity transmitted by the filter(s)
Integration	Only pocket-size device available, no OEM version available	OEM sensing board available
Powered	USB-rechargeable integrated battery	USB-rechargeable integrated battery

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5.2.6. Sample preparation for beer spectroscopy

Table II provides an overview of the seven examined samples, accompanied by their fundamental quality characteristics. Three distinct beer varieties were considered: Blanche, IPA, and Lager, respectively. Measurements were conducted in two conditions—immediately upon uncapping the bottle (gassed sample) and following ultrasonic degassing. Additionally, a sample from a previously opened bottle of Lager was included to explore potential aging effects. For comparative purposes, a water sample was also incorporated in the study.

Table II. Overview of samples analyzed, and their fundamental quality characteristics.

Code	Sample	Type	Status	Alcohol
BLG	Blanche Gas	Blanche	Gas	6.7°
BLN	Blanche No-Gas	Blanche	No-Gas	6.7°
IPG	IPA Gas	IPA	Gas	8.2°
IPN	IPA No-Gas	IPA	No-Gas	8.2°
LGG	Lager Gas	Lager	Gas	9.3°
LGN	Lager No-Gas	Lager	No-Gas	9.3°
LGO	Lager Old	Lager	Old	N/A
WAT	Water	Water	Water	0

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Glass vials were used to contain the liquid samples, facilitating convenient use with the spectral sensors and straightforward measurements. To ensure consistent and reproducible readings, a 3D-printed housing was utilized to secure the vial in front of the SCiO sensor (Figure 3, left and center). On the other hand, for measurements using the SpectraPod, the vial was positioned directly in front of the device's optical window (Figure 3, right). For every sample, 4 measurements were recorded. These approaches ensured ease of use and accuracy during the measurement process.

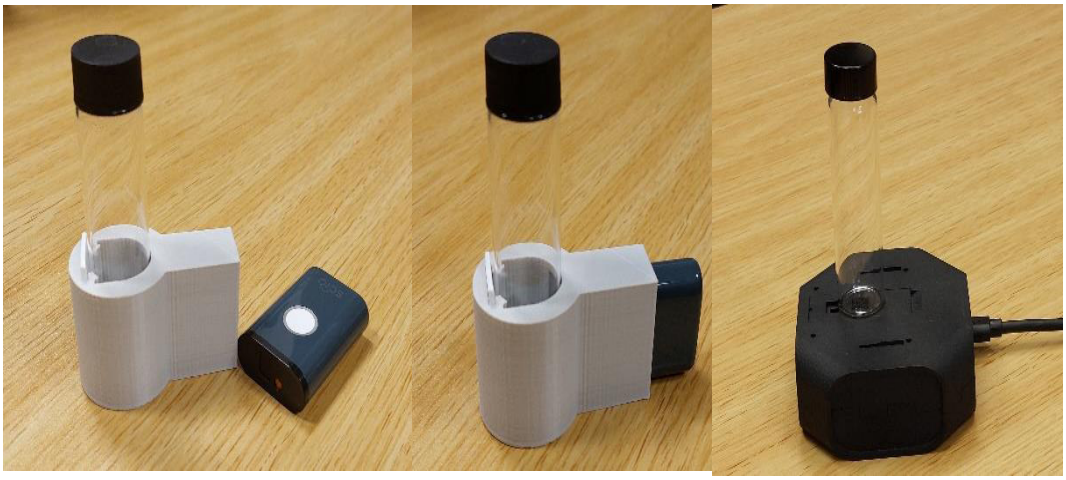


Figure 3. SCiO (left and center) and SpectraPod (right) fitted to vial.

5.2.7. Development of a new analytical protocol to discriminate the lager beers obtained by different hops using biomimetic systems (e-nose and e-tongue).

In the context of this action, lager beers created with different hops (Table III) were discriminated using the analytical protocol developed with the electronic nose and electronic tongue.

Table III. *Lager beers using different hops.*

Lager beers using different hops.	
BJ35	Barley malt, water, commercial hop variety (Cascade) grown in Calabria.
IC53	Barley malt, water, commercial hop variety (Cascade).
LZ30	Barley malt, water, native Calabrian hops.

5.2.8. Data analysis

To evaluate the ability of electronic tongue and electronic nose technologies in discerning different beers varieties, multivariate data analysis methods were applied. Qualitative analysis was conducted using Principal Component Analysis (PCA) and Discriminant Function Analysis (DFA), following the methodology outlined by Johnson and Wichern in 1992 [29].

PCA is an unsupervised method that breaks down the data matrix into principal components (PCs) to identify the most significant directions of variation within the multidimensional data space [30].

In contrast, DFA is a well-established supervised approach [30]. In DFA analysis, linear combinations of data are optimized to maximize the separation between clusters and minimize the variance within those clusters [30].

The key difference between PCA and DFA lies in their analytical approaches. PCA emphasizes the dataset's variance and doesn't consider the data's relationship to specific groupings. On the other hand, DFA integrates information about the groups and is founded on prior data classification.

The data was analyzed using AlphaSoft software, version 4.0.3.

To better highlight the differences and for an attempt of clustering the beers according to type, we carried out a multivariate data processing using the Principal Component Analysis, that is one of the most popular and robust method for unsupervised object classification [31,32]. For this processing we have used the commercially-available SIMCA software [33].

5.3. Results and Discussions

5.3.1. E-Nose

In the figures below, the results obtained from the analysis of the three beers using the Prometheus electronic nose (alphaMOS) are presented. This electronic nose comprises an array of 18 differently doped MOS sensors and is interfaced with a Mass Spectrometer.

As shown in Figure 4, the electronic nose is capable of distinguishing between the three beer recipes through Principal Component Analysis (PCA) with a discrimination index of 73%. Meanwhile, in Figure 5, Discriminant Function Analysis (DFA) achieves a discrimination index of 95%.

In Figures 6a, 6b, and 6c, the sensor responses for the analysis of white, IPA, and light beers, taken as examples, are depicted over a period of 180 seconds.

The sensor signals are typically based on the chemical sensitivity of metal oxide semiconductor (MOS) sensors, primarily used for odor measurement.

Odor molecules are detected through variations in the electrical resistance of the semiconductor. This change in resistance is attributed to combustion reactions occurring on the surface of metal oxide particles in response to the presence of chemical compounds. The output, which is an increase or mode of resistance, allows for the straightforward classification of the compound.

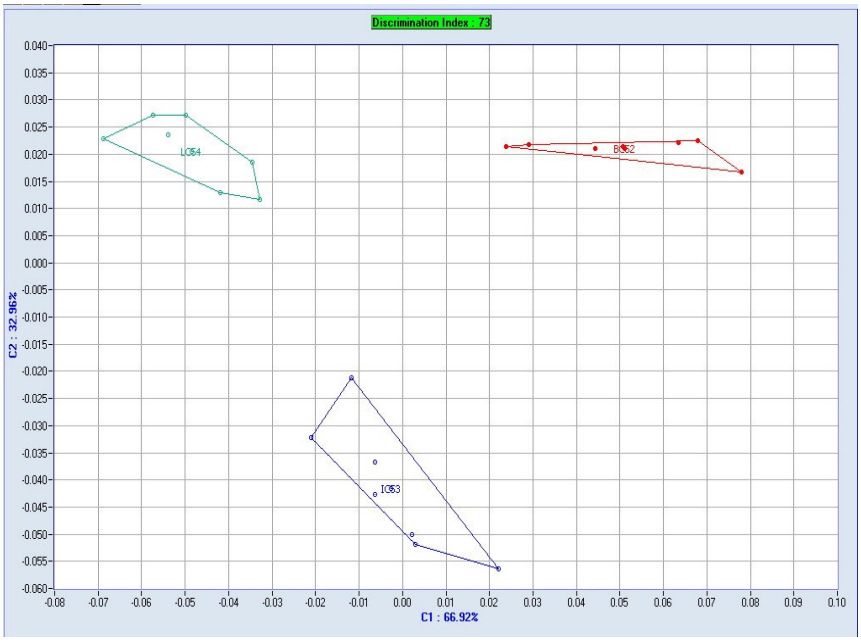


Figure 4: PCA, E-Nose beers

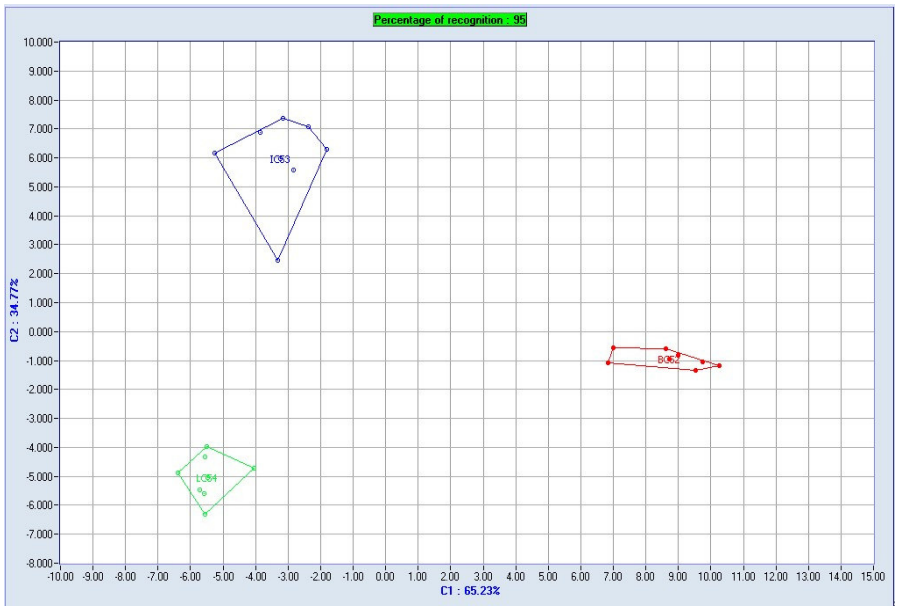
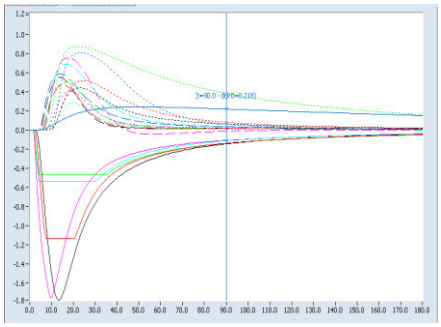
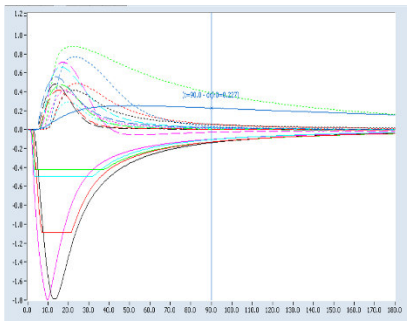


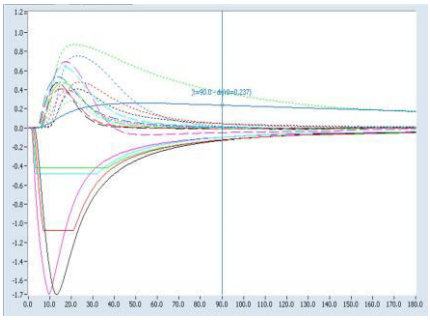
Figure 5: DFA, E-Nose beers.



a) Blanche



b) IPA



c) Lager

Figure 6: a) Blanche beer sensor response; b) response to IPA beer; a) lager beer sensor response.

5.3.2. E-Tongue

In Figures 9a, 9b, and 9c, the sensor responses are depicted for the analysis of white, IPA, and lager beers, taken as examples, over a period of 120 seconds with 1-second intervals. The sensor output results from potentiometric measurements obtained from the potential variation between the sensor's coating and the reference electrode Ag/AgCl. It is noticeable that the sensor responses are similar in white (Fig. 9a) and IPA beers (Fig. 9b), while they appear different and much less intense in lager beer (Fig. 9c).

In particular, sensors AHS, SCS, ANS, CPS, NMS, CTS, and PKS have shown responses expressed as signal intensity (mV) over time (s), all of which are linear. In white and IPA beers, sensors AHS and DPS exhibited the highest intensity values, while CPS displayed the lowest signal intensity, with the others showing lower signal intensity. However, in the case of lager beer, the difference in signal intensity is not as pronounced as in the other two, presenting a proportional signal

response. This can be easily explained since these chemical sensors, as miniaturized transducers, selectively and reversibly respond to the presence of taste-related chemical substances, generating electrical signals in real-time based on concentration.

The outputs of the sensors for different samples were processed using a multivariate statistical analysis model, PCA (Principal Component Analysis). PCA explains variance in experimental data by reducing the dimensionality of the data series from the database, represented as matrices (variables = sensor signals, objects = measurements). PCA can be seen as a repetitive process in which mutually orthogonal directions in the sensor-parameter space are calculated, along which the variance in the data series is maximized. These directions are the principal components, and PC1 is determined as the direction in the sensor-parameter space corresponding to the greatest variation in the data series. The second principal component, PC2, is the direction perpendicular to PC1, along which the remaining variation in the considered data series is maximized, and so on. For each principal component, the weight in determining the percentage of total explained variance is calculated as the percentage of data series variation along the PC.

The sensor responses were analyzed using PCA to determine if the electronic tongue could discriminate between beers produced with different recipes. From the observation of the results in Figures 7 and 8, it is evident that all beer samples are clearly separated from each other.

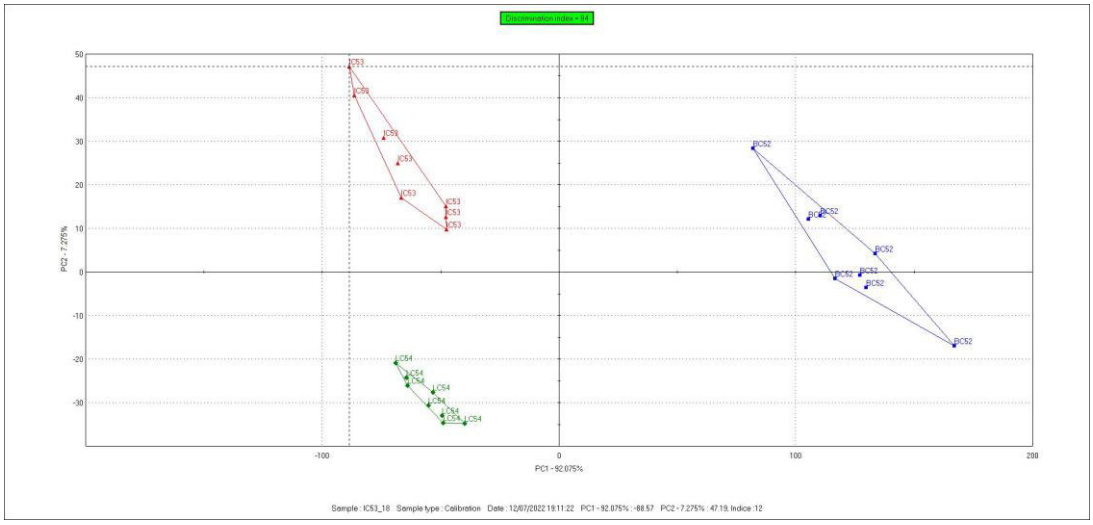


Figure 7: PCA beer, E-Tongue

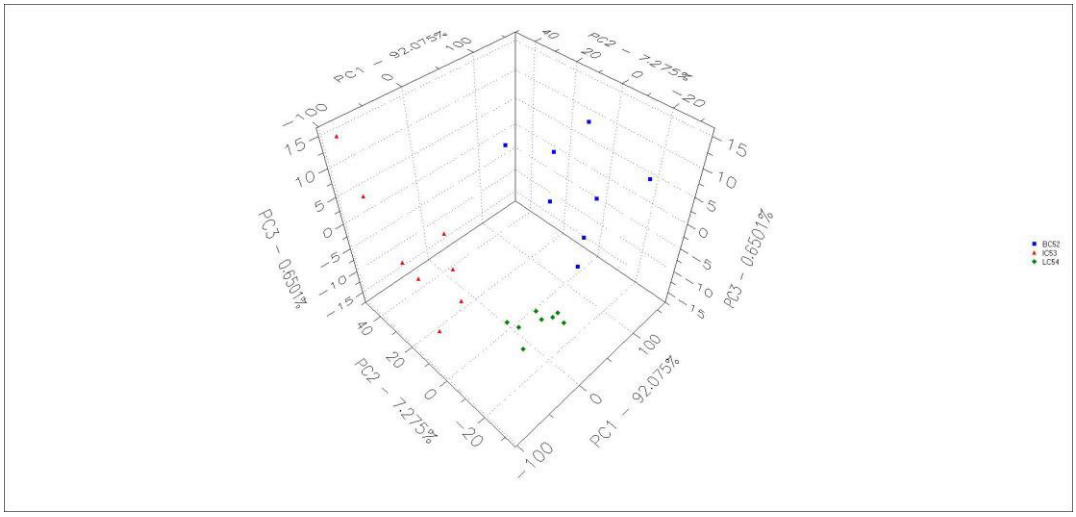
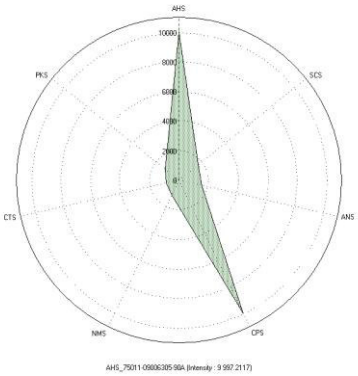
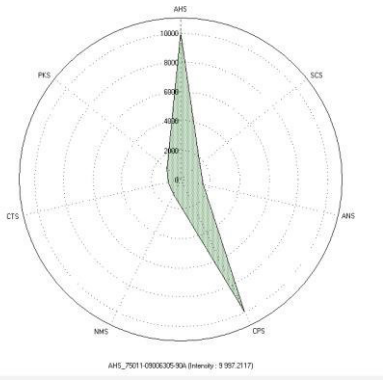


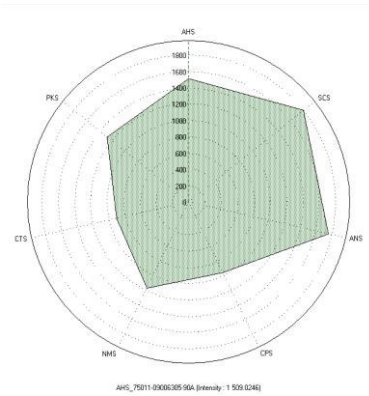
Figure 8: PCA 3d beers, E-Tongue.



a) Blanche



b) IPA



c) Lager

Figure 9: A) Blanche beer radar sensor response; B) radar response to IPA beer; C) lager beer radar sensor response.

5.3.3. SCiO and SpectraPod

Figures 10 and 11 show the measurement outcomes from SCiO and SpectraPod, respectively. Each figure showcases a pair of graphs, with beer distinguished by type (on the right) or gassed/degassed conditions (on the left).

In the case of SCiO, the initial reflectance spectra underwent transformation into absorption spectra, subsequently undergoing computation of the first derivative to mitigate variations in the baseline. Unquestionably, the spectrum pertaining to water displays distinctive dissimilarity. Furthermore, even with a cursory examination, discernible distinctions among the various beer types are apparent. The most informative band is 860-1060 nm, while the edges of the operational range of the instrument do not add anything useful.

Similarly, in the case of SpectraPod, the reflectance pattern of water manifests clear disparities compared to beers, and noteworthy distinctions are observable for both beer types and gassed/degassed conditions. Particularly pronounced variations are evident in pixels: 1, 6, 7, 10, and 13.

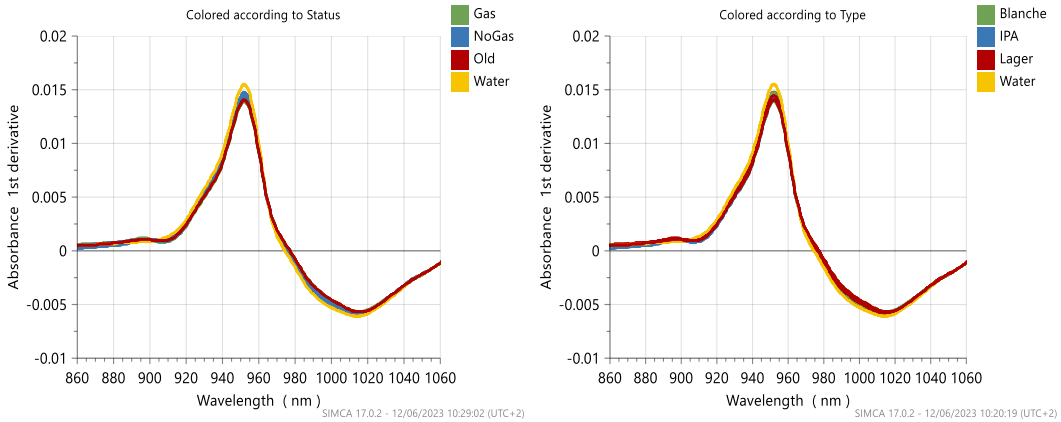


Figure 10. First-derivative absorption spectra of beers and water measured by SCiO.

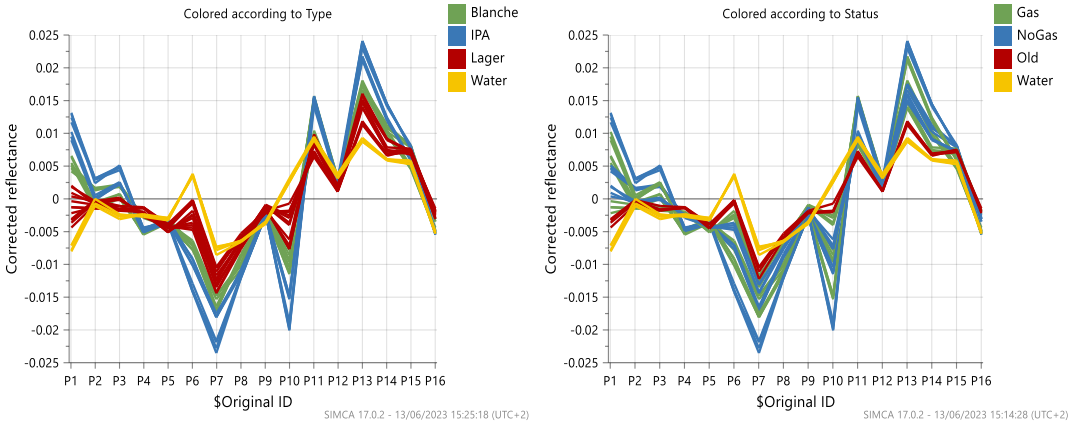


Figure 11. Reflectance patterns of beers and water measured by SpectraPod.

In order to emphasize distinctions and attempt to group the beers by type, we conducted a multivariate data analysis through Principal Component Analysis (PCA), which is a widely used and reliable method for categorizing objects without prior supervision.

Figures 12 and 13 show the outcomes of these processing for the data of SCiO and SpectraPod, respectively, that are the PCA score plots of the first two Principal Components (SIMCA names t[1] and t[2] the first and second Principal Components, respectively). As previously considered, each figure showcases a pair of graphs, with beer colored by type (on the right) or gassed/degassed conditions (on the left). The spread of dots for every beer type refers to the replica measurements.

Regarding SCiO's outcomes, the following observations can be succinctly made:

- In Figure 12 (left), the first Principal Component, responsible for 88% of the variance, effectively distinguishes between water, degassed, and gassed beer samples. This distinction is evidently influenced by the presence of carbonic acid microbubbles.
- In Figure 12 (right), the second principal component, responsible for 9.8% of the variance, notably separates IPA from Lager and Blanche types. This differentiation does not seem to be correlated with alcohol content, as the absence of IPA and Lager pairing suggests.

In the context of SpectraPod outcomes, the observations can be summarized as follows:

- In Figure 13 (left), the first Principal Component, responsible for 94% of the variance, effectively distinguishes between IPA, Blanche and Lager beers and is not influenced by the alcoholic content.
- In Figure 13 (right), the second Principal Component, responsible for 5.1% of the variance, notably separates gassed from degassed IPA and Blanche beers, but this distinction does not occur for Lager beers.

In light of the distinctive separations achieved by the first Principal Component of SCiO data for distinguishing gassed and degassed beers, and the corresponding separation of IPA, Blanche, and Lager beer types by the first Principal Component of SpectraPod data, a decision was made to combine these datasets. The subsequent representation of the first Principal Components of SCiO against that of SpectraPod in a graph has yielded promising outcomes, as depicted in Figure 14.

The left side of Figure 14 underscores the effectiveness of SpectraPod's first Principal Component (PC-1 SP) in discriminating between beer types. Conversely, the right side of Figure 14 emphasizes the robustness of SCiO's first Principal Component (PC-1 SCiO) in distinguishing between degassed and gassed beer samples. Notably, the placement of the old Lager nearly aligning with the gassed samples on Figure 14-right indicates the lingering presence of residual gas.

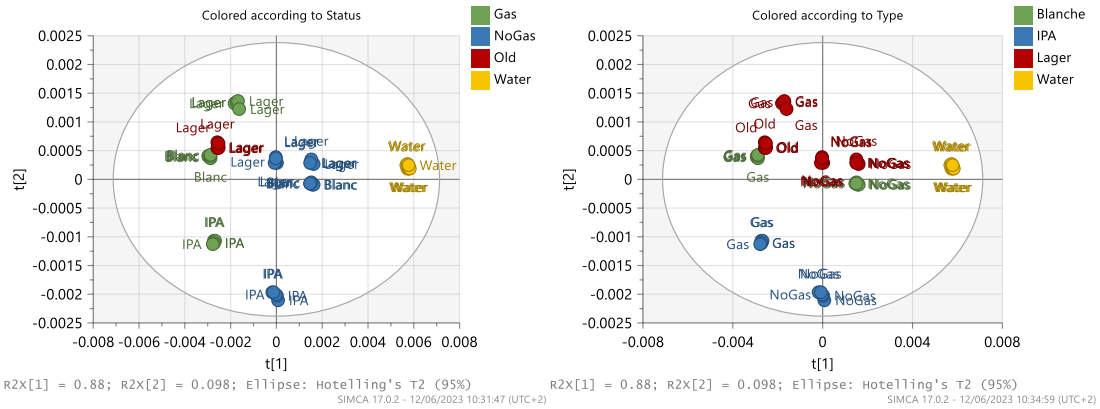


Figure 12. PCA score plots of SCiO data.

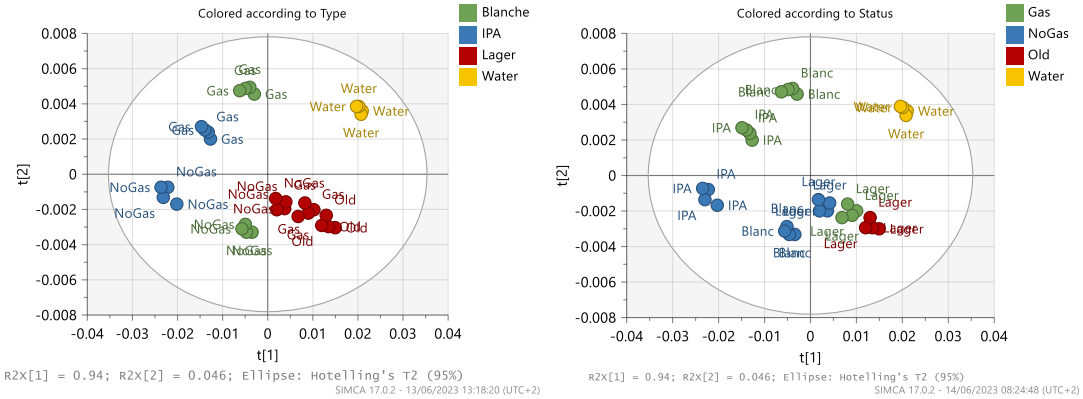


Figure 13. PCA score plots of SpectraPod data.

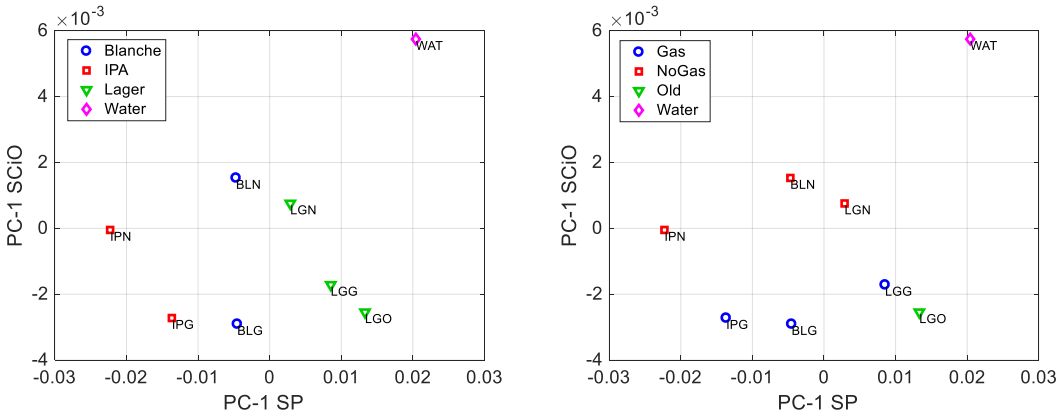


Figure 14. First Principal Components of SCiO (PC-1 SCiO) and SpectraPod (PC-1 SP).

In light of these preliminary assessments, certain observations can be drawn:

1. *Sensitivity to Gas:* The sensitivity of SCiO to gas appears to be more pronounced compared to SpectraPod. This distinction might arise from the fact that microbubbles of carbonic acid exhibit heightened scattering intensity at shorter wavelengths.
2. *Distinguishing Beer Types:* An interesting differentiation arises in terms of distinguishing beer types. SCiO exhibited limited capacity in discriminating between different beer types, primarily being able to distinguish only the white beer types. In contrast, SpectraPod demonstrated a robust ability to differentiate between various beer types, which might be attributed to the broader band measurements it employs.
3. *Alcohol Content:* Notably, both instruments did not exhibit the ability to discriminate beer types based on their alcohol content, indicating that alcohol concentration is not a defining factor in these measurements.

These initial insights pave the way for further investigations, refining methodologies, and expanding the dataset to ascertain the broader capabilities and potential applications of these spectral sensors in the context of beer analysis.

5.3.4. Development of a new analytical protocol to discriminate the lager beers obtained by different hops using biomimetic systems (e-nose and e-tongue).

In Figure 15, sensor responses for the analysis of lager beers, taken as examples (table III), over a period of 120 seconds with 1-second intervals are depicted. Specifically, sensors SCS, ANS, NMS, and PKS showed non-linear responses expressed as signal intensity (mV) over time (s). In the case of BJ35 and IC53 beers, sensor SCS exhibited the highest intensity values, while NMS and PKS showed the lowest signal intensity, with ANS showing a moderate signal intensity. However, for LZ30 beer, the difference in signal intensity is not as pronounced as in the other two, displaying a proportional signal response. This can be easily explained as these chemical sensors, as miniaturized transducers, selectively and reversibly respond to the presence of taste-related chemical substances, generating real-time electrical signals based on concentration.

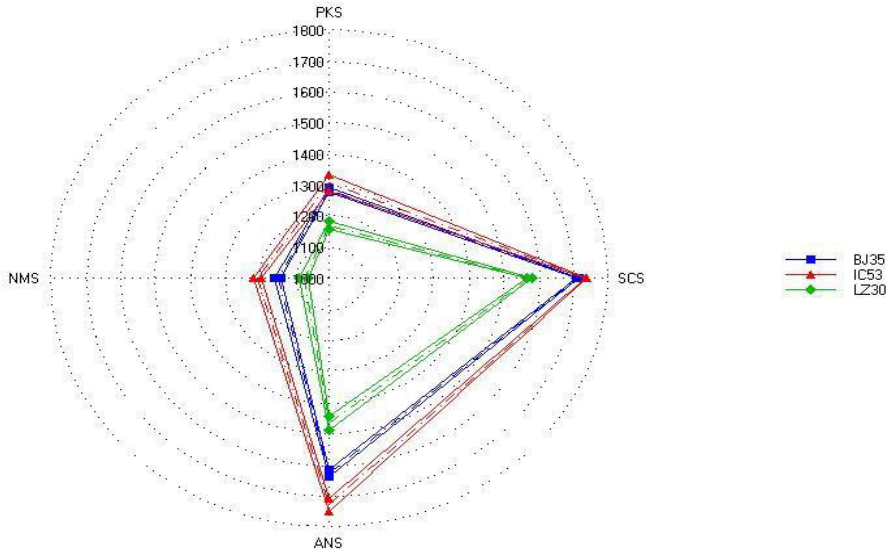


Figure 15: Radar Sensor response lager beers, E-Tongue.

The results of the sensors for different samples were analyzed using a statistical technique called Principal Component Analysis (PCA). PCA aims to explain variation in experimental data by reducing data complexity. PCA works by calculating orthogonal directions in the sensor-parameter space along which data variance is maximized. These directions are known as principal components. The first principal component (PC1) represents the direction with the greatest data variance. The second principal component (PC2) is perpendicular to PC1 and captures the remaining variation in the data. This process continues for additional principal components. The percentage of variance explained by each principal component is calculated as a weight contributing to the overall data understanding.

Sensor responses were analyzed using PCA to determine whether the electronic tongue could discriminate between beers flavored with different hops. From the observation of the results in Figure 16, it is evident that all beer samples are clearly separated from each other.

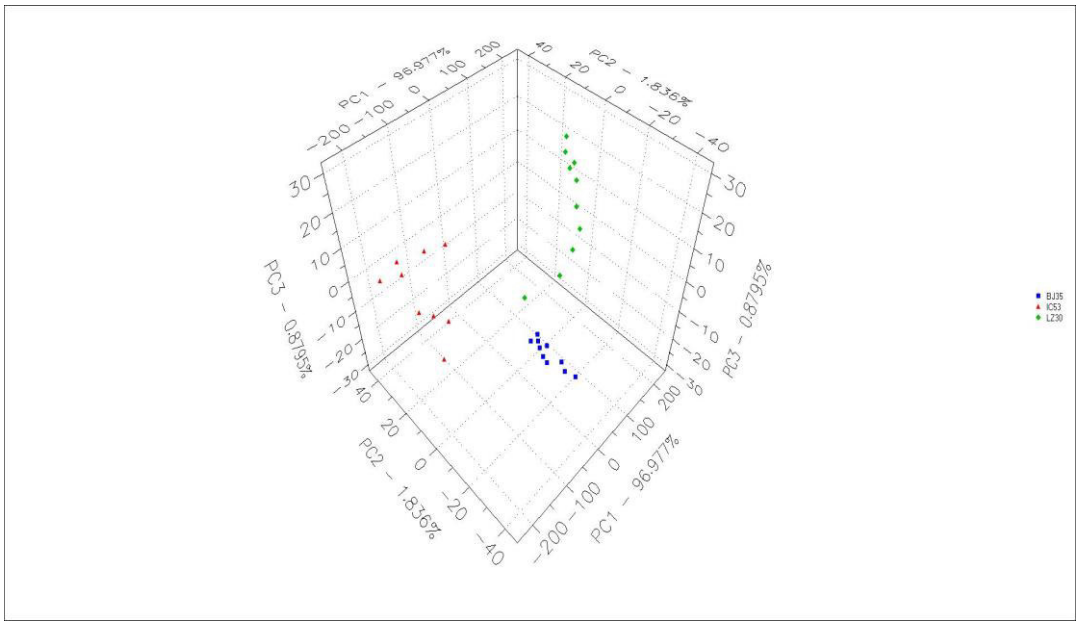


Figure 16: PCA 3d lager beers, E-Tongue.

In Figure 17, the results of the analysis of three beers with different hop profiles using the Prometheus electronic nose (alphaMOS) are presented. The electronic nose demonstrates a high capability to distinguish between the three beer recipes through Principal Component Analysis (PCA), achieving a discrimination index of 87%.

Metal oxide semiconductor (MOS) sensors detect odors by monitoring variations in the electrical resistance of the semiconductor, with this resistance change attributed to combustion reactions on the surface of metal oxide particles caused by the presence of chemical compounds. This change in resistance produces an output that allows for the direct classification of the compound.

In summary, these sensors detect odors by monitoring changes in electrical resistance due to chemical reactions on the surface of metal oxide particles caused by the presence of chemical substances. This output enables the identification of the compound.

Figure 17: PCA, lager beers, E-Nose.

5.4. Conclusions

The studies conducted aimed to develop methods based on biomimetic systems such as E-Nose, E-Tongue, Scio and SpectaPod to analyze the main characteristics of beers, including color, aroma, and taste, which respectively stimulate sight, smell, and taste. The objective was also to assess the different features in the three distinct styles of beer.

Exploratory experiments were conducted using these methods, with the ultimate goal of creating tools for non-destructive assessment of quality indicators during beer production in the future. In this preliminary pilot study, a limited number of beer samples were examined. Despite the limited quantity of samples available, it was possible to clearly distinguish between different types and styles of beer.

These initial findings, obtained through the application of the methods used in this study, strongly suggest that, even with reduced sensitivity, spectroscopy and biomimicry applied with sensors have significant potential as valuable techniques for real-time monitoring in various stages of beer production. Currently, further experiments are planned to validate and strengthen these preliminary results. This involves working with a much larger set of beer samples, including established reference data for building models of nutraceutical and quality indicators.

5.5. Acknowledgements

We extend our gratitude to Anna G. Mignani, Leonardo Ciaccheri, and Barbara Adinolfi from CNR-Istituto di Fisica Applicata "Nello Carrara" (Sesto Fiorentino - FI) for their invaluable contribution in providing access to SCiO and SpectraPod for the purpose of beer analysis. Their support extended not only to the instrumental availability but also encompassed expertise in multivariate data processing, a pivotal aspect of this research endeavour.

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CHAPTER 6

A new method for simultaneous determination of polyphenols and in beer by UHPLC-PDA

Abstract

The present study reports the development of a new method for the simultaneous identification of polyphenols in beer samples, using an ultra high-performance liquid chromatography system coupled to a photodiode array detector (UHPLC-PDA). Thirty-two polyphenols were identified simultaneously in 29 minutes, with optimized separation conditions considering the best separation for all standard compounds considered. 7-level calibration curves were constructed for the quantification of polyphenols and the method was validated for these analytes with the parameters of LOD, LOQ, R2, repeatability and reproducibility.

Three beers with different styles were analyzed by validating a direct sample injection system.

Keywords: *Polyphenols, HPLC, DAD, Lod, Loq, Craft Beer.*

6.2. Introduction

Beer is obtained with the alcoholic fermentation of the must produced with four main raw materials: malted and unmalted cereals, water, hops (*Humulus lupulus L.*) and yeast [1]. The chemical composition of beer, therefore taste, aroma and color, changes with the variation of raw materials and production processes [2].

The taste of beer is determined by numerous compounds, including polyphenols, which are 80% obtained from cereals and 20% from hops [3]. The polyphenolic composition of beer can change significantly due to the use of particular types of

cereals and hops, but also for "characterizing foods" such as fruit, herbs and juices used for the production of flavored beers.

Roasted malts are used to produce dark beers such as Stout and Imperial Stout, they have a strong and decisive flavor caused by the decarboxylated phenols and caramelized sugars [2,4].

Beers produced with citrus juices, such as beers with bergamot, or with grape must (Italian Grape Ale or IGA) are characterized by phenols do not present in classic beers since they come from "characterizing foods".

The phenols, in addition to determining the taste of the beer, stabilize the fermented product, improving its state of conservation over time. Polyphenols such as flavon-3-oils naturally chelate the metals present in solution, while numerous families of phenols inhibit the oxidative processes that induce the formation of diketones, sulfur compounds, aldehydes and fatty acids with low molecular weight, all molecules which cause the maturation of the beer [5,6].

The polyphenolic profile of craft beers, primarily associated with barley and hops, is not only a significant indicator of the nutritional and antioxidant quality of beers, colloidal stability, and the ability to interact with proteins, but it also profoundly influences their sensory characteristics (color, aroma, and flavor). Therefore, it is crucial to find a fast, easy, cost-effective method that is readily available in common analysis laboratories to unequivocally trace the polyphenolic fingerprint of beers.

Often beers with a high amount of bitter acids, such as Indian Pale Ale (IPA) and American Pale Ale (APA), are perceived by the consumer as less bitter than beers with a medium amount of bitter acids, such as Bitter and Strong Bitter. This phenomenon is due to the type of bitter acids present in beer and the presence of phenols, which balance the bitter taste [1,3,7].

Chromatographic methods have been developed for the identification and quantification of phenolic acids and flavonoids [4,12,13,14]. Some methods involve a sample preparation with liquid liquid extraction (LLE) with organic

solvents [15,16], other procedures involve the injection of the sample without treatments, determining only a part of phenols contained in the samples [17,18].

Total phenols YYin beers are generally quantified by different spectrophotometric methods developed by the American Society of Brewing Chemists (ASBC) and European Brewery Convention (EBC) [3,8,9,10,11]. Chromatographic methods have been developed for the identification and quantification of phenolic acids and flavonoids [4,12,13,14]. Some methods involve a sample preparation with liquid liquid extraction (LLE) with organic solvents [15,16], other procedures involve the injection of the sample without treatments, determining only a part of phenols contained in the samples [17,18].

Few studies have been published for the identification of the different bitter acids in beers using chromatographic methods, all have a liquid-liquid extraction method (LLE) using organic solvents [19,20,21,22].

The aim of this project was to develop and validate an ultra-high-performance liquid chromatography method coupled to a photodiode array detector (UHPLC-PDA) for the simultaneous analysis of different classes of phenols present in beers by making a quick and simple sample preparation.

6.3. Materials and methods

6.3.1. Chemical substances

Ultrapure water was obtained using a Milli-Q system (Millipore, Milan, Italy), formic acid and acetonitrile (ACN) with HPLC grade of purity were purchased from Sigma-Aldrich (Milan, Italy), while the phenol standards (*p*-Coumaric acid, 4-Hydroxybenzoic acid, Caffeic acid, Ethylgallate, Ferulic acid, Kampferol, Naringin, Protocatechuic acid, Rutin, Syrengin acid, Vanillic acid, Hesperidin, Sinensetin, Neodiosmin, Neoeriocitrin, Hesperetin, (-)Epicatechin, Eriocetrin, Isorhamnetin, Myricetin, Neohesperidin, Diosmin, Narirutin, Rhamnetin, Tangeretin, Apigenin, Chlorogenic acid, Nobiletin, Naringenin) were purchased from Extrasynthese (Genay Cedex, France).

6.3.2. Samples

Three beers were manufactured in a pilot-scale facility at the Wild Orange Fermented Beverage Technology Room in Vibo Valentia, Calabria, Italy, through a collaboration between the FoCuSs Laboratory and the Pedology Laboratory of the Mediterranean University of Reggio Calabria.

These craft beers belong to the most popular beer styles in Europe (Table I). The craft beer samples were as follows:

***Table I**. Craft Beer Samples*

Craft beers	
BC52	*water, barley malt, hops, unmalted wheat, bitter orange peel, and coriander seeds.*
IC53	*water, hops, barley malt, wheat, oat flakes, and bergamot peels.*
LC54	*water, hops, and barley malt.*

The analysis was carried out in triplicate for each batch and for each type of Craft Beer. It's worth noting that while the barley malt, yeasts, and hops were consistent across all three beer styles, the grains and flavor bases were modified.

The beers were stored in a cool dark place at 4 °C and were analyzed well before the deadline.

The beers are handcrafted in compliance with Italian legislation [23], therefore, they are: not pasteurized, not microfiltered and produced by small independent breweries with production below 200,000 hL/year.

6.3.3. Sample preparation

The beers were degassed at a temperature of 20 °C with magnetic stirring (500 rpm) for 5 hours. A representative amount of each beer (5mL) was filtered with a hydrophilic membrane with a pore size of 0.45 μm (Aisino Corporation

regenerated cellulose membrane). The analyzes were conducted on the same day as the degassing and filtration process.

6.3.4. UHPLC-PDA instrumentation

The analyzes were carried out with a Shimadzu Nexera UHPLC-PDA system (Shimadzu, Kyoto, Japan), composed of a controller (CBM-20A), a degasser (DGU-20A5R), dual-plunger parallel-flow pumps (LC-30AD), an autosampler (SIL-30AC), a column oven (CTO-20AC), a photodiode detector (SPD-M30A). LC data processing was performed with LCsolution software (Version 5.71, Shimadzu).

6.3.5. UHPLC-PDA condition

The analytical conditions used for the analyzes have been optimized to obtain the best chromatographic separation for the classes of molecules considered, phenols and bitter acids.

Ten microliters of degassed and microfiltered sample were injected without performing preliminary extraction procedures. The chromatographic separation was carried out with a Kinetex C18 column (50 mm × 3 mm × 1.7 µmd.p) and a Kinetex C18 pre-column, the columns are manufactured by Phenomenex (Torrance, California, United States). The oven temperature was set at 40°C, a flow rate of 0.6 mL/min was used with mobile phases composed of water with 0.1% formic acid (v/v) (mobile phase A) and acetonitrile with 0.1% formic acid (v/v) (mobile phase B). The gradient used was: 5 minutes with 1% B (isocratic mode), 15 minutes from 1% to 30% B, 3.5 minutes from 30% to 47% B (gradient mode for the separation of polyphenols), 30 seconds from 47% at 60% B, 2 minutes with 60% B (isocratic mode for the separation of bitter acids), washing the system with 100% B and reconditioning with 1% B.

The photodiode detector was set with 8 nm divided width, 256 spectrum resolution, 40 Hz sampling rate, 40°C cell temperature and 190-450 nm analysis range.

6.3.6. Validation method to quantify polyphenols

Seven concentration levels of the polyphenolic standards were prepared with methanol from a 1000 mg/L stock solution with concentration range of 0.5-120 mg/L. Five analyzes were performed for each concentration level with the HPLC-PDA system under optimized chromatographic conditions. Seven-level calibration curves were constructed using the least squares method by obtaining the equations of the regression lines (table II). Mandel's test confirmed the linearity of each calibration curve in the considered range. The limits of quantification (LoQs) and limits of detection (LoDs) (table II) were calculated by multiplying the standard deviation (SD) of the lowest level of the calibration curve (n = 7) ten and three times, respectively, and dividing the result for the slope of the calibration curve. The repeatability and reproducibility values (table II) were expressed as percentage coefficient of variation (CV%) and calculated using the average of the areas of the lowest level of the calibration curve (n=7) divided by the corresponding standard deviations. Finally, retention time, instrumental recovery and percentage relative standard deviation (RSD%) were determined using the fourth level (n=4) of each calibration curve (table III).

***Table II**: Validation parameters values determined for the polyphenols.*

Compounds	***Linear range (ppm)***	***Regression equation***	***R2***	***LOD (ppm)***	***LOQ (ppm)***	***Repertability (%)***	***Riproducibility (%)***
Coumaric acid	*0.5-100*	*y = 101.919.0398x - 19.710.9815*	*0.9998*	*0.033*	*0.097*	*1.81*	*1.49*
4-Hydroxybenzoic acid	*0.5-100*	*y = 66.976.5781x + 8.811.2215*	*0.9996*	*0.047*	*0.156*	*3.46*	*3.42*
Caffeic acid	*0.5-100*	*y = 80.403.9809x – 5.618.2263*	*0.9998*	*0.050*	*0.167*	*3.892*	*2.89*
Ethylgallate	*0.5-100*	*y = 43.307.1523x + 2.133.0116*	*0.9997*	*0.075*	*0.250*	*5.68*	*4.30*
Ferulic acid	*0.5-100*	*y = 76.190.7484x – 15.485.2230*	*0.9999*	*0.062*	*0.205*	*4.72*	*3.50*
Gallic acid	*0.5-100*	*y = 58.838.4809x – 34.304.6944*	*0.9996*	*0.065*	*0.218*	*5.16*	*5.19*
Kampferol	*0.5-100*	*y = 35.708.4046x – 8.738.4609*	*0.9997*	*0.051*	*0.171*	*5.41*	*3.82*
Naringin	*0.7-110*	*y = 23.180.1541x – 18.258.1645*	*0.9994*	*0.034*	*0.081*	*1.71*	*6.00*
Protocatechuic acid	*0.5-100*	*y = 56.968.3550x + 10.041.8594*	*0.9996*	*0.079*	*0.264*	*5.57*	*4.19*
Rutin	*0.7-110*	*y = 26.417.8491x – 27.143.8067*	*0.9993*	*0.048*	*0.159*	*3.40*	*3.79*
Syrengin acid	*0.5-100*	*y = 45.154.2555x – 19.290.0310*	*0.9998*	*0.081*	*0.270*	*5.81*	*5.01*
Vanillic acid	*0.5-100*	*y = 39.046.0740x – 17.589.1592*	*0.9998*	*0.076*	*0.254*	*5.69*	*6.00*
Hesperidin	*0.5-*	*y = 25.158.0962x*	*0.9999*	*0.040*	*0.107*	*1.22*	*2.39*

	100	*– 8.615.2595*					
Sinensetin	*0.5-100*	*y = 52.911.3069x – 17.330.2806*	*0.9995*	*0.029*	*0.076*	*1.01*	*0.99*
Neodiosmin	*0.5-100*	*y = 22.188.5733x – 11.478.9917*	*0.9996*	*0.039*	*0.083*	*1.45*	*4.38*
Neoeriocitrin	*0.5-100*	*y = 21.193.8726x – 8.292.7441*	*0.9998*	*0.043*	*0.086*	*1.66*	*2.13*
Hesperetin	*0.5-100*	*y = 61.439.7309x – 25.498.1206*	*0.9998*	*0.053*	*0.175*	*1.24*	*2.56*
(-)Epicatechin	*0.7-110*	*y = 8.995.0663x – 8.543.3500*	*0.9993*	*0.075*	*0.251*	*4.90*	*4.05*
Eriocetrin	*0.5-100*	*y = 22.104.7983x – 5.509.6099*	*0.9997*	*0.040*	*0.096*	*1.39*	*4.19*
Isorhamnetin	*0.5-100*	*y = 44.346.4593x – 26.794.1428*	*0.9997*	*0.028*	*0.093*	*2.45*	*3.98*
Myricetin	*0.5-100*	*y = 44.789.0025x – 37.079.9968*	*0.9997*	*0.057*	*0.190*	*6.38*	*5.41*
Neohesperidin	*0.5-100*	*y = 10.709.8118x + 3.447.8446*	*0.9993*	*0.032*	*0.106*	*1.36*	*1.65*
Diosmin	*0.5-100*	*y = 4.214.8056x + 1.093.5404*	*0.9987*	*0.077*	*0.255*	*1.19*	*1.24*
Narirutin	*0.5-100*	*y = 22293x + 2686.4*	*0.9995*	*0.106*	*0.355*	*4.78*	*5.53*
Rhamnetin	*0.5-100*	*y = 41523x – 22496*	*0.9995*	*0.048*	*0.159*	*5.10*	*4.15*
Tangeretin	*0.5-100*	*y = 63271x – 8409.3*	*0.9995*	*0.050*	*0.110*	*0.67*	*2.02*
Apigenin	*0.5-100*	*y = 68.168.9349x – 21.240.6742*	*0.9994*	*0.036*	*0.120*	*2.81*	*3.23*
Chlorogenic acid	*0.75-120*	*y = 37088x – 53084*	*0.9992*	*0.084*	*0.281*	*5.08*	*5.34*
Nobiletin	*0.5-100*	*y = 48.288.0608x – 24.771.5556*	*0.9995*	*0.038*	*0.078*	*1.21*	*2.52*
Naringenin	*0.5-100*	*y = 36.851.3110x – 17.639.1841*	*0.9997*	*0.033*	*0.077*	*1.69*	*3.69*

Table III. *R*etention time, instrumental recovery and percentage relative standard deviation of polyphenols.

Compounds	***Rt***	***±SD***	***RSD%***	***Recovery (%)***
Coumaric acid	*12.396*	*0.008*	*0.496*	*102.1*
4-Hydroxybenzoic acid	*4.060*	*0.014*	*1.310*	*103.8*
Caffeic acid	*7.751*	*0.011*	*0.601*	*102.0*
Ethylgallate	*9.836*	*0.020*	*1.396*	*102.7*
Ferulic acid	*11.323*	*0.007*	*0.258*	*101.9*
Gallic acid	*1.07*	*0.012*	*0.208*	*94.6*
Kampferol	*18.551*	*0.014*	*0.767*	*100.8*
Naringin	*14.125*	*0.010*	*0.358*	*93.2*
Protocatechuic acid	*2.157*	*0.009*	*0.769*	*102.3*
Rutin	*13.01*	*0.012*	*0.668*	*93.2*
Syrengin acid	*8.996*	*0.008*	*0.283*	*97.3*
Vanillic acid	*7.344*	*0.015*	*1.172*	*95.9*
Hesperidin	*14.531*	*0.007*	*0.321*	*95.8*
Sinensetin	*21.488*	*0.006*	*0.385*	*108.2*
Neodiosmin	*15.191*	*0.014*	*0.356*	*95.5*
Neoeriocitrin	*12.986*	*0.011*	*0.772*	*95.1*
Hesperetin	*18.558*	*0.018*	*0.246*	*96.0*
(-)Epicatechin	*9.880*	*0.013*	*0.868*	*93.9*
Eriocetrin	*12.597*	*0.015*	*0.657*	*97.0*
Isorhamnetin	*19.087*	*0.019*	*1.357*	*95.6*
Myricetin	*14.332*	*0.019*	*1.066*	*95.0*
Neohesperidin	*14.925*	*0.042*	*0.604*	*100.8*
Diosmin	*14.864*	*0.011*	*2.077*	*86.9*
Narirutin	*13.721*	*0.016*	*0.931*	*105.1*
Rhamnetin	*20.826*	*0.008*	*0.368*	*102.3*
Tangeretin	*23.290*	*0.019*	*0.964*	*104.4*
Apigenin	*18.489*	*0.009*	*0.655*	*102.8*
Chlorogenic acid	*8.637*	*0.015*	*0.837*	*95.9*
Nobiletin	*22.432*	*0.006*	*0.077*	*99.7*
Naringenin	*17.644*	*0.011*	*0.101*	*99.6*

6.3.7 Statistical analysis

The data were analyzed using XLSTAT software (Version 2022.4.5, Addinsoft, Paris, France). All the data were subjected to analysis of variance (ANOVA). ANOVA was applied to the antioxidant profile. The means were separated using the Tukey test only when the F-test for treatments and interactions was significant at the $p \leq 0.05$ probability level.

6.4. Results and discussions

6.4.1. Instrumental optimization

The method for simultaneous identification of thirty-two polyphenols has been refined by optimizing various analytical parameters, including the choice of columns, acidifiers for the mobile phase, flow rate, and oven temperature. In previous literature, C18 columns were commonly employed for chromatographically separating polyphenols (Quifer-Rada et al., 2015; López-Fernández et al., 2020). Our study found that the Kinetex C18 50 mm × 3 mm × 1.7 µm d.p. column outperformed the Kinetex C18 100 mm × 2.1 mm × 2.6 µm d.p. column, primarily due to the use of sub-2 core-shell particles as the stationary phase. Subsequently, two different acidifiers were tested for mobile phase acidification. Formic acid is typically employed to enhance chromatographic separation of polyphenols [12]. Notably, when acid used at a 0.1% v/v concentration, no substantial differences were observed, leading to the selection of formic acid as the optimal acidifier. Following this, parameters such as flow rate, oven temperature, and injection volume were fine-tuned.

Chromatographic separation was further enhanced by adjusting the mobile phase composition, resulting in a gradient mode for polyphenols.

Accordingly, the chromatographic representations of the beers are reported (Figure 1).

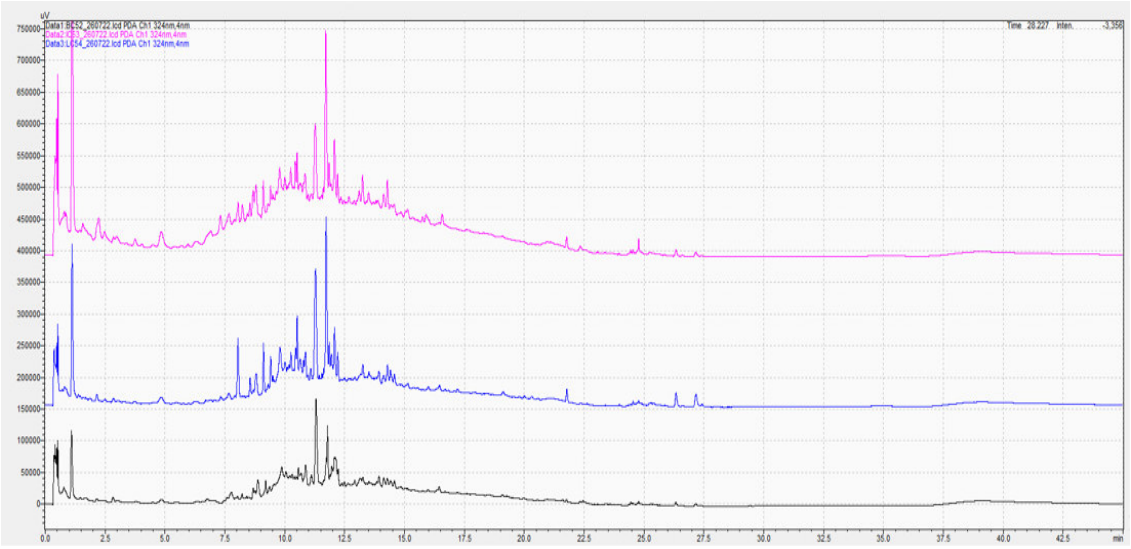

Figure 1: Chromatographic profile of polyphenols in beers. From top to bottom: Blanche (flavored with bitter orange peels and coriander) IPA (flavored with bergamot peels) and Lager.

6.4.2. Determination of polyphenols in beers

Polyphenols determined in three Italian craft beers as an application of the proposed method. The beers were chosen for the different raw materials used, such as: malted and unmalted cereals, roasted and unroasted malts, hops with different degrees of bitterness and aromaticity and “characterizing foods” such as bergamot peel, coriander and bitter orange peel.

The identification of the compounds in the beer samples was made considering the retention times and the profile of the UV absorption spectra.

Regarding the qualitative and quantitative analysis of polyphenols present in craft beers (Figure II), significant differences have emerged. Total concentrations of polyphenols obtained by HPLC-PAD were compared to evaluate relative phenolic

content between beer styles. The three beer styles showed high concentrations of polyphenols, with an average amount of 238.81 mg/l for bergamot-flavored IPA, 115.66 mg/l for Lager-style beer, and 94.58 mg/l for Blanche-style beer flavored with coriander and bitter orange peel. The IPA beer, obtained by adding barley malt, oats, and bergamot peels, contained the highest overall phenolic content among the analyzed beers.

Epicatechin and gallic acid were higher than other polyphenols in all beer styles. Compared to other beers, Lager-style beer was characterized by a higher amount of gallic acid with an average concentration of 30.04 mg/l, while IPA showed higher levels of (-) epicatechin with an average value of 47.87 mg/l. This value is significantly higher than Blanche (18.86 mg/l) and even more than Lager beer (7.94 mg/l).

The most prevalent phenols are characteristic of citrus fruits such as Vanillic Acid, Naringin, Neoeriocitrin, Neohesperidin, and Tangeretin, compounds typical of bergamot (Citrus Bergamia) and bitter orange (Citrus Aurantium), identified with literature data [24,25]. The compounds Melitidin and Brutieridin were quantified through naringenin (dehydrosylated aglycone precursor).

In the three craft beers, hydroxycinnamic acids like ferulic acid, p-coumaric acid, and caffeic acid are present in small amounts, while hydroxybenzoic acids are more concentrated, such as protocatechuic acid, hydroxybenzoic acid, and syringic acid. Blanche beer, produced with citrus peels in infusion, contains phenols mainly found in citrus fruits such as Vanillic Acid, Neohesperidin, and Chlorogenic Acid.

The results obtained with the proposed method are in agreement with literature data. Nardini et al. [26] quantified more hydroxybenzoic acids than hydroxycinnamic acids in Italian beers, while the average values published by Floridi et al. and Garcia et al. [27,28] are consistent with the presented average values.

In particular, numerous studies have identified chlorogenic acid as one of the major phenolic acids found in oranges and other citrus fruits [29], as observed in

our bergamot-flavored IPA beers in particular, but also in the Blanche flavored with bitter orange.

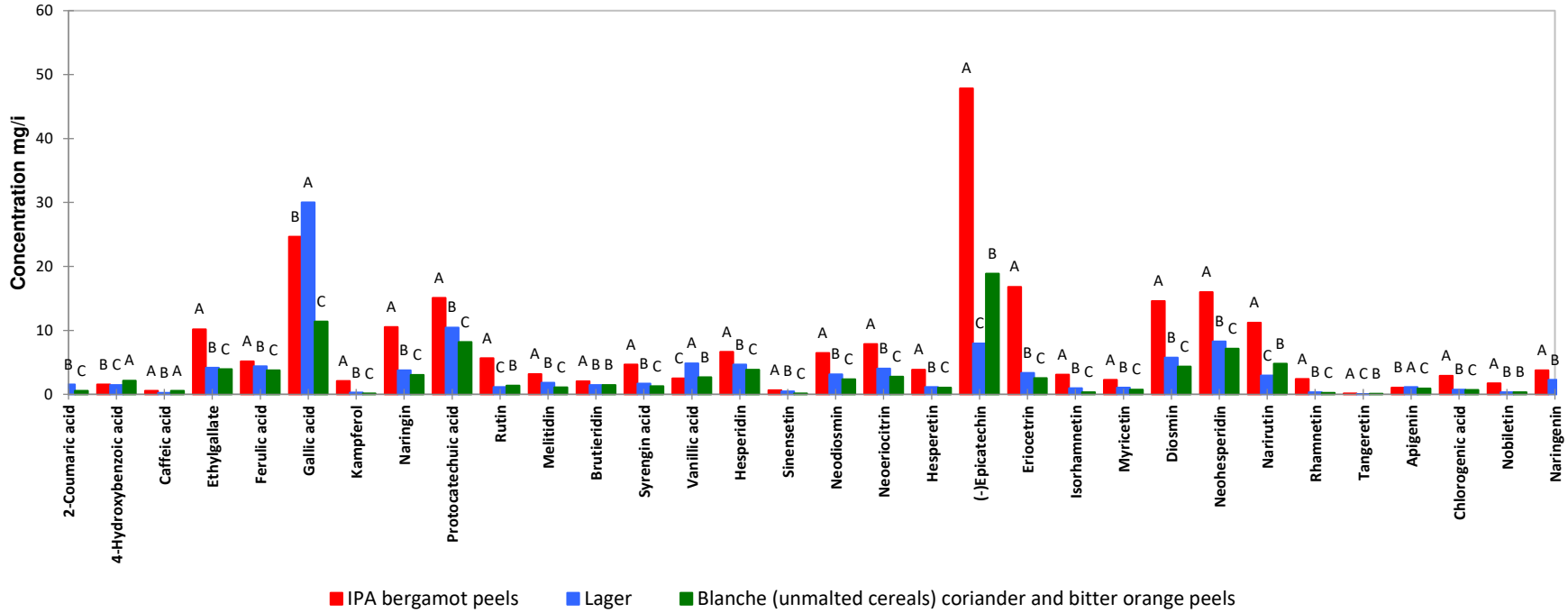

Figure II. Poliphenols concentration (means between three replicates) of beers samples. Different capital letters (A-C) indicate significant differences ($p<0.05$) between the samples.

The dataset underwent multivariate analysis with the aim of studying variations among specific beer styles and identifying the compounds that discriminate between different types. The results of the Principal Component Analysis (PCA) with the Loading Plot are displayed in Figure III.

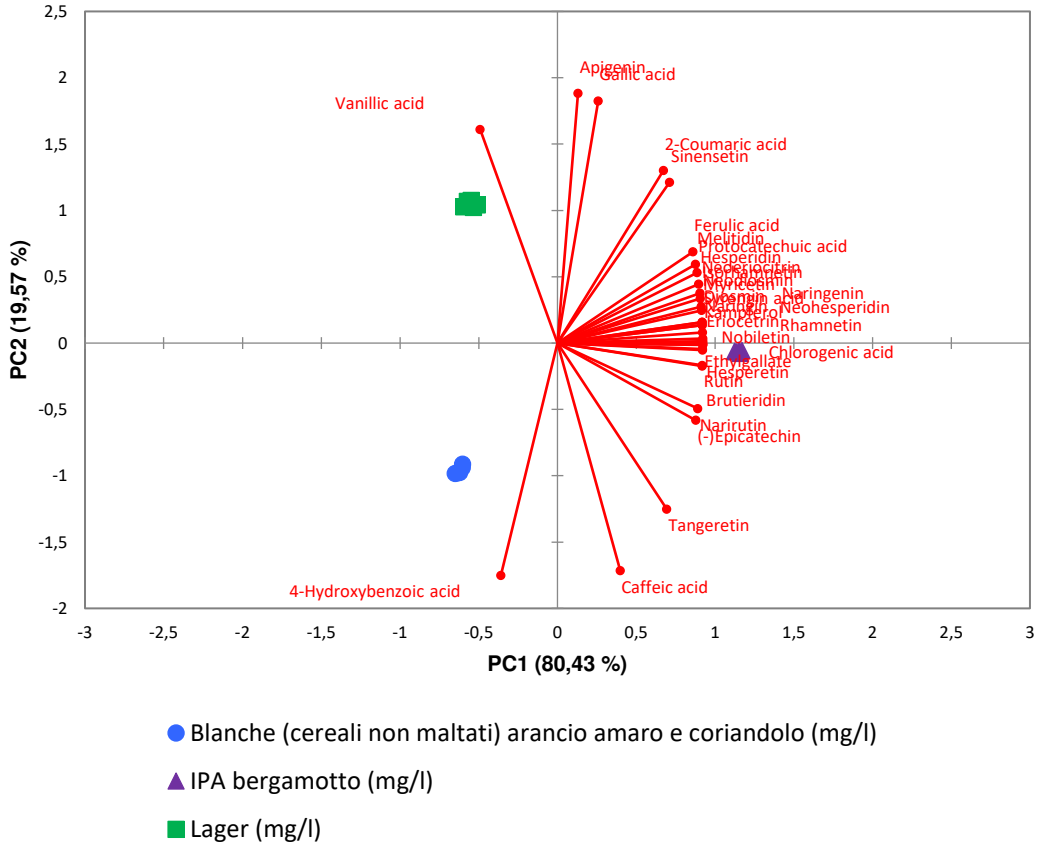

Figure III. Biplot PCA Beer Samples (GREEN, BLUE and PURPLE beer samples, RED variables).

The Principal Component Analysis (PCA) results, as depicted in Figure III, reveal a remarkable distinction among the three beer types under consideration. The first principal component (PC1) accounts for a substantial discrimination index of 80.43%, emphasizing its pivotal role in capturing the major variations. Simultaneously, the second principal component (PC2) contributes with a discrimination index of 19.57%, collectively providing a comprehensive overview of the dataset.

The figure further illuminates the specific polyphenolic compounds that significantly contribute to this pronounced separation. Notably, Blanche exhibits a distinctive pattern primarily attributed to the concentration of 4-Hydroxybenzoic acid, signifying its unique phenolic profile. On the other hand, Lager is discerned by the prominence of Vanillic Acid, emphasizing its specific chemical fingerprint in comparison to the other beer styles.

The most intriguing differentiation is observed in the case of IPA, where the characteristic phenolic compounds associated with bergamot, including Epicatechin, Nobiletin, Naringin, Naringenin, and Neohesperidin, play a crucial role in defining its distinct position in the multivariate space. This underlines the influence of specific polyphenols, particularly those derived from bergamot, in shaping the aromatic and compositional characteristics of IPA.

In essence, the PCA not only successfully segregates the beer styles but also provides valuable insights into the key polyphenolic contributors that contribute to their individuality. This analytical approach proves instrumental in unraveling the intricate chemical nuances that define the sensory profiles of Blanche, Lager, and IPA, thus enhancing our understanding of the unique compositional attributes that set them apart.

6.5. Conclusions

In this study, an RP-UHPLC-PDA method was developed and validated for the simultaneous determination of thirty-one polyphenols in craft beer's samples, using a direct sample injection system. The method developed allowed to determine numerous polyphenols with excellent chromatographic separations and to quantify typical phenols of beers, such as p-Coumaric acid, 4-Hydroxybenzoic acid and Protocatechuic acid, but also compounds derived from "characterizing foods" such as Naringina, Neohesperidine, (-)-Epicatechin and Tangeretin. UHPLC system with sub-2 core-shell column allowed to separate compounds with very similar structure such as cis-trans pairs. The method was applied to

three different beers, the quali- quantitative results for the results for the polyphenols are in line with the data in the literature.

The validated method can be used by breweries to carry out a kidnapped screening of polyphenols of a beer in the production phase to perfect taste, flavor and style or to improve production techniques and use of raw materials. Furthermore, the method described can be a powerful tool to determine food fraud (use of non-natural essences) and to determine the conservation status of beers. The ease and speed of analysis with an no expensive instrument make this method great for many beer supply chain applications.

Further experiments are currently planned to validate and strengthen these preliminary results. This involves working with a much larger set of beer samples, including established reference data for building nutraceutical and quality indicator models.

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CHAPTER 7

Development of a new analytical protocol for the aromatic fingerprinting tracking of craft beers using GC/MS and GCxGC/Q-TOF coupled with mass spectrometry, and Head Space-SPME extraction.

Abstract

In this study, we utilized Headspace Solid-Phase Microextraction (HS-SPME) combined with Gas Chromatography-Mass Spectrometry (GC/MS) to extract and identify volatile compounds in various styles of craft beer. The HS-SPME extraction, using a divinylbenzene/carboxen/poly(dimethylsiloxane) fiber, was specifically tailored for extracting target analytes, which included aldehydes, alcohols, acids, terpenoid esters, and other compounds.

Simultaneously, we replicated the same analysis using an advanced technique: Gas Chromatography coupled with Quadrupole and Time-of-Flight Mass Spectrometry (GC-QTOF/MS). This advanced method is particularly valuable as it combines the separation capabilities of GC with the structural information obtained from high-resolution mass spectrometry.

We adopted a multidimensional qualitative approach for compound identification, which encompassed library searches, comparison of retention indices, accurate mass measurements, and area normalization. Ultimately, our analyses identified a total of 21 compounds through GC/MS and 33 compounds through GC-QTOF-MS in the beers.

Similarly, headspace solid-phase microextraction (HS-SPME) followed by gas chromatography-mass spectrometry (HS-SPME-GC/MS) is commonly employed to study volatile profiles. This approach is further enhanced when coupled with time-of-flight mass spectrometry (GC-TOF/MS), enabling the generation of highly representative aromatic profiles of reference matrices.

The new protocol developed in this article for studying the aromatic components of craft beers appears to yield satisfactory results for tracking aromatic fingerprinting. Our results have demonstrated the efficiency of HS-SPME in extracting polar volatile compounds and the accuracy of gas chromatography coupled with mass spectrometry in identifying volatile compounds in complex matrices.

Keywords: Aromatic fingerprinting, volatiles, Head Space, SPME
quadrupole time-of-flight mass spectrometry.

7.2 Introduction

Headspace solid-phase microextraction (HS-SPME) represents a rapid and solvent-free approach to extract compounds from a sample matrix, employing a fiber coated with an adsorbent phase. [1-4]

To improve sensitivity in the detection of target compounds, it is essential to coat SPME fibers with specific sorbents that exhibit a high affinity for these compounds [5]. Presently, there is a widening range of commercially accessible fibers, such as polyacrylate (PA), polydimethylsiloxane (PDMS), PDMS/divinylbenzene (DVB), carboxen (CAR)/PDMS, CAR/DVB, and DVB/CAR/PDMS, and more. Each type of fiber is selected based on its particular use and intended application. [6-7].

The DVB/CAR/PDMS fiber has demonstrated its effectiveness in the extraction and release of volatile compounds characterized by diverse chemical properties and polarities [8]. As the emphasis has been on enhancing the extraction efficiency of analytes with varying polarities from various matrices, the evolution of SPME fiber coatings has contributed to its extensive application in the analysis of volatile compounds derived from sources like herbs, food items, and environmental samples. [9-13]

Gas chromatography-mass spectrometry (GC-MS) is a potent instrument for performing both qualitative and quantitative analyses [14-18]. Nevertheless, one of the key challenges associated with GC-MS is the precise identification of the

numerous isomers and isobars that exist within the volatile compounds found in alcoholic beverages. To tackle this challenge, various qualitative techniques have been devised, including library searches, comparing retention indices (RI), and utilizing diverse MS scan technologies to recognize unidentified compounds [19-23]. By combining these methods, they can collectively deliver a reliable means of identifying volatile compounds, even when present in trace amounts. Nevertheless, the recent introduction of high-resolution quadrupole time-of-flight mass spectrometry (HR-QTOF MS) has further elevated the precision of identification [24-28].

Compared to low-resolution MS instruments such as quadrupole or ion trap MS, HR-QTOF MS offers not only excellent full-spectral sensitivity with high mass resolution but also enables the elucidation of molecular structures through high-resolution product ion spectra. With these advanced features that enhance selectivity, sensitivity, and precision, GC-QTOF MS demonstrates significant potential for qualitative analysis, especially when dealing with intricate components.

Gas chromatography (GC) and mass spectrometry (MS) are among the most crucial analytical techniques employed in volatile organic compound (VOC) analysis. However, the advanced approach of coupling gas chromatography with quadrupole mass spectrometry (GC-QTOF / MS) is particularly advantageous as it combines the advantages of GC separation with the detailed structural insights provided by high-resolution MS. , the utilization of solid-phase microextraction sampling (HS-SPME) in headspace analysis followed by GC coupled with MS (HS-SPME-GC / MS) is a common method for studying volatile profiles. This method is further enhanced when coupled with time-of-flight mass spectrometry (GC-TOF / MS), enabling the extraction of highly representative aromatic profiles from the reference matrices.

Beer is unique for its status as one of the oldest fermented beverages that remains readily available in today's market. Furthermore, it stands out as one of the most widely consumed alcoholic beverages globally. [29].

The taste of beer plays a crucial role in determining whether consumers will embrace or reject the product. This flavor is the result of complex interactions

among a wide range of chemical compounds [30,31]. Beer contains a diverse array of flavor components, including alcohols, esters, aldehydes, ketones, carboxylic acids, organic acids, sulfur compounds, amines, phenols, and various concentrations of these compounds. All of these components contribute to the aroma and taste of the final product [32,33]. Some of these volatile compounds are key in shaping the overall beer flavor, while others provide subtle background notes to the taste of the product [34]. These compounds have various sources, including the grains used in fermentation, yeast metabolic processes, bittering hops, and flavoring ingredients.

The purpose of this study was to develop a new analytical protocol using HS-SPME extraction via GC/MS and to compare the volatile and semi-volatile compounds among different styles of Calabrian craft beers produced with various malt bases, hops, and flavoring ingredients. The preliminary study and the results obtained aimed to create aromatic fingerprinting by leveraging the selectivity and sensitivity of GC/MS-QTOF, identifying even smaller compounds.

7.3. Reagents and materials

7.3.1. Samples

Three beers were manufactured in a pilot-scale facility at the Wild Orange Fermented Beverage Technology Room in Vibo Valentia, Calabria, Italy, through a collaboration between the FoCuSs Laboratory and the Pedology Laboratory of the Mediterranean University of Reggio Calabria.

These craft beers belong to the most popular beer styles in Europe (Table I). The craft beer samples were as follows:

Table I. *Craft Beer Samples.*

Craft beers	
BC52	water, barley malt, hops, unmalted wheat, bitter orange peel, and coriander seeds.
IC53	water, hops, barley malt, wheat, oat flakes, and bergamot peels.
LC54	water, hops, and barley malt.

The analysis was carried out in triplicate for each batch and for each type of Craft Beer. It's worth noting that while the barley malt, yeasts, and hops were consistent across all three beer styles, the grains and flavor bases were modified.
Sodium Chloride was purchased Chem-Lab NV (Zedelgem, EU, Belgium).

7.3.2. Instrumentation and analytical conditions

For the headspace composition was investigated by Headspace solid-phase micro-extraction (HS-SPME) coupled to gas chromatography (GC) separation and mass spectrometry (QTOF/MS) detection. The extraction of volatile compounds was carried out with a 50/30 μm fiber Divinylbenzene / Carboxene / Polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, Bellefonte, PA, USA). The GC (Agilent 7890A) coupled into a QTOF accurate mass selective detector (Agilent, 7200) was used to analyze the sample headspace components. Volatile compounds were separated using a capillary column coated with HP-5MS (crosslink 5% PH ME siloxane, 30 cm *0.25 mm i.d., 0.25um film thickness). MassHunter software B.08 was employed to control the equipment and acquire data. MassHunter Qualitative Analysis and MassHunter Quantitative Analysis, version B.08 (Agilent Technologies, Germany), were utilized for data analysis. Compound identification was performed by comparing mass spectra and referencing NIST11 library data.
The carrier gas was helium (purity ≥ 99.999%) with a constant flow of 1.0 mL/min, and the split ratio was 18:1. The initial temperature of the column oven was maintained at 40°C for 3 minutes, then raised to 250°C with a temperature program, as reported in Table II, designed based on the chemistry of the molecules present. Mass spectrometry (MS) detection was performed in electron ionization mode (70 eV). The temperature of the ion source, injector, and transfer line was 280, 250, and 280°C, respectively. The MS analyzer operated in full scan mode (between m/z 40 and 600), and the solvent delay time was set to 1 minute. The mass resolution was 2000 (FWHM), and the collision energy was 15 eV.
Analysis of comparison was performed on a GC-MS QP 2010 by Shimadzu equipped with a split/split less injector. The instrument was placed under the same analytical conditions.

Table II. *Programmed temperature*

RATE	T °C	HOLD
-	40	3
6	200	0
15	250	10

7.3.3. Sample Preparation

For sampling, 5 mL of each craft beer sample were taken and placed in 20 mL headspace glass vials and supplemented with 1.75 g of sodium chloride.
In HS mode, the mixture was agitated at 250 rpm using a magnetic stirrer in a heating bath for 20 minutes at 40°C. This phase was carried out manually for GC/MS injection, while it occurred automatically in the Q-Tof system.
At the end of the 20 minutes, the fiber was exposed for the adsorption phase in the vial for 5 minutes. The adsorption phase took place in the injector at 250°C. The fiber was preconditioned before each injection for 20 minutes at 250°C in the injector port.

7.4. Results and discussions

The main goal of this study was to develop a new analytical approach through HS-SPME extraction combined with GC/MS to compare the volatile and semi-volatile compounds present in different variations of craft beers produced in Calabria, using a variety of malts, hops, and aromatic ingredients. After a preliminary study phase and the analysis of the obtained results, the focus was on creating detailed aromatic profiles, leveraging the selective and perceptual capabilities offered by GC/MS-QTOF, thus enabling the detection of even smaller compounds.
The conditions for HS-SPME were optimized and met the requirements of this study. NaCl was used to reduce the solubility of analytes in the aqueous phase and improve their extraction (1-4).
It should be noted that the study initiated, and the results reported, represent only a preliminary investigation, and further tests will be required to confirm the data obtained. The conducted analyses, however, yielded rapid and high-quality MS

and MS/MS spectra, allowing the confirmation of many target and non-target compounds. By combining the results from library searches and calculated formulas for molecular ions and fragment ions, using the MassHunter data management software, it was possible to identify, quantify, and confirm the chemical metabolites comprising the different samples. It should be clarified that, being an initial study, quantification in GC/MS was conducted as a percentage area referred to the individual areas of the total ion current (TIC) peaks in Table III.

Using the SureMass application optimized for EI data at 70 eV, compound detection was carried out, as evidenced by the figures displaying the chromatographic separation profile of the different processed samples with their corresponding identification.

Table III. *Qualitative comparison of the volatile composition of beers (GC/MS).*

		BC52		**IC53**		**LC54**	
COMPOUNDS	**RT TIME**		**AREA %**		**AREA %**		**AREA %**
Carbamic acid, monoammonium salt	0.719	**X**	4,41	**X**	3,40	**X**	4,45
Ethanol	0.816	**X**	18,49	**X**	26,02	**X**	30,73
Ethyl Acetate	1.170	**X**	2,16	**X**	2,15	**X**	3,82
1-Propanol, 2-methyl-	1.234	**X**	3,46	**X**	3,74	**X**	5,07
1-Butanol, 3-methyl-	2.144	**X**	9,29	**X**	12,65	**X**	12,67
1-Butanol, 2-methyl-	2.183	**X**	7,84			**X**	10,44
1-Butanol, 3-methyl-, acetate	5.199	**X**	1,60	**X**	2,59	**X**	3,65
1-Butanol, 2-methyl-, acetate	5.264	**X**	0,34	**X**	0,55		
Styrene	5.431	**X**	0,65	**X**	4,22	**X**	2,82
Hexanoic acid, ethyl ester	8.687	**X**	2,56	**X**	0,61	**X**	1,69
1-Octanol	10.734	**X**	0,19	**X**	0,25	**X**	0,46
beta.-Linalool	11.506	**X**	21,20	**X**	11,97	**X**	2,44
Phenylethyl Alcohol	11.795	**X**	9,07	**X**	12,91	**X**	8,54
Terpinen-4-ol	13.433	**X**	0,28	**X**	0,14		
L-.alpha.-Terpineol	13.807	**X**	0,68	**X**	0,80		
Octanoic acid, ethyl ester	13.887	**X**	7,35	**X**	5,58	**X**	4,10
UNKNOW	14.810	**X**	1,08	**X**	0,34	**X**	0,25
Acetic acid, 2-phenylethyl ester	15.440	**X**	4,75	**X**	1,86	**X**	1,37
Ethyl 9-decenoate	18.569	**X**	0,40	**X**	0,54	**X**	0,39
Decanoic acid, ethyl ester	18.755	**X**	0,74	**X**	8,76	**X**	6,43
Humulene	20.025	**X**	3,46	**X**	0,91	**X**	0,67

Figure I represent the tracking of the aromatic profile of the three beers traced by GC-MS, while Figure II shows the aromatic fingerprinting of the three beers traced by GC-QTOF.

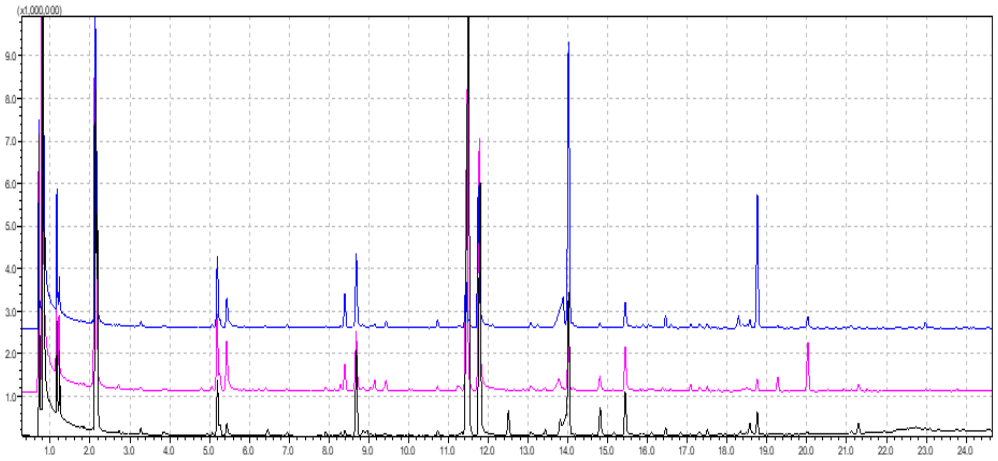
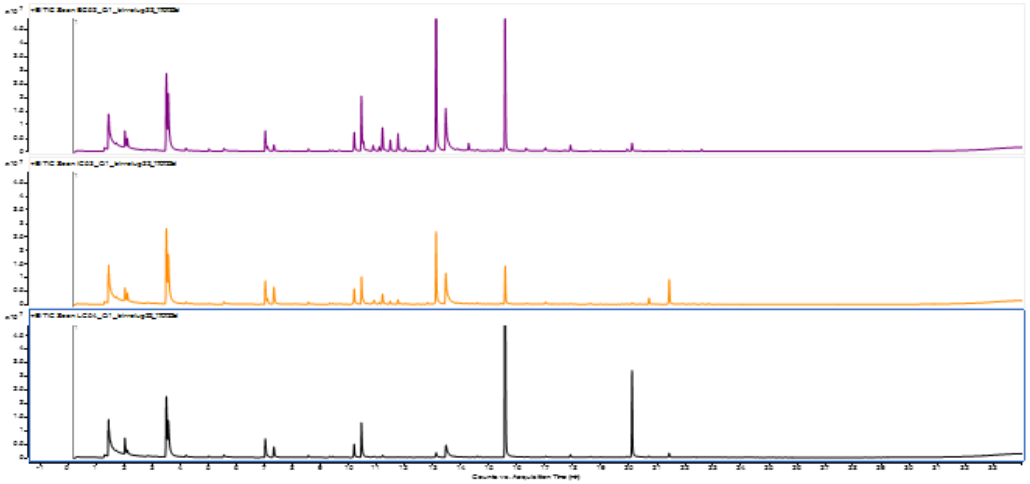


Figure I. *Aromatic fingerprinting overlaid beers GC/MS. From top to bottom: BC52 in pink, IC53 in blue and LC54 in black.*



Figures II. *Aromatic fingerprinting of overlapping beers GC-QTOF/MS. From top to bottom: BC52 in purple, IC53 in orange and LC54 in black.*

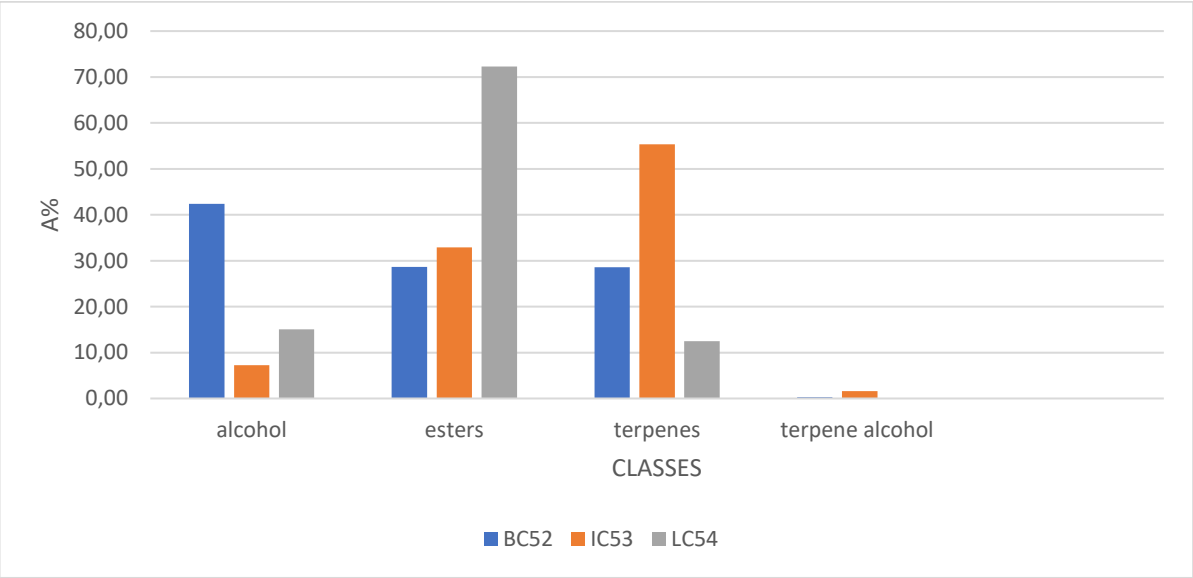
The identification by GC/MS-QTOF was achieved through comparison with MS data obtained from the NIST literature. Thirty-three compounds were defined, all recognized with a match factor (MF) exceeding 75%. In Table IV, the isolated volatile compounds and their respective recognition index and retention time are

reported. As evident, these compounds are primarily terpenic, alcoholic, and ester compounds.

Table IV. *Qualitative comparison of the volatile composition of beers.*

					BC52		IC53		LC54	
	RT	**MF**	**NAME**	**CLASSES**		**Area %**		**Area%**		**Area%**
1	1,7157	87,5	3-Pentanol	alcohol	X	0,95	X	0,15	X	0,33
2	2,0223	77,2	Ethyl Acetate	ester	X	8,28	X	3,14	X	22,02
3	2,1084	82,8	1-Propanol, 2,2-Dimethyl-	alcohol	X	1,18	X	0,12	X	0,38
4	3,5018	80,6	1-Butanol, 2-methyl-	alcohol	X	6,21	X	0,98	X	3,79
5	3,5707	86,4	1-Butanol, 3-methyl-	alcohol	X	3,83	X	0,72	X	4,17
6	5,0199	87,5	Butanoic acid, ethyl ester	ester	X	0,14	X	0,14	X	0,46
7	7,0308	84,8	1-Butanol, 3-methyl-, acetate	ester	X	7,15	X	4,25	X	9,12
8	7,1058	80,5	1-Butanol, 2-methyl-, acetate	ester	X	1,31	X	0,44	X	2,39
9	7,3366	95,2	Styrene	terpene	X	10,14	X	0,23	X	0,74
10	8,0911	89,2	Propanoic acid, 2-methyl-, butyl ester	ester	X	0,13	X	0,03	X	0,58
11	10,202	85,6	trans-.beta.-Ocimene	ester	X	2,70	X	6,83	X	7,99
12	10,4564	91,1	Hexanoic acid, ethyl ester	ester	X	2,60	X	3,51	X	14,01
13	10,5327	84,8	.alpha.-Phellandrene	terpene	X	0,55	X	1,50	X	0,32
14	10,8755	83,9	3,4-Dimethylbenzyl alcohol	alcohol	X	0,19	X	1,02	X	0,13
15	10,9138	89	Propanoic acid, 2-methyl-, 3-methylbutyl ester	ester	X	0,76	X	0,24	X	2,61
16	11,0971	93,9	o-Cymene	terepene	X	0,62	X	0,88	X	0,27
17	11,2057	88,7	D-Limonene	terpene	X	1,85	X	5,72	X	0,74
18	11,4866	83,5	alpha- Pinene	terpene	X	0,60	X	3,69	X	0,18
19	11,7637	84,9	beta-Ocimene	terpene	X	0,92	X	3,69	X	0,26
20	12,0318	83,7	.gamma.-Terpinene	terpene	X	0,16	X	0,80	X	0,05
21	12,8185	90,4	Terpin-2-ol	terpene alcohol	X	0,26	X	1,35	X	0,09
22	13,1237	88,8	3-Carene	terpene	X	10,59	X	38,78	X	2,36
23	13,4656	90,8	Phenylethyl Alcohol	alcohol	X	29,83	X	4,06	X	6,20
24	13,7795	85	Octanoic acid, methyl ester	ester		0,00		0,00		0,46
25	15,1132	77,6	Terpinen-4-ol	terpene alcohol		0,00	X	0,22		0,00
26	15,4408	89,4	UNKNOW	UNKNOW	X	0,15	X	2,95	X	0,08
27	15,5826	88,9	Octanoic acid, ethyl ester	ester	X	3,58	X	6,46	X	5,35
28	16,3303	83,1	6-Octen-1-ol, 3,7-dimethyl-, (R)-	alcohol	X	0,17	X	0,23	X	0,03
29	17,0247	90,7	Acetic acid, chloro-, 2-phenylethyl ester	ester	X	1,20	X	6,65	X	2,89
30	17,9179	84,4	Nonanoic acid, ethyl ester	ester	X	0,05	X	0,00	X	0,06
31	20,1122	83	Nonanoic acid, 5-methyl-, ethyl ester	ester	X	0,14	X	1,19	X	3,47
32	20,7205	86,8	9,12-Octadecadiynoic acid, methyl ester	ester	X	0,63		0,00	X	0,90
33	21,4349	77,3	Humulene	terpene	X	3,60	X	0,01	X	6,84

The obtained results were processed in the form of a bar graph (Graph I), where, to make the distinctions clearer, the volatile compounds were classified into different chemical families to which they belong.



Graph I: *Histogram distribution of compound families.*

Graph 1 and Table IV highlight how the composition percentage varies depending on the state of the beers.

The aromatic differences among the analyzed samples are quite evident. It can be easily inferred that the profiles, concerning the reference VOC markers related to the ingredients used, can be attributed to the different recipes used in the analyzed beers. Specifically, the Lager-style beer (LC54) had a high percentage of esters, the Blanche-style beer (BC52) had a high percentage of alcoholic compounds, while the IPA-style beer (IC53) had the highest percentage of terpenic compounds.

The class of terpenic compounds is most prevalent in IC53, followed by BC52, which is undoubtedly due to the fact that these two styles of beer included citrus-based flavorings in their recipes (bitter orange peel and bergamot). Additionally, terpenic alcohols, including Terpin-2-ol found in all three beer styles, with the highest concentration in the IPA style, and Terpinen-4-ol identified only in the IPA-style beer, were also detected.

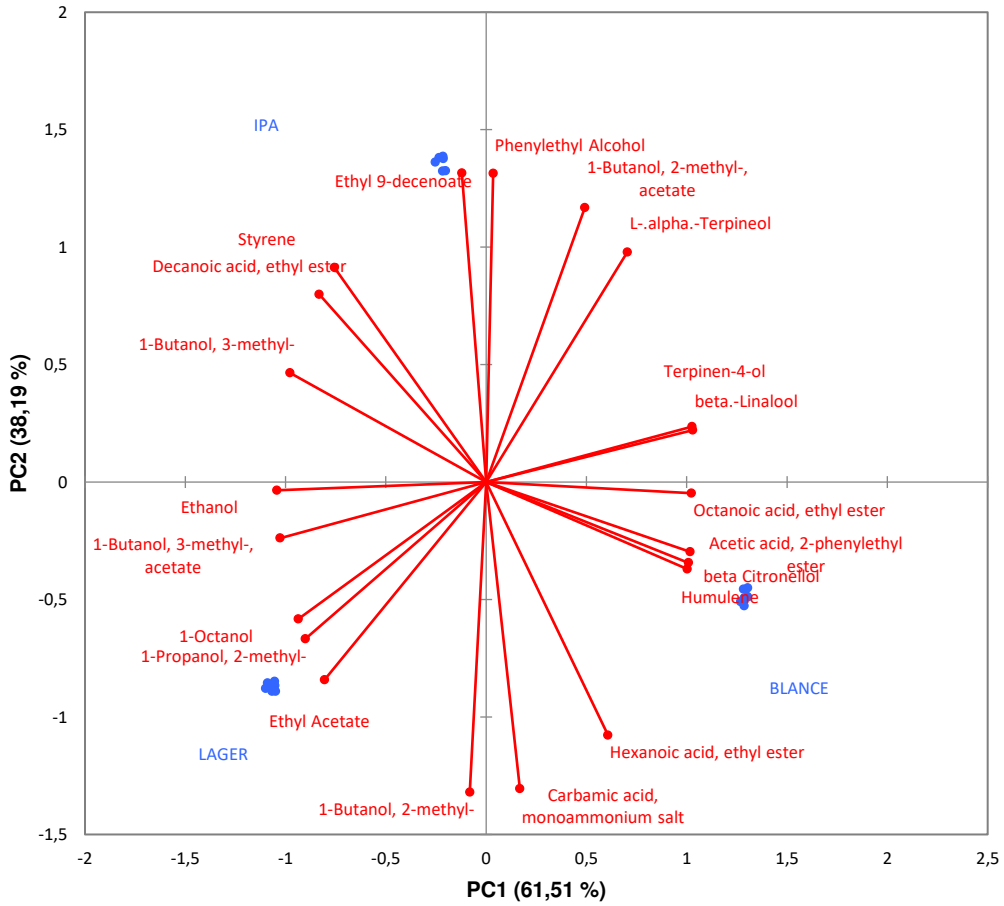


Figure II. Biplot PCA Beer Samples ((Box 1 top left: IPA, Box 2 top right: main compounds present in the 3 beers; Box 3 bottom left: LAGER, Box 4 bottom right: Blanche). RED variables.

The outcomes of the Principal Component Analysis (PCA), as depicted in Figure II, indicate a significant differentiation among the three types of beer in question. The primary component (PC1) plays a pivotal role, contributing significantly to a discrimination index of 61.51% and capturing the major variations. Concurrently, the second principal component (PC2) adds to the discrimination with a 38.19% index, offering a comprehensive overview of the dataset.

The figure provides further insights into specific aromatic compounds identified through GC/MS that contribute significantly to this distinctive separation.

Particularly noteworthy is the differentiation observed in Blanche, characterized by a unique pattern primarily associated with specific aromatic compounds such as Hexanoic acid ethyl ester, Octanoic acid ethyl ester, Humulene, Acetic acid 2-

phenylethyl ester, and notably Beta-Citronellol, linked to this beer style flavored with coriander seeds.

Other crucial and distinguishing compounds include L-Terpin-4-ol, Beta-Linalool, and L-alpha-Terpinol, likely associated with the aromatic profile influenced by the flavoring ingredients in different beer types.

In summary, PCA not only effectively distinguishes between beer styles but also offers valuable insights into the major contributors to aromatic fingerprinting, contributing to their unique characteristics. This analytical approach proves essential in unraveling the complex chemical nuances defining the sensory profiles of Blanche, Lager, and IPA, thereby enhancing our understanding of the distinct compositional features that set them apart.

7.5. Conclusions

TOF MS analyzers provided a complete and accurate mass spectrum for each compound, thanks to their high resolution and scan frequency. This is particularly advantageous compared to quadrupole MS, as the high mass resolution of TOF allows for the creation of extracted-ion chromatograms using narrow mass windows and the precise measurement of characteristic ions in mass spectra. These narrow mass windows significantly improve sensitivity by reducing background noise in chromatograms and enhancing the signal-to-noise ratio.

The ongoing study highlights how the developed analytical method serves as a valuable tool for identifying the aromatic profile of the analyzed compounds. In fact, the analysis of the volatile fraction of the samples shows that the protocol followed can detect even trace amounts of compounds quickly and simply, without the need for any sample pretreatment. However, to definitively confirm the presence or absence of a specific molecule, further optimization of the analytical method and MS characterization through the study of a significant number of samples will be necessary.

Thanks to the tandem mass spectrometer, GC-QTOF MS can perform MS2 scans following the separation of the precursor ion in the quadrupole and its subsequent collision-induced dissociation. The presence of abundant accurate product ions

has allowed for the confirmation of the identity of suspected compounds and has enhanced the accuracy of identification.

The DVB/CAR/PDMS fiber has demonstrated excellent extraction capacity for compounds and, when combined with the HS-SPME mode, has yielded outstanding results in the extraction of various analytes and reproducibility through adsorption for craft beers of different styles.

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CHAPTER 8

Development of a liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the simultaneous qualitative and quantitative screening of craft beers

Abstract

A new method based on liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) for simultaneous qualitative and quantitative determination of 9 mycotoxins was developed. A new multianalyte IAC (AOFZDT2TM column), containing antibodies for aflatoxins (AFB1, AFB2, AFG1, AFG2), Ochratoxin A (OTA), fumonisins (FB1, FB2), Deoxynivalenol (DON) and HT-2 was used as single extraction and clean up step. The Shimadzu Nexera X2 chromatograph coupled with LCMS-8050 Triple Quad LC/MS detector (Shimadzu Corporation, Milan, Italy) was used for separation of mycotoxins. The MS/MS system was equipped with an ESI source operating in the positive ion mode [1]. The optimization of ionization source and MS parameters, individually for each analytes, have executed using the direct standard infusion of the standard solutions. Detection has done in MRM mode, in order to get high sensitivity and selectivity for each analyte, based on in-source generation of the protonated molecular ions of the mycotoxins, as well as collision-induced production of mycotoxin-specific fragment ions [2]. The specific MRM mycotoxins transitions as well as their corresponding fragmentor voltages, collision energies and dwell were determined individually for each analytes.

The chromatographic elution was conducted using a LUNA C18 (3um,150 X 3 mm) as stationary phase ad a gradient MeOH:H20 as mobile phase. The method was applied to craft beer and freeze-dried craft beer and the method performance characteristics were determined after spiking the beers with mycotoxins as model matrix at multiple concentration levels according legal limit (Commission Recommendation of March 27 (2013/165 / EU), Official Journal of the European

Union.) [3]. The investigation of the spiked model matrix confirmed linearity and precision of the method. The limits of detection were below the regulated values of mycotoxins in beer. Although this is only a preliminary study, which therefore requires further investigation, at the moment it can be concluded that the developed LC-MS/MS multi-mycotoxins method can be used as a tool to obtain a comprehensive screening of the range of mycotoxins potentially co-occurring in the final craft beer.

Keywords: Aflatoxins, Fumonisins, Ochratoxin A, Deoxynivalenol, Zearalenone, HT-2, LC-ESI MS/MS.

8.2 Introduction

Beer is the most widely consumed alcoholic beverage in the world. The presence of mycotoxins in beer is a significant public health concern, particularly for individuals who consume it in large quantities. Beer production involves a range of processes that can either increase or decrease the initial levels of mycotoxins. The intricate nature of these processes does not provide brewers with absolute control over the chemical and biochemical reactions occurring in each batch. However, it's important to note that the most effective measure to prevent mycotoxin accumulation remains the prevention of mold growth in raw materials.

Mycotoxins are naturally occurring compounds with a low molecular weight, produced by filamentous fungi as secondary metabolites that hold no biochemical significance for fungal development. When subjected to favorable conditions for mycotoxin synthesis, they generate a toxic environment capable of causing diseases in both animals and humans [4]. The mycotoxins that significantly impact both agro-economics and public health include aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT), trichothecenes (deoxynivalenol DON, nivalenol NIV, HT-2 toxin, T-2 toxin), zearalenone (ZEN), fumonisins (FUM), tremorgenic toxins, and ergot alkaloids (Hussein & Brassel, 2001). Genera such as Aspergillus (AFs, OTA, PAT), Penicillium (OTA and PAT), and Fusarium (DON, NIV, HT-2, T-2, ZEN) are the main producers of these mycotoxins [5].

Mycotoxin formation can occur through the contamination of various commodities and products utilized in the food and feed industry by mycotoxin-producing fungi. Commonly contaminated commodities include cereals, peanuts, milk, dairy products, coffee, wine, beer, cottonseeds, fresh and dried fruits, vegetables, and nuts [6].

Barley, a crucial ingredient in beer production alongside water, hops, and yeast, significantly influences the overall quality and market acceptance of beer [7]. It's noteworthy that beer can be susceptible to mycotoxin contamination, originating from infected raw materials such as barley, malt, hops, or adjuncts [8].

Alternaria, Aspergillus, Penicillium, and Fusarium are the most common fungal genera in malting barley that produce a wide range of mycotoxins at the same time, fusarium fungi isolated from barley grains were able to generate alternaria toxins, aflatoxins, ochratoxin A, deoxynivalenol, and zearalenone, with almost 30% of Alternaria being the most capable, as well as 88% of Fusarium fungi. [9].

Numerous studies on beer have primarily focused their research on deoxynivalenol (DON), as it is the most prevalent mycotoxin and poses the most significant public health concern related to beer consumption [10,11,12,13,14].

Globally, there are two well-defined categories of beer distinguished by their fermentation techniques: ale, characterized by top fermentation, and lager, distinguished by bottom fermentation. In addition to the use of different yeast strains, various fermentation attributes, including the generation of secondary products and the types of sugars subjected to fermentation, play a role in shaping the distinct characteristics of these beer styles [15].

The current European regulations on mycotoxins establish maximum allowable levels in food products for 13 different compounds (EC 1881/2006; Commission Recommendation, 2013/165/EU) [3]. These limits are set as follows for cereal-based products (Including beer):

Table I: Legal mycotoxin limit for cereal products.

MYCOTOXIN	**LEGAL LIMIT**
AFB1	2 µg/kg
AFs,	4 µg/kg
DON	750 µg/kg
FUMB1+ FUMB2	400 µg/kg
OTA	5 µg/kg

Beer, being a widely consumed beverage globally, has the potential to contribute to mycotoxin intake, particularly among heavy consumers. Mycotoxin contamination can occur at various stages of the brewing process. Some mycotoxins, such as AFs, ZEN, and DON, with high thermal stability, and DON and FUM, with water solubility, can be transferred from cereals to malt and, subsequently, into beer. Regardless of their origins, numerous surveys worldwide have analyzed the occurrence of mycotoxins in beer, covering different styles of beer production [17]. Several studies have specifically focused on the presence of various Fusarium mycotoxins in beer [18,19,14,20,21]. Additionally, some investigations grouped beer samples based on the production style applied to the malting barley used, revealing that wheat beer contains higher levels of deoxynivalenol (DON) and its derivatives compared to barley beer. This variation is attributed to the distinctive characteristics of wheat and barley matrices, influencing the composition of fungal species.

The majority of samples were found to contain detectable levels of other mycotoxins. These included deoxynivalenol (DON), fumonisin B1, B2, and ochratoxin A. So, it's clear that researchers are consistently striving to develop quick and dependable methods for identifying mycotoxins in both raw materials, like cereals, and final products [22].

To identify the new analytical procedures that evaluate their presence in food products and in particular in beer, was fundamental. A new method based on liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) for simultaneous qualitative and quantitative determination of 9 mycotoxins was developed. A new multianalyte IAC (AOFZDT2TM

column), containing antibodies for aflatoxins (AFB1, AFB2, AFG1, AFG2), Ochratoxin A (OTA), fumonisins (FB1, FB2), Deoxynivalenol (DON) and HT-2 was used as single extraction and clean up step.

LC-MS is the dominant technique applied for the separation and characterization mycotoxins due to its ability to quantify and identify analytes at low concentrations in the presence of interferences [23], of which beer has many. In this respect, LC-MS may be a more appropriate alternative to the standard fluorimetric or UV detection methods currently used in order to understand the presence of specific mycotoxins [24]. Using LC-QQQ-mass analyzer allows for higher accuracy ion mass measurements and introduces the potential for predicting the compounds that may be responsible to evaluate the potential toxicity of beer [25]. The usage of advanced analytical techniques in the brewing industry could potentially assist to improve quality control practices, or provide brewers with a better understanding of how the ingredients and processes affect their finished product [25]. Of course, LC-MS represents a much larger capital investment than a Classc HPLC-UV-RF instrument, and it is more complicated to operate. However, if one desires toparse the vast degree of chemical variation in the products produced by today's craft brewing industry, instrumentation with much higher specificity is needed, and LC-MS is one obvious choice for this task [25].

8.3 Materials and method

8.3.1 Chemical and reagent

Acetonitrile, methanol, toluene (all HPLC grade) and glacial acetic acid were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Milli-Q system (Millipore, Bedford, MA, USA). Ammonium acetate (for mass spectrometry), AFB1, AFB2, AFG1, AFG2, FB1, FB2, OTA, DON, and HT-2 were purchased from Sigma-Aldrich (Milan, Italy). Whatman GF/A glass micro fiber filter papers were obtained from Whatman International (Maidstone, UK). DONtestTM HPLC, T-2testTM HPLC and AOFZDT2TM immunoaffinity

columns were obtained from VICAM (Watertown, MA, USA). Phosphate-buffered solution at pH 7.4 (PBS) was prepared by dissolving commercial phosphate-buffered saline tablets (Sigma-Aldrich) in distilled water.

8.3.2. Sample preparation

Beer samples (Lager Style beer showed in table II) stored at +4°C were degassed (3 cycles of 10 minutes each at +10°C to avoid excessive sample heating). Craft beer as it is, and the same beer freeze-dried were used.

Table II. Sample

Sample	Ingredients
lager style beer	Water, barley malt, hops and yeast.
lager style beer frieze-dried	Water, barley malt, hops and yeast.

The beer samples degassed (10 ml) were first extracted with 50 mL PBS, by shaking for 60 min. After centrifugation at 3000 g for 10 min, 35 mL of PBS extract (extract A) were collected and filtered through a glass microfiber filter. Then 35 mL methanol were added to the remaining solid material, containing 15 mL PBS, and the sample was extracted again by shaking for 60 min. After centrifugation (3000 g, 10 min), 10 mL of methanol/PBS extract were diluted with 90 mL PBS to reduce the organic fraction and filtered through a glass microfiber filter (extract B). Aliquots of the two extracts were separately submitted to cleanup through the same multianalyte IAC. In particular, 50 mL of extract B were passed through the AOFZDT2TM column at 1–2 drops per second; the column was then washed with 20 mL PBS to completely remove methanol residues. After passing 5 mL of extract A that was eluted at 1–2 drops per second, the column was washed with 10 mL distilled water to remove PBS residue and matrix interfering compounds. Toxins were eluted from the column with 3 mL

methanol in two steps of 1.5 mL each at 1 drop per second. After the first step, a 5 min interval was allowed to favor methanol-antibody contact; complete elution of all toxins was obtained with the second elution step followed by air flushing through the column. The methanolic eluate was dried under an air stream at 50°C and reconstituted with 250 µL methanol/water 40:60, containing 1 mM ammonium acetate and 0.1% acetic acid (Mobil Phase). The sample was stored at -18° in the dark until analysis. Inject 100 µL were injected to be analyzed by LC/MS/MS.

Figure 1. Analytical procedure for multy-Mycotoxins analysis.

The same extraction procedure was carried out using previously freeze-dried beer. 500 mg of lyophilized sample was made up to volume with 5 ml of water. As control, the same beer doped with known standard concentrations of mycotoxins (Ochratoxin A, Afla B1, Afla B2, Afla G1, Afla G2, Fumonisin B1, Fumonisin B2, Deoxynivalenol and HT-2) according to table III, was also extracted.

Table III. Mycotoxins Level of doped craft beers

MYCOTOXIN	Legal Limit	Concentration in the Sample
AFLATOXIN B1	4 µg/L	5 µg/L
AFLATOXIN B2	4 µg/L	5 µg/L
AFLATOXIN G1	4 µg/L	5 µg/L
AFLATOXIN G1	4 µg/L	5 µg/L
FUMONISIN B1	400 µg/L	500 µg/L
FUMONISIN B2	400 µg/L	500 µg/L
OCRATOXIN A	5 µg/L	10 µg/L
DEOXYNIVALENOL	750 µg/L	1000 µg/L
HT2	n.d.	1000 µg/L

8.3.3. Standard and matrix-matched calibration

Stock solutions of each mycotoxin were prepared at different concentrations by diluting the as-purchased solutions in the appropriate solvent. DON, HT-2 were dissolved in acetonitrile, AFs in toluene/acetonitrile 99:1, OTA in toluene/acetic acid 99:1, and FBs in acetonitrile/water 1:1. Subsequently, a mix containing all the mycotoxins to be analyzed at the maximum concentration allowed by legal limits was prepared. This mix was then diluted differently and used to prepare calibration solutions and the spiking solution. Standard calibration curves at five points were established using calibration solutions prepared in the LC mobile phase. The mobile phase consisted of methanol/water (40:60), containing 1 mM ammonium acetate and 0.1% acetic acid. The appropriate amounts of the starting mix solution were dissolved in the calibration solutions to achieve the desired concentrations for calibration at each point.

8.3.4. LC-ESIMS/MS method

The Shimadzu Nexera X2 chromatograph coupled with LCMS-8050 Triple Quad LC/MS detector (Shimadzu Corporation, Milan, Italy) was used for separation of mycotoxins. The UHPLC system consisted of a binary pump, automatic degasser, column heater and autosampler. The MS/MS system was equipped with an ESI source operating in the positive ion mode according with the parameters showed in the table (IV).

Table IV. LC-ESI-MS/MS condition.

ESI SOURCE	
NEBULIZING GAS FLOW L/min	3
HEATING GAS FLOW L/min	10
INTERFACE TEMPERATURE °C	300
DL TEMPERATURE °C	250
HET BLOCK TEMPERATURE °C	400
DRYING GAS FLOW L/min	10

The optimization of ionization source and MS parameters, individually for each analytes, was operated using the direct standard infusion of the standard solutions.

The chromatographic elution was conducted using a LUNA 3µm C18 (100 Å-size 150 X 3 mm) preceded by a Gemini C18 guard column (4 mm x 2 mm, 5 µm particles) as stationary phase and a gradient MeOH:H20 (Table V) as mobile phase.

Table V. Concentration gradient.

Gradient		
Time	**Concentration**	**Value**
0.10	Inject	20
3.00	B Conc.	40
40.00	B Conc.	63
58.00	B Conc.	63
60.00	Stop	

8.4. Results and discussion

A new method for the multiresidual analysis of mycotoxins with a single sample preparation for the determination was developed. The method was applied to craft beer and freeze-dried craft beer and the method performance characteristics were determined after spiking the beers with mycotoxins as model matrix at multiple concentration levels according legal limit.

The investigation of the spiked model matrix confirmed linearity and precision of the method. The limits of detection were below the regulated values of mycotoxins in beer.

Figure 3 shows the different profiles related to the doped beer (obtained by adding the standard mixes) used as a reference against the same beer (not doped).

Figure. 6 shows the scan of OtA standard and the Q1scan in which the same precursor ion (403 + H +) is highlighted.

The developed method allowed to detect the "co-ocurrence" of different mycotoxins and, at the same time, to determine the quantity of each mycotoxin, in a single analysis.

The development of the method involved the extraction and purification of the mycotoxins, the optimization of the mass parameters followed by the chromatographic optimization.

The MS detection was carried out in MRM mode, in order to obtain a high sensitivity and selectivity for each analyte, based on the in-source generation of the protonated molecular ions of the mycotoxins, as well as on the collision-induced production of fragments of specific ions of the mycotoxin (table VI). The specific MRM mycotoxins transitions as well as their corresponding fragmentor voltages, collision energies and dwell were determined individually for each analyte.

Table VI. Fragmentation mycotoxins

	Precursor (m/z)	Product (m/Z)	Dwell (msec)	Q1Prebias (Volt)	CE	Q3 Prebias (Volt)
OTA	403,8	239,15	100	-14	-24	-18
	403,8	357,95	100	-14	-15	-28
HT2	442	263,2	100	-12	-15	-14
	442	157,05	100	-12	-23	-12
FUMO B2	706,1	336,3	100	-20	-38	-18
	706,1	318,4	100	-20	-41	-17
FUMO B1	722,1	334,2	100	-20	-41	-18
	722,1	352,35	100	-20	-37	-19
AFLA B1	312,8	241	100	-15	-38	-18
	312,8	185,2	100	-14	-51	-21
AFLA B2	314,8	287	100	-11	-25	-22
	314,8	259,1	100	-11	-30	-20
AFLA G1	328,8	311	100	-11	-21	-24
	328,8	245,1	100	-11	-28	-18
AFLA G2	331	313,1	100	-11	-11	-17
	330,8	245,1	100	-15	-30	-19
DON	296,9	249,05	100	-13	-11	-19
	296,9	279	100	-13	-11	-22

The chromatographic method for simultaneous identification of mycotoxins has been refined by optimizing various analytical parameters, including the choice of columns, the mobile phase, flow rate, and oven temperature. Following this, parameters such as flow rate, oven temperature, and injection volume were fine-tuned.

Chromatographic separation was further enhanced by adjusting the mobile phase composition, resulting in a gradient mode.

Our method achieved elution times of approximately 48 minutes for 9mycotoxins.

The assessment of potential interferences in compound quantification, arising from the direct injection of beer, was conducted through the evaluation of matrix effects (ME).

Matrix calibration curves were established using five levels. ME was computed leveraging the slopes of the calibration curves in both solvent and matrix. The matrix effect remains well within the acceptable range of 80% to 120%.

Regarding the development of the multi-mycotoxin method in beer, in figs. 2, 3, 4 and 5 the chromatographic results and mass spectra are shown.

In detail, a method that allows, through a single analysis, the determination of 9 mycotoxins: Ochratoxin A, Afla B1, Afla B2, Afla G1, Afla G2, Fumonisin B1, Fumonisin B2, Deoxynivalenol and HT-2 (fig. 1) has been developed.

Figure 3 (3a and 3b) shows the different profiles related to the *doped* beer (obtained by adding the standard mixture to the model beer) used as a reference with respect to the same beer (undoped).

Figure 3 shows the profile for freeze-dried and doped beer (obtained by adding the standard blend to the freeze-dried model beer).

Fig. 5 shows, by way of example, the scan of the OTA standard and Figure 6, the Q1scan in which the same precursor ion ($403 + H^+$) is highlighted.

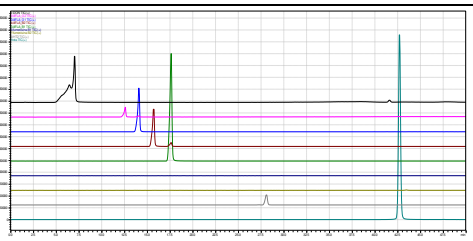

Fig. 2: *SCAN standard mycotoxins (Ochratoxin A, Afla B1, Afla B2, Afla G1, Afla G2, Fumonisin B1, Fumonisin B2, Deoxynivalenol and HT-2)*

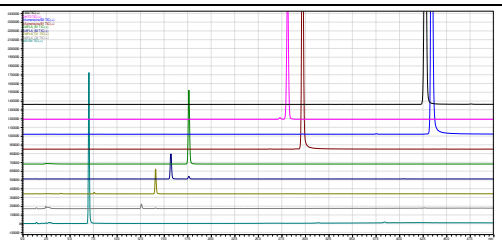

Fig. 3a: *SCAN doped beer with standards mix*

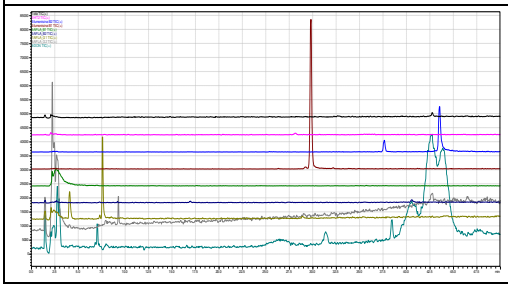

Fig. 3b: *SCAN undoped beer*

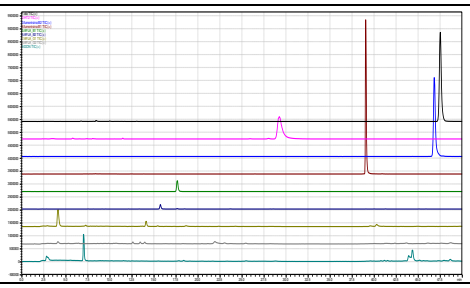

Fig. 4: *SCAN freeze-dried doped beer with standards mix*

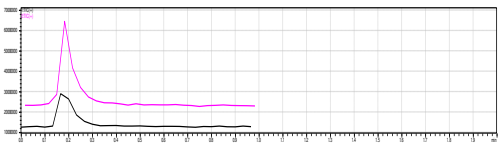

Fig. 5: *SCAN optimization Ochratoxin A*

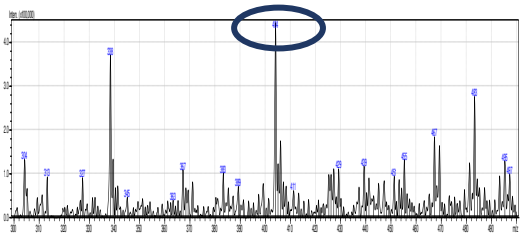

Fig. 6: Optimization of Ochratoxin A-SCAN Q1

Five concentration levels of mycotoxins standards were prepared from a stock solution at different concentration range. Five analyzes were performed for each concentration level with the LC-MS 8050 system under optimized chromatographic conditions. Five-level calibration curves were constructed using the least squares method by obtaining the equations of the regression line. Mandel's test confirmed the linearity of each calibration curve in the considered range. The limits of quantification (LoQs) and limits of detection (LoDs) (table

VII) were calculated by multiplying the standard deviation (SD) of the lowest level of the calibration curve (n = 7) ten and three times, respectively, and dividing the result for the slope of the calibration curve. The repeatability and reproducibility values (table VII) were expressed as percentage coefficient of variation (CV%) and calculated using the average of the areas of the lowest level of the calibration curve (n=5) divided by the corresponding standard deviations. Finally, retention time, instrumental recovery and percentage relative standard deviation (RSD%) were determined using the fourth level (n=4) of each calibration curve (table VIII).

Tab.VII. Chromatographic optimization

Compounds	**Linear range (ppb)**	**R2**	**LOD (ppb)**	**LOQ (ppb)**	**Repertability (%)**	**Riproducibility (%)**
OCHRATOXIN A	2-10	0.9998	0.4	1.233	1.89	1.41
AFLA B1	1-5	0.9996	0.3	0.863	3.36	3.37
AFLA B2	1-5	0.9998	0.3	0,842	4.542	2.78
AFLA G1	1-5	0.9997	0.4	0.950	5.48	4.27
AFLA G2	1-5	0.9999	0.5	1.005	4.46	3.62
FUMONISIN B1	10-500	0.9996	1.1	3.18	4.98	5.25
FUMONISIN B2	10-500	0.9997	0.4	2.171	5.361	3.89
DON	10-1000	0.9994	4.2	9.081	1.681	5.87
HT-2	10-1000	0.9996	1.9	6.264	5.29	3.95

Table VII. Retention time. instrumental recovery and percentage relative standard deviation of mycotoxins

Compounds	Rt	RSD%	Recovery (%)
OCHRATOXIN A	43,08	11	82
AFLA B1	17,55	10	104
AFLA B2	15,48	6	98
AFLA G1	14,01	6	102.7
AFLA G2	12,42	7	95
FUMONISIN B1	28,89	13	101
FUMONISIN B2	42,96	8	96
DEOXYNIVALENOL	44,05	0.2	79
HT-2	28,15	1	180

8.5. CONCLUSION

During this study a new rapid and simple analytical method for the simultaneous determination of 9 mycotoxins in beer was developed and validated. Although this is only a preliminary study, which therefore requires further investigation, at the moment it can be concluded that the developed LC-MS/MS multi-mycotoxins method can be used as a tool to obtain a comprehensive screening of the range of mycotoxins potentially co-occurring in the final craft beer.

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CHAPTER 9

Conclusions and Future Perspectives

The research focuses on characterizing the quality of hops in Mediterranean climatic conditions, highlighting a significant gap between studies on hop quality and agronomic aspects. This imbalance is attributed to the growing interest of the craft beer industry in unraveling the unique characteristics ("terroir") associated with different cultivation locations. In 2021, Rossini et al. highlighted the feasibility of organic hop cultivation in Italy, emphasizing the need for comprehensive agronomic strategies due to the absence of registered chemicals for hop protection. A forthcoming study plans to conduct a nutraceutical evaluation of native Calabrian hops, representing a pioneering exploration of the agronomic factors specific to this region.

Subsequent studies on the nutraceutical evaluation of both native and non-native Calabrian hops have demonstrated significant potential in the examined cultivars based on their composition, especially due to their documented antioxidant and antimicrobial properties. These cultivars possess untapped potential in terms of volatile compounds and other chemical constituents, suggesting a transformative impact on the beer industry. If fully explored, this unused resource could revolutionize beer production by providing a natural means of preservation and introducing new raw materials, thus contributing to the expansion of the growing beer sector.

Regarding the production of craft beers, the subject of analysis in this PhD, it requires a combination of the type of added product, the stage of addition, and the diversification of the starting raw materials used. An interesting observation pertains to the low DPPH content and, consequently, a high value of Total Polyphenols for all three samples, suggesting that the use of aromatic ingredients is not the sole factor responsible for the variability of these indices; rather, these data could also be related to hops and malt. For example, in the Blanche-style beer, unmalted grains were also used, and thus the development of melanoidins did not occur, influencing the antioxidant result of the sample.

The beer that showed the best results seemed to be the IPA, in which oats and wheat were used in addition to barley malt. This could explain the excellent results obtained both from an antioxidant perspective and in the overall chemical-physical characterization.

The conducted studies aim to develop methods based on biomimetic systems such as E-Nose, E-Tongue, Scio, and SpectaPod to analyze the main characteristics of beers, including color, aroma, and taste, which respectively stimulate sight, smell, and taste. The goal is also to assess the different features of the three distinct styles of beer. Exploratory experiments were conducted using these methods, with the ultimate aim of creating tools for the non-destructive assessment of quality indicators during beer production in the future. Despite the limited quantity of samples available in this preliminary pilot study, it was possible to clearly distinguish between different types and styles of beer. These initial findings suggest that, even with reduced sensitivity, spectroscopy and biomimicry applied with sensors have significant potential as valuable techniques for real-time monitoring in various stages of beer production. Currently, further experiments are planned to validate and strengthen these preliminary results, working with a much larger set of beer samples, including established reference data for building models of nutraceutical and quality indicators.

In this study, a new method was developed and validated for the simultaneous determination of over thirty polyphenols in craft beer samples using a direct sample injection system with RP-UHPLC-PDA. The developed method allowed for the determination of numerous polyphenols with excellent chromatographic separations and the quantification of typical phenols in beers, such as p-Coumaric acid, 4-Hydroxybenzoic acid, and Protocatechuic acid, as well as compounds derived from "characterizing foods" such as Naringin, Neohesperidine, (-)-Epicatechin, and Tangeretin. The UHPLC system with a sub-2 core-shell column enabled the separation of compounds with very similar structures, such as cis-trans pairs. The method was applied to three different beers (Blanche, IPA, and Lager), and the qualitative and quantitative results for the polyphenols are in line with the literature data. The validated method can be used by breweries to carry out a rapid screening of polyphenols in beer during the production phase to refine

taste, flavor, and style or to improve production techniques and the use of raw materials. Furthermore, the described method can be a powerful tool to detect food fraud (use of non-natural essences) and to determine the conservation status of beers. The ease and speed of analysis with an inexpensive instrument make this method suitable for many applications in the beer supply chain. Further experiments are currently pla nned to validate and strengthen these preliminary results, working with a much larger set of beer samples, including established reference data for building models of nutraceutical and quality indicators.

TOF MS analyzers provided a complete and accurate mass spectrum for each compound, thanks to their high resolution and scan frequency. This is particularly advantageous compared to quadrupole MS, as the high mass resolution of TOF allows for the creation of extracted-ion chromatograms using narrow mass windows and the precise measurement of characteristic ions in mass spectra. These narrow mass windows significantly improve sensitivity by reducing background noise in chromatograms and enhancing the signal-to-noise ratio.

The ongoing study highlights how the developed analytical method serves as a valuable tool for identifying the aromatic profile of the analyzed compounds. In fact, the analysis of the volatile fraction of the samples shows that the protocol followed can detect even trace amounts of compounds quickly and simply, without the need for any sample pretreatment. However, to definitively confirm the presence or absence of a specific molecule, further optimization of the analytical method and MS characterization through the study of a significant number of samples will be necessary. Thanks to the tandem mass spectrometer, GC-QTOF MS can perform MS2 scans following the separation of the precursor ion in the quadrupole and its subsequent collision-induced dissociation. The presence of abundant accurate product ions has allowed for the confirmation of the identity of suspected compounds and has enhanced the accuracy of identification. The DVB/CAR/PDMS fiber has demonstrated excellent extraction capacity for compounds and, when combined with the HS-SPME mode, has yielded outstanding results in the extraction of various analytes and reproducibility through adsorption for craft beers of different styles.

Regarding the developed multi-mycotoxin LC-MS/MS method, it can be used as a tool to obtain a comprehensive screening of the range of mycotoxins potentially present in the final craft beer.

The final working model was conceived by creating a basic lager-style beer composed of water, barley malt, and yeast. This beer was subsequently flavored with various hops, including those indigenous to Calabria, commercial Cascade, and Cascade cultivated in Calabria. The objective was to evaluate discriminatory aromatic differences among the following samples.

In the course of this study, the new method developed using biomimetic systems, specifically electronic nose and tongue, capable of emulating human taste and olfactory systems, was employed. The subsequent analysis of the samples, supported by statistical analysis, yielded distinctive and discriminatory results for the three samples. However, it is important to note that this analysis represents only a preliminary phase, and it would be desirable to expand the analytical models by focusing on various specific aspects such as the chemical-physical, nutraceutical, aromatic, and toxicological properties of the finished product.

At the conclusion of the research activities, an attempt was made to establish possible correlations based on the robustness of the obtained data through a logical analysis.

Specifically, in the selection of taste sensors for use in the nose-tongue prototype, an effort was made to identify a potential correlation between the response of the electronic tongue sensors and the polyphenolic profile of the three different types of beer. For this purpose, Partial Least Squares (PLS) analysis was chosen. Partial Least Squares regression is a fast, efficient, and optimal regression method based on covariance. It is recommended in cases of regression where the number of explanatory variables is high, and multicollinearity among the variables is likely, meaning that the explanatory variables are correlated. Partial Least Squares (PLS) regression is a technique that reduces the number of variables utilized for prediction to a smaller set of predictors. These selected predictors are subsequently employed for regression analysis. Constructing a multiple linear regression model enables the quantification of the relationship between dependent variables (y) and a set of explanatory variables (x).

The PLS analysis applied to our data generates the correlations shown in Figure I and Table I.

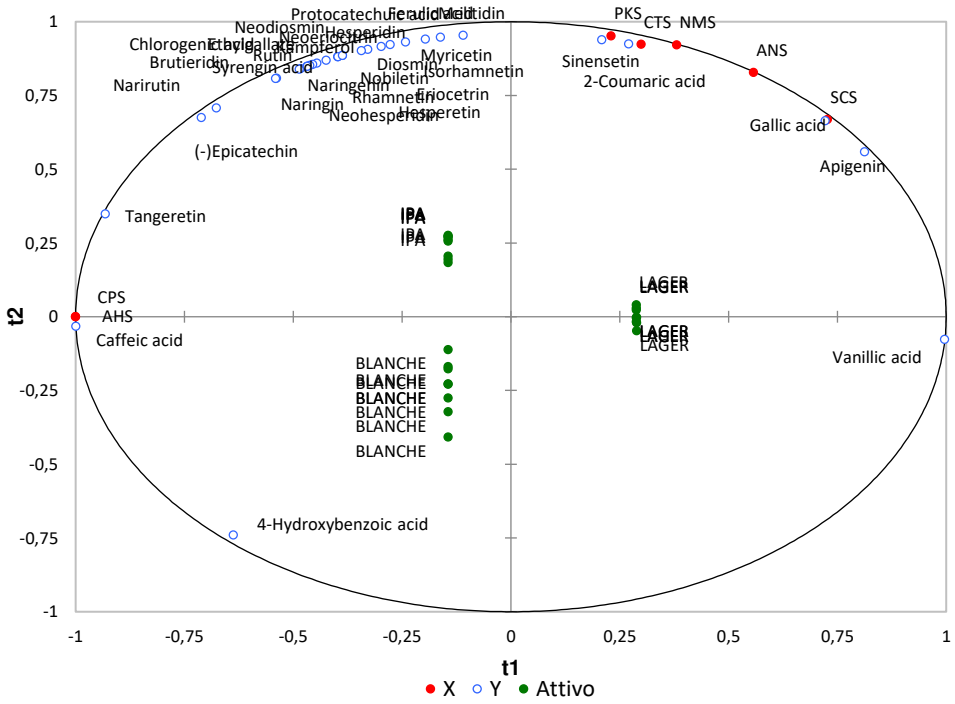

Figure. I: PLS Bi-plot of Sensor Response E-Tongue vs Polyphenol Content of Three Beer Prototypes

The main objective is to analyze the correlations between the sensor responses and individual polyphenolic compounds obtained through HPLC analysis. In this regard, the correlation coefficient plays a crucial role as it reflects the strength of the bond between two quantitative variables, whether it is of a positive or negative nature. The gathered data comprises continuous variables, and consequently, the Pearson correlation coefficient will be employed to assess the magnitude of the linear association between these variables. A value near -1 signifies a robust negative relationship, suggesting that as one variable increases, the other decreases. Conversely, a value near 1 indicates a strong positive relationship, signifying that both variables increase or decrease concurrently. A correlation coefficient of 1 represents a perfect positive linear relationship between the variables. Conversely, if the coefficient approaches 0, it suggests the absence of a significant relationship between the two variables.

Table I: Correlation matrix (Pearson) e-tongue sensors vs polyphenolic compounds.

Variables	2-Coumaric acid	4-Hydroxybenzoic acid	Caffeic acid	Gallic acid	Sinensetin	Apigenin
AHS	-0,271	0,637	**0,999**	**-0,722**	-0,209	**-0,813**
PKS	**0,950**	**-0,858**	-0,261	**0,804**	**0,950**	**0,724**
CTS	**0,892**	**-0,840**	-0,328	**0,799**	**0,887**	**0,734**
NMS	**0,964**	**-0,932**	-0,411	**0,894**	**0,954**	**0,831**
CPS	-0,271	0,637	**0,999**	**-0,722**	-0,209	**-0,813**
ANS	**0,907**	**-0,961**	-0,583	**0,946**	**0,885**	**0,911**
SCS	**0,856**	**-0,991**	**-0,750**	**0,999**	**0,821**	**0,990**

Values in bold are different from 0 at the alpha=0.05 significance level.

From Table I and Figure II, it can be observed that the AHS sensor is strongly positively correlated with caffeic acid and negatively correlated with gallic acid, vanillic acid, and apigenin. The PKS sensor is positively correlated with coumaric acid, gallic acid, sinensetin, and apigenin, and negatively correlated with hydroxybenzoic acid. Both CTS and NMS sensors are positively correlated with coumaric acid, gallic acid, sinensetin, and apigenin, and negatively correlated with hydroxybenzoic acid. The CPS sensor is positively correlated with caffeic acid and negatively correlated with gallic acid and apigenin. The ANS sensor is positively correlated with coumaric acid, gallic acid, sinensetin, and apigenin, and negatively correlated with hydroxybenzoic acid. Lastly, the SCS sensor is positively correlated with coumaric acid, gallic acid, sinensetin, and apigenin, and negatively correlated with hydroxybenzoic acid and caffeic acid.

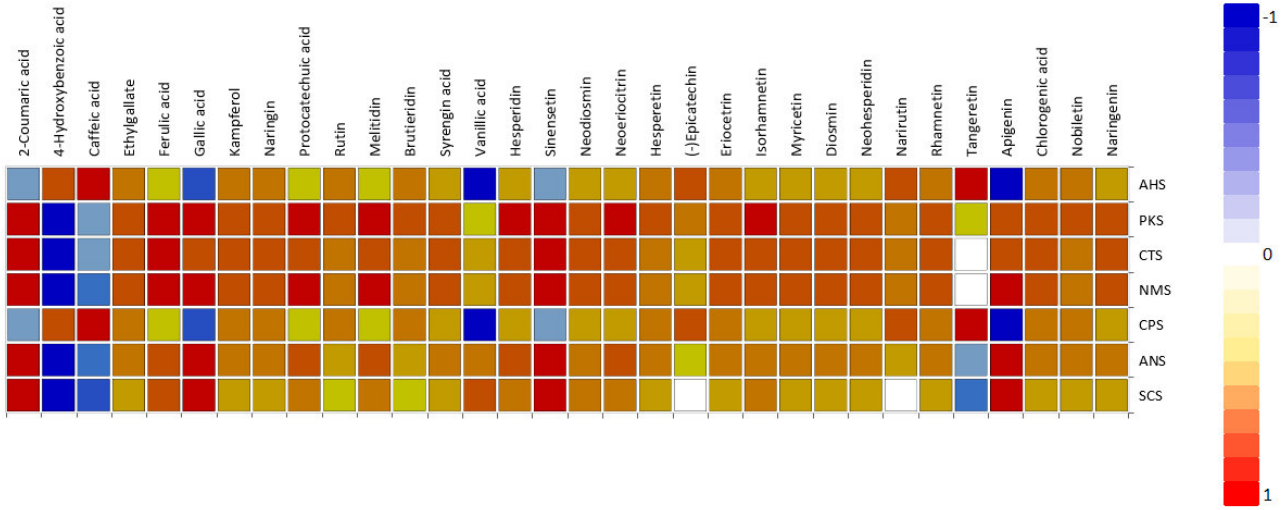

Figure II - Map of tongue sensor correlations vs polyphenolic compounds using a blue-red (cold-hot) scale to visualize the correlations. The color blue corresponds to a correlation close to -1, and the color red corresponds to a correlation close to 1.

Regarding the possibility of integrated product traceability/authentication, the research has been focused on the identification of authentication markers. In particular, a data fusion of the results obtained from both biomimetic sensors and the results from the polyphenolic and aromatic profiles obtained with GC/MS has been carried out. A principal component analysis (PCA) was conducted on the entire dataset, and the result obtained is shown in Figure III.

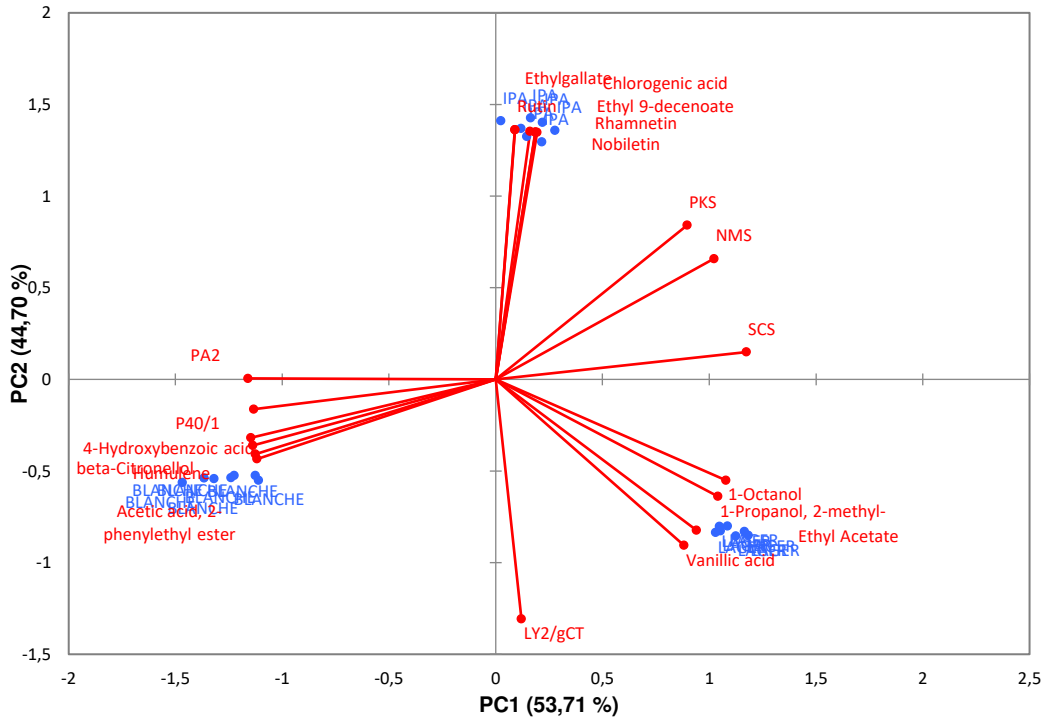

Figure III. PCA Biplot on model beers (in blue, samples of BLANCHE beer with non-malted cereals, bitter orange, and coriander; IPA with bergamot, and LAGER; in red, the variables).

As seen in the biplot (Fig. III), data fusion proves to be a fundamental tool in the analysis of the sensory characteristics of model beers. Through this technique, we can obtain crucial information to identify both polyphenolic and aromatic markers that allow discrimination among the three different sample beers and the olfactory and gustatory sensors most suitable for this task.

Finally, in Table II, the variables that discriminate for different types of beer are expressed.

Table II. Beer discriminating variables.

Discriminating variables		Beer sample		
Compounds	**Classes**	**BLANCHE**	**IPA**	**LAGER**
4-Hydroxibenzioc acid	Polyphenol	**X**		
Ethylgallate	Polyphenol		**X**	
Chlorogenic acid	Polyphenol		**X**	
Rhamnetin	Polyphenol		**X**	
Nobiletin	Polyphenol		**X**	
Vanillic acid	Polyphenol			**X**
beta-citronellol	Flavor GC-MS	**X**		
Acetic acid 2-phenylethyl ester	Flavor GC-MS	**X**		
Ethyl 9-decenoate	Flavor GC-MS		**X**	
1-octanol	Flavor GC-MS			**X**
1-Propanol, 2-methyl	Flavor GC-MS			**X**
Ethyl Acetate	Flavor GC-MS			**X**
P40/1	Sensor e-Nose	**X**		
LY2/gCT	Sensor e-Nose			**X**
PKS	Sensors E-Tongue		**X**	
NMS	Sensors E-Tongue		**X**	
SCS	Sensors E-Tongue		**X**	

These conclusions highlight the diverse aspects of the research, ranging from hop cultivation and organic farming to beer production techniques, quality indicators, and analytical methods for compound identification. Validation and further expansion of studies are crucial to advance the understanding of these complex processes and their implications in the beer industry.